DETECTION AND FATE OF ADENOVIRUS IN THE ENVIRONMENT

by

Chi Yuen (Kelvin) Wong

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ABSTRACT

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The prevalence of adenovirus (AdV) in fecal-related materials has been recognized by the scientific community and numerous studies have reported the presence of human adenoviruses (HAdV) in the water environment. One of the major reservoirs of AdV in the environment is solid material such as biosolids and manure. However, the quantitative levels and fate of HAdV in land applied waste such as manure and biosolids is still poorly understood. The overall objective of this study is to quantify adenovirus and other virus levels in land applied solid materials (biosolids, manure) and develop an understanding of the factors influencing the transport and sorption of biosolid-associated viruses, particularly HAdV.

Among five different enteric viruses tested in this study, HAdV had the highest concentration and occurrence in the mesophilic anaerobic digested (MAD) biosolids. The infectious HAdV was also more prevalent than the infectious enterovirus. Bovine adenovirus (BAdV) was detected frequently in manure samples; however, the occurrence and quantitative levels of BAdV were lower than bovine polyomavirus (BPyV), and a high genetic diversity was observed in the BAdV isolated from different samples. These results suggest that biosolids are the major reservoir of HAdV and a more vigorous sludge treatment may be needed to avoid the risk of water contamination by enteric viruses at the land application site. Also, BAdV may be less suitable than BPyV as bovine fecal indicator due to its lower prevalence and higher genetic diversity.

Despite the high loads of HAdVs observed in biosolids, no indigenous viruses (HAdV and somatic phage) were observed in any of the leachate (lysimeter effluent) samples in a large-scale field experiment, which indicate that the sandy-loam soil system described in this study could effectively remove/sorb the biosolid-associated pathogens. P-22 bacteriophage (microbial tracer) was found in leachate samples collected from three of the lysimeters with a breakthrough occurring at less than 1.0 pore volume, and early breakthrough indicates that preferential flow plays a critical role in virus transport in subsurface. A first order decay model was fit to the measurements of somatic phage and P-22 in the surface water samples and results indicate the biosolid-associated viruses could survive up to ten days after land application.

Based on the Freundlich constants obtained from isotherm curves, both bonded organic matter (OM) and dissolved OM (DOM) inhibited the sorption of HAdV to soil, and soil with higher OM also enhanced desorption of HAdV. A series of experiments provided evidence that the loss of virus in the polyethylene (PE) vials was due to sorption rather than inactivation, and similar to soil, the sorption of HAdV to polyethylene (PE) was inhibited by DOM. Glass containers are preferable to containers made with plastic materials such as PE for HAdV-soils sorption experiment since the sorption of HAdV was significantly reduced in glass tubes. The overall results suggested that OM plays an important role on sorption and desorption of HAdV.

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ABBREVIATIONS

Adenovirus- AdV

American Type Culture Collection - ATCC

Analysis of variance - ANOVA

Beef extract - BE

Bovine adenovirus - BAdV

Bovine polyomavirus - BPyV

Cation exchange capacity - CEC

Complementary DNA - cDNA

Concentrated animal feeding operations - CAFOs

Contaminant Candidate List - CCL

Cytopathic effects - CPE

Deoxyribonucleic acid - DNA

Duplex fluorescence resonance energy transfer - FRET

Enteroviruses - EV

Environmental Protection Agency - EPA

Fecal coliform - FC

Genome equivalent copies - GEC

Hepatitis A - HAV

Hepatitis E - HEV

Human adenoviruses - HAdV

Humic acid - HA

Integrated cell-culture PCR - ICC-PCR

Limit of quantification - A_{LOD}

Mesophilic anaerobic digested - MAD

Messenger RNA - mRNA

Microbial source tracking - MST

Noroviruses - NV

Organic matter - OM

Phage forming unit - PFU

Polyethylene - PE

Polyomaviruses - HPyV

Pore volumes - PV

Real-time quantitative PCR - qPCR

Ribonucleic acid - RNA

Soil extracted solution - SES

Total organic carbon - TOC

Triple-phase boundary - TPB

Virus lysis buffer - AVL

Wastewater treatment plant - WWTP

CHAPTER 1

INTRODUCTION

The occurrence of enteric virus in water environment

The emergence of microbial contaminants, such as human viruses in water and the potential associated disease in humans is an urgent global health threat. Waterborne disease statistics estimate a growing global burden of infectious diseases from contaminated drinking water. It has been estimated that 1.5-12 million people die per year from waterborne diseases (Gleick, 2002). Most of the waterborne disease outbreaks in the US that occurred between 1991 and 2004 were related to microbial agents, i.e. viruses, bacteria, and parasites (Moore *et al.* 1993; Kramer *et al.* 1996; Levy *et al.* 1998; Barwick *et al.* 2000; Lee *et al.* 2002; Blackburn *et al.* 2004; Liang *et al.* 2006), and the majority of the outbreaks involved un-identified agents. The failure to identify etiologic agents of a waterborne disease is most likely due to lack of sensitive analytical techniques including appropriate concentration and isolation methods. The Environmental Protection Agency (EPA) suspects that many of the outbreaks due to unidentified sources were caused by enteric viruses (USEPA 2006).

Occurrence of human pathogenic viruses in environmental waters (surface waters, groundwater, drinking water, recreational water, and wastewater) raises concerns regarding the possibility of human exposure and waterborne infections. Commonly observed waterborne viruses include adenoviruses, enteroviruses, and noroviruses, and they have been appeared on the Drinking Water Contaminant Candidate List (CCL) one, two and three, which were announced by EPA in the year of 1998, 2005 and 2007,

respectively (Table 1.1). Common waterborne viruses and associated-disease are summarized in Table 1.2.

Table 1.1. Microbial agents on CCL list (viruses are shown in bold letters).

CCL 1	CCL 2	CCL 3
Acanthamoeba	**Adenoviruses**	**Adenoviruses**
Adenoviruses	*Aeromonas hydrophila*	**Caliciviruses**
Aeromonas hydrophila	**Caliciviruses**	*Campylobacter jejuni*
Caliciviruses	Coxsackieviruses	**Enterovirus**
Coxsackieviruses	Cyanobacteria (blue-green algae), other freshwater algae, and their toxins	*Escherichia coli* 0157
Cyanobacteria (blue-green algae), other freshwater algae, and their toxins	**Echoviruses**	*Helicobacter pylori*
Echoviruses	*Helicobacter pylori*	**Hepatitis A virus**
Helicobacter pylori	Microsporidia (*Enterocytozoon* & *Septata*)	*Legionella pneumophila*
Microsporidia (*Enterocytozoon* & *Septata*)	*Mycobacterium avium intracellulare* (MAC))	Microsporidia (*Enterocytozoon* & *Septata*)
Mycobacterium avium intracellulare (MAC)		*Naegleria fowleri*
		Salmonella enterica
		Shigella sonnei

Table 1.2. Commonly found waterborne viruses and associated disease (Maier *et al.* 2008; Wong 2008).

Virus	Nucleic acid characteristic	Associated disease
Adenoviruses	double stranded DNA	Gastroenteritis, upper and lower respiratory system infections, conjunctivitis
Astrovirus	positive single stranded RNA	Infantile gastroenteritis
Enteroviruses	positive single stranded RNA	Respiratory infections, gastrointestinal infections, skin infections, neurological infections.
Hepatitis A	positive single stranded RNA	Gastroenteritis, fever
Noroviruses	positive single stranded RNA	Gastroenteritis, fever
Rotavirus	double stranded RNA	Infantile gastroenteritis

Viruses are the smallest of all microorganisms and their size facilitates transport in environmental media. In addition, viruses have very low die-off rates and low infectivity doses. The ability to effectively detect waterborne viruses is the basis for microbial risk assessment and management of water sources with the ultimate goal to protect public health. However, precise detection, quantification, and infectivity determination of viruses has always been a challenge in water quality laboratories.

Only with the recent advancement of molecular biology techniques it has become possible to test environmental samples for non-culturable viruses. Table 1.3 presents the latest reported groundwater enteric viral pollution in the US. A groundwater-associated outbreak affected approximately 1,450 residents and visitors of South Bass Island, Ohio in 2004, and two out of 16 wells that provide potable water to public water systems tested positive for adenovirus (Fong *et al.* 2007). In a nationwide study, samples for 448 groundwater sites in 35 states were analyzed by PCR for enteroviruses, rotavirus,

hepatitis A virus, and noroviruses. Infective viruses and viral nucleic acid were present in 4.8 and 31.5% of samples respectively (Abbaszadegan *et al.* 2003). Fout *et al*. (2003) analyzed 29 groundwater sites for 1 year for enteroviruses, hepatitis A virus, Norwalk virus, reoviruses, and rotaviruses. Human enteric viruses were detected in 16% of the groundwater samples analyzed. Borchardt *et al.* (2003) tested 50 private household wells in Wisconsin four times per year and found that four wells (8%) were virus positive. Three wells were positive for hepatitis A virus and another well was positive for rotavirus, norovirus, and enterovirus. In an earlier study (Lieberman *et al*. 1995), 30 public water supply wells were examined. The authors reported 24% of the samples were positive for culturable viruses. Also, the US Geological Survey (USGS 1998) reported about 8% of wells positive for culturable human viruses.

Table 1.3. Enteric viral pollution in US groundwater.

Waterborne virus	Reference
Adenovirus	Fong *et al.* 2007
Enterovirus, Hepatitis A virus, Norwalk virus, Reovirus, Rotavirus	Fout *et al.* 2003
Enterovirus, Hepatitis A virus, Rotavirus, Norwalk virus	Abbaszadegan *et al.* 2003
Enteroviruses, Hepatitis A virus, Rotavirus, Norwalk-like Viruses	Borchardt *et al.* 2003
Enteric Viruses	USGS, 1998
Culturable Viruses	Lieberman *et al.* 1995

Adenoviruses in water and human health

The importance of adenoviruses and the potential health risks associated with their waterborne transmission has been recognized by the scientific community. Adenoviruses are a common cause of gastroenteritis, upper and lower respiratory system infections, and conjunctivitis. Other diseases associated with adenoviruses include acute

and chronic appendicitis, cystitis, and nervous system diseases. Adenoviruses are considered important opportunistic pathogens in immunocompromised patients (Wadell 1984). There are 51 different types of human adenoviruses (Jiang 2007).

Enteric and non-enteric adenoviruses are shed in feces and can contaminate water systems. Initial infection may occur by the respiratory route, but fecal-oral transmission accounts for most adenovirus infections in young children because of prolonged shedding of the viruses in feces (Maier *et al.* 2008). Enteric adenoviruses were identified as one of the etiological agents causing acute gastroenteritis in a waterborne outbreak in Finland (Kukkula *et al.* 1997). Outbreaks have been associated with recreational exposure in swimming pools (Foy *et al.* 1968; Martone *et al.* 1980; Papapetropoulou and Vantarakis 1998).

Enteric adenoviruses have been reported to cause 5-20% of the infant and child acute gastroenteritis (Albert 1986). Adenovirus types 40 and 41 have also been associated with gastroenteritis in infants and children by Krajden *et al.* 1990; and Bon *et al.* 1999. The enteric adenoviruses 40 and 41 have been reported to be almost as important etiological agents of viral gastroenteritis in children as rotavirus (Uhnoo *et al.* 1986; Cruz *et al.* 1990). Among 153 children with diarrhea in a case-control study conducted in central Wisconsin, 7 children (5%) were positive for adenovirus 40/41 (Borchardt *et al.* 2003). Gastroenteritis in children has also been associated with adenovirus 31 (Adrian *et al.* 1987; Krajden *et al.* 1990) and with adenovirus type 2 (Swenson *et al.* 2003).

The potential of transmission of adenoviruses through water is also suggested by the findings of several researchers. Enriquez *et al.* (1995) concluded that enteric adenoviruses are more stable in tap water and wastewater than poliovirus. Irving and

Smith (1981) reported that adenoviruses are more likely to survive conventional sewage treatment than enteroviruses. In addition, Hurst *et al.* 1988, estimated that most adenoviruses detected in waste water may be enteric adenoviruses.

Latest virus detection technologies

Cupples A., Rose J.B., Xagoraraki I. (2010). New Molecular Methods for Detection of Waterborne Pathogens. In: Environmental Microbiology (Mitchell R., Gu J.D., Editors), Wiley-Blackwell, Hoboken, NJ

Traditionally, cell culture has been recognized as the gold standard for virus detection. In the last few decades, however, polymerase chain reaction (PCR) has rapidly emerged as a method of virus detection in environmental samples. Compared to cell culture, the main advantages of PCR methods for virus detection include: fast result, less intensive labor, high specificity and sensitivity, and capability of detecting difficult-to-culture or non-culturable viruses (for examples, human noroviruses and adenovirus 40/41). However, PCR is not free from problems. The main disadvantage of PCR methods is that they are not able to determine infectivity.

Currently, enteric waterborne viruses that have been widely studied are adenoviruses, noroviruses, enteroviruses, hepatitis A and E, rotaviruses, and astroviruses. Different PCR and combination techniques have been developed for detecting these viruses. The techniques include cell-culture/cell-infectivity, conventional PCR, nested PCR, multiplex PCR, integrated cell-culture PCR (ICC-PCR), and real-time quantitative PCR (qPCR). Table 1.4 describes characteristics of the different virus detection techniques.

Table 1.4. Virus detection methods.

Method	Outcome	Characteristics
Cell Culture	Presence/Absence, Infectivity Determination	Infected cell cultures undergo morphological changes called cytopathogenic effects (CPEs) that are observed microscopically. The method is labor-intensive and time-consuming. Some viruses do not show CPEs.
PCR	Presence/Absence	Able to detect non-cultural viruses. High sensitivity and specificity. Requires design of primers that amplify specified DNA regions. Prone to environmental inhibition. Gel electrophoresis is required for confirmation of results.
RT-PCR	Presence/Absence	For RNA viruses, a reverse transcription step (RT) is required before PCR amplification, for the conversion of RNA to cDNA. Following RT, the same steps as with conventional PCR are followed.
Nested PCR	Presence/Absence	Requires two sets of primers. Inner primers amplify the target sequence within the amplicon generated by outer primers. This technique has higher sensitivity than conventional PCR.
Multiplex PCR	Presence/Absence	Uses multiple primer sets in a single PCR reaction to detect multiple targets simultaneously. It is time-efficient and reduces the cost of reagents. The design and optimization of multiplex assays could be more challenging than of conventional PCR assays.
ICC-PCR	Presence/Absence, Infectivity Determination	This is a combination of the traditional cell-culture / cell-infectivity method with PCR. PCR is performed on cell culture supernatant. The method has higher sensitivity than conventional PCR.
Real Time PCR	Quantitative	This is the only quantitative method (except for the MPN dilution method). No post PCR handling step is required to confirm the results. It is very target-specific and sensitive method. The cost of thermocycler and reagent are higher than the ones used in conventional PCR.

Conventional PCR is the most basic molecular technique, in which two primers bind to target deoxyribonucleic acid (DNA) and amplification takes place during the PCR cycles. For detecting ribonucleic acid (RNA) viruses, a reverse transcription step is needed to convert RNA to complementary DNA (cDNA) before amplification. Nested PCR was developed for increasing the assay sensitivity by having another set of primers to amplify a target DNA sequence within the first amplicon. The multiplex PCR technique incorporates multiple primer sets in one reaction, and the main advantage of using this technique is the ability to detect multiple viruses simultaneously.

The main drawback of using PCR to detect pathogens is that it cannot differentiate between viable/infectious and non-viable targets. Therefore, ICC-PCR, a method combining cell culture with PCR, was developed to detect infectious viruses. Beside the advantage of only detecting the infectious viruses, it also has higher sensitivity than conventional PCR due to the amplification of viruses during cell culture. Recently, researchers developed an mRNA RT-PCR method for the detection of infectious adenoviruses in cell culture (Ko *et al*. 2005). The rationale behind this method is that only the infectious adenoviruses can enter cells and transcribe mRNA during replication. Therefore, the positive messenger RNA (mRNA) RT-PCR result indicates the presence of infectious adenoviruses in the samples. Table 1.5 summarizes methods using the combination of cell culture and PCR to detect infectious adenoviruses in different water samples.

Table 1.5. Summary of cell-culture PCR methods for human adenoviruses in environmental waters.

Sample type	Volume	ICC-PCR method	Cell line	Reference
Lake water	250-350 L	ICC-nested-PCR	BGMK	Xagoraraki *et al*. 2007
Marine water	114-151 L	ICC-nested-PCR	BGMK	Ballester *et al*. 2005
Surface water, Tap water	70–300 L, 1000-3000 L	ICC multiplex-nested PCR	BGMK	Lee *et al*. 2005
River water	2 L	ICC multiplex nested RT-PCR	A549 and BGMK	Lee *et al*. 2004
Source water, Drinking water	200 L, 1500 L	ICC-nested-PCR	BGMK	Lee and Jeong 2004
Raw water, Treated water	100-1000 L	ICC-nested-PCR	PLC/PRF/5	Van Heerden *et al*. 2003
Sewage	1L	ICC-PCR	A549 and BGMK	Greening *et a*l. 2002
River water	10 L			
Tap water	1000-3000 L	ICC-multiplex-nested PCR	BGMK	Lee and Kim 2002
Surface water	None	ICC-nested PCR	BGMK	Chapron *et al*. 2000

BGMK = buffalo green monkey kidney cell
A549 = adenocarcinomic human alveolar basal epithelial cells
PLC/PRF/5 = human hepatoma cell line

Real time PCR (qPCR) is currently the most advanced technology for virus detection. Two commonly used qPCR methods require the use of fluorescent dyes binding to target DNA or modified oligonucleotide probes that would bind to the target DNA and fluoresce during primers extension. The most important advantage of qPCR over other PCR methods is that the result generated by qPCR is both qualitative and quantitative. Most of the qPCR assays described in literature use hydrolysis or Taqman probes labeled with fluorescence dyes. The sensitivity of qPCR is equal to or greater than the sensitivity of traditional PCR and nested-PCR. Most of the qPCR assays reported in the literature have a detection limit of 10 copies or less of the target gene. Another advantage of qPCR is that it does not require post PCR handling, such as gel electrophoresis, to view and confirm the results. Multiplex assays can be applied to qPCR method. Wolf *et al*. (2007) used a multiplex qPCR assay to simultaneously detect norovirus type 1, 2, and 3 in environmental samples. Other studies also used multiplex qPCR to detect enteric viruses but the application is in clinical samples. With the rapid advancement of qPCR technology, it is expected that the limitations of using multiplex real time PCR, such as limited available fluorophoric labels and the significant overlap in emission spectra would be overcome soon.

The design of primers and probes is an important step since it affects the sensitivity and specificity of the PCR assay. The general procedure for primer and probe design begins with retrieving the nucleotide sequences of specific target organisms from the gene bank, and then aligning them using software like ClustalW. Primers and probes could be designed from the consensus or variable region of the genes depending on the specificity and types of PCR assay. Numerous software programs are available for

different types of PCR assay design. Some of the software is available online like Primer3 Input 0.4.0. Other software such as Primer Express 2.0 and Lightcycler Probe Design 2 can be purchased from PCR design software companies. After the primers and probes are designed, a BLAST search on the primers and probes is performed to confirm target specificity. Assay optimization for increasing sensitivity is usually required; assays are optimized by adjusting annealing temperature, primer and probe concentration, and magnesium chloride concentration.

Adenoviruses as fecal indicator

Fecal coliform, *Escherichia coli* (*E. coli*), enterococci and *Clostridium perfringens* have been the gold standard for fecal pollution indication. National Research Council (NRC 2004) separates the ideal fecal indicator criteria outlined by Bonde (1966) into desirable attributes of the biological indicators and desirable attributes of the methods to detect the indicators. These attributes, as summarized by Yates (2007), are shown in Table 1.6.

Table 1.6. Desirable attributes of biological indicators and desirable attributes of methods to detect the indicators (Yates 2007).

The desirable attributes of biological indicators	**Desirable attributes of the methods**
Correlated to health risk	Specificity (independent of matrix effects)
Similar (or greater) survival to pathogens under environmental conditions	Broad applicability
Similar (or greater) transport to pathogens	Precision
Present in greater numbers than pathogens	Adequate sensitivity
Specific to a fecal source or identifiable as to source of origin	Rapidity of results
	Quantifiable
	Measures viability or infectivity
	Logistical feasibility

The traditional fecal indicators and the conventionally used methods posses most of the attributes describe in Table 1.6. However, it is difficult to differentiate human and animal fecal sources using the traditional indicator culture methods. The public health threat from human fecal source is generally more of concern but zoonotic pathogens originating from agricultural animal fecal material could also cause serious disease in humans. For example, a recent outbreak in Canada indicates that the potential risk for human infections caused by zoonotic pathogens is real. More than 2,300 people in the town of Walkerton, Ontario suffered gastrointestinal illness where seven people perished when the town's shallow water supply was contaminated by manure pathogens from a nearby farm after more than five inches of rain fell over a five day period in May, 2000 (Hrudey and Hrudey 2004). Microbial source tracking (MST) has been developed recently and various approaches have been introduced to differentiate the fecal materials from different sources. These approaches can be divided into library-dependent culture based, library-independent culture based, and library-independent culture independent methods.

Enteric viruses have been proposed as one of the library-independent culture independent MST tools. The main advantages of using enteric virus for MST is that viruses are generally very host specific (Scott *et al.* 2002) and there is evidence showing that enteric virus are strongly associated with gastroenteritis (Wilhelmi *et al.* 2003). The main disadvantage of using enteric virus for MST is low concentration in the environment (Scott *et al.* 2002), which may result in levels of virus below the detection limit of the assay. With the advancement of detection and sampling technology over the last 20 years, many of the problems associated with low detection of viruses in the

environments have been addressed. Studies have shown the possibility of using enteric virus for MST, and these studies are summarized in Table 1.7. As shown, adenoviruses have been proposed in several studies for human, bovine, ovine, and porcine fecal indication. One of the advantages of using adenoviruses for fecal indication is that adenoviruses have high resistance to ultraviolet (UV) radiation due to their double stranded DNA structure. This resistance should reduce their decay rate in the natural environment and make adenovirus more detectable in water environment as compared to other RNA viruses. Also, a study has found human adenoviruses have the highest concentration in wastewater influent and effluent as compared to enterovirus and norovirus (Katayama *et al.* 2008). Therefore, using human adenovirus as a fecal pollution indicator and a MST organism seems promising based on the criteria of abundance and survival ability. However, the study on the occurrence of bovine adenovirus in environment is significantly limited compared to human adenovirus, and the concentration of bovine adenovirus in the cow fecal and manure samples has never been reported in the literature.

Table 1.7. List of studies used enteric virus for fecal source tracking.

Enteric virus	Origin	Authors
Adenovirus	Human	Jiang *et al.* 2007
		Noble *et al.* 2003
		Wolf *et al.* 2010
	Bovine	de Motes *et al.* 2004
		Hundesa *et al.* 2006
		Wolf *et al.* 2010
	Ovine	Wolf *et al.* 2010
	Porcine	de Motes *et al.* 2004
		Hundesa *et al.* 2009
		Wolf *et al.* 2010
Enterovirus	Human	Jiang *et al.* 2007
		Noble *et al.* 2003
	Bovine	Fong *et al.* 2005
		Jimenez *et al.* 2005
		Ley *et al.* 2002
Norovirus	Human	Wolf *et al.* 2010
	Porcine	Wolf *et al.* 2010
	Bovine, ovine	Wolf *et al.* 2010
Polyomavirus	Bovine	Hundesa *et al.* 2006
		Hundesa *et al.* 2010
	Human	McQuaig *et al.* 2006
		McQuaig *et al.* 2009
		Harwood *et al.* 2009
Teschovirus	Porcine	Jimenez *et al.* 2003

Major sources of viral pollution in agricultural areas

Most of the waterborne disease outbreaks worldwide and in the US are associated with rural drinking water systems. According to Craun *et al.* (2006), in the US during the 12 year period of 1991-2002, 207 waterborne disease outbreaks and 433,947 illnesses were reported; 42% of these outbreaks occurred in non-community water systems, 22% occurred in individual systems such as private wells, and only 36% occurred in community systems.

The major sources of viral pollution in agricultural areas include land application of biosolids and manure. Land application of biosolids has been increasingly practiced

worldwide. In the US, approximately 5 million dry tons of biosolids were generated annually and 60 percent of the biosolids were applied on land (National Research Council 2002). Pathogens generally tend to attach to solid surfaces (Maier *et al.* 2000) and the majority of pathogens in wastewater utilities is likely associated with sludge particles and are expected to end up in wasted sludge. The most common class B sludge stabilization process in US is mesophilic anaerobic digestion (MAD). Table 1.8 illustrates the studies on the occurrence of enteric viruses in biosolids. Compared to existing data of enteric viruses in water, limited data of enteric viruses in biosolid has been reported in the literature and most of these studies focused on enteroviruses. Despite limited data, there is evidence that treated biosolids may contain high loads of enteric viruses.

Table 1.8. Enteric viruses in treated biosolids.

Treatment Type	Enteric Virus	Detection Method	Level of Occurrence (PCR)	Level of Occurrence (cell culture)	Reference
Aerobic digested	Adenovirus	qPCR	2.43×10^{2} to 1.02×10^{4} copies/g		Bofill-Mas *et al*. 2006
Aerobic digested	Adenovirus	qPCR	5×10^{5} copies/g		Viau and Peccia 2008
Aerobic digested plus composting	Adenovirus	qPCR	2.5×10^{4} copies/g		
Lime stabilized	Astrovirus	ICC-PCR	5 out of 5 positive		Chapron *et al*. 2000
Lime stabilized	Enterovirus	ICC-PCR	5 out of 5 positive		
Digested-dewatered	Enterovirus	cell culture		35 PFU/10g	Guzman *et al*. 2007
Dewatered				27 PFU/10g	
Anaerobic digested	Enterovirus	qPCR	1.2×10^{4} copies/g		Monpoeho *et al*. 2004
		cell culture		38.2 MPN/g	
Lime stabilized	Enterovirus	qPCR	1.03×10^{4} copies/g		
		cell culture		37 MPN/g	
Composting	Enterovirus	qPCR	non-detected		
		cell culture		non-detected	
Heat treated	Enterovirus	qPCR	4.5×10^{4} copies/g		
		cell culture		non-detected	
Aerobically digested	Reovirus	ICC-PCR	1 out of 3 positive		Gallagher *et al*. 2007
Aerobic digested	Polyomavirus		6.20×10^{2} to 7.71×10^{3} copies/g		Bofill-Mas *et al*. 2006

Animal manure is often land applied without prior treatment and there is a growing concern regarding transmission of pathogens by manure land application due to the increase of concentrated animal feeding operations (CAFOs). According to USEPA (2006) fecal contamination from livestock manure handling and storage facilities is one of the most prevalent sources of groundwater microbiological pollution. Besides zoonotic protozoa (*Cryptosporidium parvum, Giardia* spp.) and bacteria (*Listeria monocytogenes, E. coli* O157:H7, *Salmonella* spp., and *Mycobacterium paratuberculosis*), zoonotic viruses could also present in livestock manure. Rotavirus and Hepatitis E (HEV) have been suggested as potential zoonotic viruses (Cook 2004; Vasickova *et al.* 2007). Several studies have shown animal rotavirus could cause infection in humans and the genetic sequences of human and animal rotavirus are similar (Cook 2004). HEV is classified as waterborne virus and causes diseases in developing countries in Asia, Africa, and South and Central America (Vasickova *et al.* 2007). Infection of domestic swine with human strains of HEV has been experimentally shown (Balayan *et al.* 1990). The close relationship between the HEV genetic sequences of human and animal in some geographic regions support the hypothesis of HEV as a zoonotic virus (Meng *et al.* 1997; Tsarev *et al.* 1998; Yazaki *et al.* 2003).

Transmission of viruses through natural soil system

The potential for groundwater contamination in Michigan is considerable due to the prevalence of shallow groundwater. The seasonal high water table in many of the agricultural areas of Michigan and the Upper Midwest is within ten feet of the soil surface. Viruses originating from land application sites can be transported to shallow

groundwater and beyond in the aquifer. There is a need to evaluate the fate and transport of viral contaminants in agro-ecosystems and develop biosolids and manure management systems which protect groundwater quality and minimize health risks.

The extremely small size (between 10 and 300 nm) of viruses allows them to infiltrate soils, potentially reaching aquifers. Presumably, most microbial transport occurs in saturated soil (Powelson and Mills 2001) or by preferential flow (Shipitalo and Gibbs 2000; Mawdsley *et al.* 1995). Liquid manure was detected in tile drains within minutes of subsurface injection due to preferential flow through macropores (large, continuous openings in the soil formed by plant roots), soil fauna, cracks, fissures and other natural phenomena (Shipitalo and Gibbs 2000). Shelton *et al.* (2003) reported that the average velocity of bacteria was seven times faster than pore water velocity indicating most of the flow bypassed the soil matrix.

Penetration of viruses to depths as great as 67 m (220 ft) and horizontal migration as far as 408 m (1,339 ft) in glacial till and 1,600 m (5,240 ft) in fractured limestone has been reported (Keswick and Gerba 1980; Robertson and Edberg 1997). Viruses and other microorganisms can survive for several months in soil and groundwater when temperatures are low and soils are moist (Yates *et al*. 1985; Jansons *et al.* 1989; Straub *et al.* 1993; Robertson and Edberg 1997) and the risk of groundwater contamination is increased from long survival time because there is increased potential for the virus to travel farther to the depth of the groundwater table

In the Great Lakes region, biosolids and manure are typically applied when the root system is in decay, or to a growing crop with an active root system. Below the surface, root systems evoke physical changes in the soil that affect microbial movement.

Root systems create root channels through which microorganisms are transported with water movement, thereby reducing the filtering capacity of the soil (Mawdsley *et al.* 1995). Rassett *et al.* (1995) reported that corn roots increased the soil saturated hydraulic conductivity (K_{sat}) while the re-growth of cereal rye roots caused a decrease. The die-off and decomposition of both corn and cereal rye root systems led to a large increase in K_{sat}, and a cereal rye cover crop grown after the corn roots had decomposed reduced the K_{sat} to near pre-decomposition levels. Presumably, factors that increase the rate of water movement in soil also increase the rate of viral and pathogen movement.

Virus sorption to soils

Sorption to soil is one of the most important factors attributing to the removal and transport of viruses and other water-transmitted pathogens at the land application sites (Schijven and Hassanizadeh 2000). Since viruses can travel further than either bacteria or protozoa due to their smaller size (Scheuerman *et al.* 1986), it has been selected as the biological agent for modeling the transport of waterborne pathogens in the subsurface with variation of the factors influencing the adsorption characteristic (Keswick and Gerba 1980; Herbold-Paschke 1991).

The sizes of viruses are within the size range of colloids; therefore, virus sorption has been described by the theories that elucidate colloidal behavior (Gerba 1984). The stability of colloids is controlled by the balance between repulsive double-layer interactions and attractive van der Waals forces (Verwey and Overbeek 1948). Chattopadhyay and Puls (1999) proposed that the total force contributing to the adhesion

of virus to a solid surface can be divided into three groups, which are: 1) electrostatic (EL) interactions, 2) Lihshitz-van der Waals electrodynamic forces (LW), which include van der Waals-Keesom or orientation forces, van der Waals-Debye or induction forces, and van der Waals-London or dispersion forces, and 3) polar forces or acid-base interaction (AB). The force of LW and AB are determined by interfacial tension, which is related to hydrophobicity of the sorbates and sorbents.

Jin and Flury (2002) suggested that protein sorption would be similar to virus sorption since the virus are composed of RNA and DNA that is surrounded by a protein capsid. Yuan *et al.* (2000) outlined the possible types of protein sorption: irreversible and reversible sorption. Irreversible sorption take place when the adhesion between the charged particle and the surface with opposite charge is very strong and it would maintain in a stable position at the surface. On the other hand, particle would not bind to surface permanently and the particle-surface interaction is weak during the reversible sorption. These particle may bind to various part of the surface or undergo desorption. Larger particle is likely to have irreversible sorption based on energetic considerations

Batch experiments have been used to investigate the factors affecting the virus-soil sorption behavior. Jin and Flury (2002) summarized the batch studies done in last 20 years (24 studies were summarized), and most of the studies have focused on the effect of pH and ionic strength of the solution, presence of compounds that compete for binding sites on sorbents (e.g. organic matter), isoelectric point and hydrophobicity of the virus, and properties of the sorbent. Enterovirus and bacteriophage (MS-2 and ΦX174) were used in most of these studies. The sorbents used in these studies were mostly soil (sand,

silt, and clay) with the exception of one study which used activated carbon (Powell *et al.* 2000).

The Freundlich isotherm model was used to describe the sorption between virus and different kinds of sorbent (Bales *et al.* 1991; Bitton *et al.* 1976; Burge and Enkiri 1978; Drewry and Eliassen 1968; Gerba and Lance 1978; Jin *et al.* 1997; Moore *et al.*; 1981; Murray and Parks 1980; Powell *et al.* 2000, Powelson and Gerba 1994; Thompson *et al.* 1998). The constant values of the Freundlich isotherm are the Freundlich constant (K_F) and the slope of the logarithm plot of the isotherm curve (1/N). K_F is roughly related to sorbent capacity and N relates to the intensity of sorption (Burge and Enkiri 1978). Based on these values, studies have determined clayey soils have higher virus sorption capacity (Burge and Enkiri 1978). Also, an increase in cation concentration in solution would increase virus sorption (Bales *et al.* 1991; Drewry and Eliassen 1968). pH also has the effect on virus sorption since pH in the solution could change the net charge on the virus surface. MS-2 is a virus with high hydrophobicity and the values of K_F was ten times larger with hydrophobic than hydrophilic silica (Bale *et al.* 1991).

Adenovirus has recently received increased attention and is included in the Environmental Protection Agency's contaminant candidate list (CCL) one, two and three. The research presented here showed the levels of adenovirus are significantly higher than enterovirus in biosolids by about 1 to 2 logs ($P \leq 0.05$). Adenovirus was also found to have the highest levels in wastewater influent and effluent compared to other enteric virus like enterovirus and norovirus (Katayama *et al.* 2008). Also, different viruses could have different responses to factors influencing their sorption based on their physical properties. Zhuang and Jin (2003) compared the effect of OM on the transport and

sorption of MS2 and ΦX174, and results showed OM only significantly promoted the transport of MS2 but not ΦX174. The authors explained the difference between the transport/sorption behavior of these two viruses based on the fact that MS2 is a more hydrophobic virus than ΦX174 (Shields and Farrah 1987). Therefore, there is a need to determine the sorption characteristic of adenovirus to soil

The presence of organic matter (OM) is a major factor responsible for the uncertainty associated with predicting virus transport in soils and groundwater. If soil-bonded OM or dissolved OM (DOM) in the solution inhibits the sorption of viruses to soil particles, facilitation of the virus transport in the subsurface by OM would be expected. Previous works have indicated that DOM could enhance the virus transport (Bixby *et al.* 1979; Powelson *et al.* 1991; Zhuang and Jin 2003; Bradford *et al.* 2006). Most of these studies concluded that DOM compete with viruses for the favorable sites on soils and lead to promotion of virus transport. However, there is controversial discussion on the effect of bonded-OM on virus sorption. Bales *et al.* (1991) and Kinoshita *et al.* (1993) reported OM coated on grain surface could enhance the virus sorption by increasing the hydrophobicity of the solid surfaces. However, a decrease of virus sorption or increase of virus transport was observed in soils with higher OM from other studies (Fuhs *et al.* 1985; Moore *et al.* 1981; Zhuang and Jin 2003). The results from these studies create some ambiguity and hinder our ability to draw conclusions about the effect of bonded-OM on virus sorption.

Hypotheses and objectives

The prevalence of adenovirus in fecal-related materials has been recognized by the scientific community and studies have reported high concentration of adenovirus present in domestic wastewater. The high concentration of adenovirus provides a huge advantage in using adenovirus as a fecal indicator and potentially a microbial source tracking tool. However, the concentration of adenovirus in animal fecal material and its relative concentration compared to other fecal indicators in animal manure are still unknown. Also, quantitative information on concentrations of adenovirus as well as other enteric viruses in biosolids is still limited and the infectivity of adenovirus in treated biosolids has never been reported. To date, no study has reported the fate and transport of adenovirus at biosolid and manure application sites, and the sorption behavior between adenovirus and soils have never been characterized.

The overall objective of this study was to develop an understanding on the quantitative levels of adenovirus in land applied solids (biosolids and bovine manure) and the fate and transport of adenovirus at land application sites through field study and sorption experiments. The results of this study will fill the gaps of knowledge in the following areas: quantitative data on adenovirus present in manure and biosolids, the removal and transport of biosolid-associated adenovirus/bacteriophage by the natural soil system, and the sorption characteristics of adenovirus under the influence of organic matter. The specific objectives of this study are to design the molecular assay to investigate the occurrence and quantitative levels of bovine adenovirus in manure and fecal materials (presented in Ch. 2.1), to evaluate the possibility of utilizing bovine adenovirus and polyomavirus as bovine fecal indicators based on their quantitative levels

in manure and fecal samples (presented in Ch. 2.2), to investigate the quantitative levels of adenovirus and other emerging enteric virus in biosolids by the combination of molecular and cell culture methods (presented in Ch. 3), to evaluate the leaching and ponding of adenovirus/bacteriophage following land application on sandy loam soils using a large scale lysimeter and artificial rainfall simulator (presented in Ch. 4), and to investigate the sorption characteristics of adenovirus to a polyethylene surface and soil particles under the influence of organic matter (presented in Ch. 5).

The main hypotheses are (1) bovine adenovirus is detected frequently in manure and fecal samples, but bovine polyomavirus and bacterial indicators have a higher occurrence and concentration than bovine adenovirus does; (2) adenovirus has the highest concentration in the mesophilic anaerobic digested sludge when compared to other enteric viruses, and the occurrence of infectious adenovirus is higher than the occurrence of infectious enterovirus; (3) most of the pathogens originating from biosolids will be removed by the natural infiltration systems and the preferential flow governs the breakthrough of viruses in the lysimeter; and (4) bonded organic matter (OM) increases the hydrophobicity of the solid surfaces and thus enhances the sorption of virus by lowering the interfacial tension. On the other hand, soluble OM will compete with viruses for favorable sorption sites and thus hinder the sorption.

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CHAPTER 2.1

Wong, K., and I. Xagoraraki, I. (2010) Quantitative PCR assays to survey the bovine adenovirus levels in environmental samples. Journal of Applied Microbiology. 109:605-612.

QUANTITATIVE PCR ASSAYS TO SURVEY THE BOVINE ADENOVIRUS LEVELS IN ENVIRONMENTAL SAMPLES

Abstract

Aims: Previous studies suggested Bovine Adenoviruses (BAdV) could be used as cattle fecal indicators. The main aim of this study was to survey the levels of BAdV in environmental samples by quantitative polymerase chain reaction (qPCR).

Methods and Results: Two qPCR assays were developed to identify and quantify BAdVs in environmental samples. BAdVs were detected in all dairy manure and in most cases the sample concentrations were around10^3 to 10^4 copies/ml. Farm tile drainage samples were also detected but the concentrations were about 1 to 3 $\log_{10}$ lower than the BAdV concentrations in the manure samples. The genome equivalent copies (GEC) levels of BAdV and the phage forming unit (PFU) levels of somatic phage in manure samples were comparable. Four out of twenty individual cattle feces were positive with concentrations similar to that found in the manure samples. Sequencing results confirmed the presence of BAdV in the environmental samples and phylogenetic analysis indicated that BAdV 2 and 4 were the most prevalent serotypes in all the manure samples tested. The qPCR assays developed in this study showed higher sensitivity in detecting BAdV 1 and 2 than the previous published nested assay.

Conclusion: The high levels of BAdV in the environmental samples may suggest it could be used for bovine fecal indicator. The significant levels of BAdV in the drainage

samples may indicate the potential of surface water pollution by the manure applied to farm fields.

Significance and Impact of the Study: This is the first study that reports the quantitative level of BAdV in environmental samples. These results could be useful when it comes to determining whether BAdV could be utilized as a bovine fecal indicator.

Keywords: Bovine adenoviruses, fecal indicator, quantitative PCR

Introduction

Animal manure is often land applied without prior treatment and there is a growing concern regarding transmission of pathogens by land application due to the increase of concentrated animal feeding operations (CAFOs). According to USEPA (2006) fecal contamination from livestock manure handling and storage facilities is one of the most prevalent sources of groundwater microbiological pollution. A recent outbreak in Canada indicates that the potential of human infections caused by zoonotic pathogens is real. More than 2,300 people in the town of Walkerton, Ontario, Canada suffered gastrointestinal illness where seven people perished when the town's shallow water supply was contaminated by manure pathogens from a nearby farm after more than five inches of rain fell over a five day period in May, 2000 (Hrudey and Hrudey 2004).

Bovine adenoviruses (BAdV) and enteroviruses (BEV) were suggested to be suitable agents for animal fecal indication since they were reported to be environmentally stable (Pell 1997), and have been detected in surface waters near farms (Jimenez-Clavero *et al.* 2005; Hundesa *et al.* 2006). Currently, the serotypes of BAdV reported in literature are divided into two subgroups. Subgroup 1 includes BAdV 1, 2, 3, and 10, which belong to *Mastadenovirus*, and subgroup 2 includes BAdV 4-8, which belongs to *Atadenovirus*. de Motes *et al.* 2004 developed a nested-PCR assay to detect BAdV in fecal samples. However, no quantitative results of BAdV in environmental samples have yet to be reported yet. According to Yates (2007), one indicator characteristic is higher occurrence than the pathogens. Quantitative results of BAdV in environmental samples may prove to be useful in determining whether BAdV would be an ideal indicator for bovine fecal contamination.

The main objective of this study was to survey the levels of BAdV in cattle manure and feces. Also, the levels of BAdV in drainage samples after heavy runoff were investigated to determine the potential of surface water pollution by drainage from the manure applied farm fields. The occurrence and levels of BAdV in manure, feces and drainage was investigated by using two qPCR assays developed for this study. Somatic phage concentration in manure samples were measured and compared with BAdV concentration. Randomly selected positive farm samples were sequenced to confirm the results. Also, phylogenetic trees were generated to compare sequence homologies. Finally, the sensitivity of previously published nested PCR assay and qPCR assays was compared.

Materials and Methods

Environmental sampling. Thirteen dairy manure samples from Green Meadow Farms (Elsie, MI) were obtained from April to August 2007. Samples were collected in 25 ml sterilized polyethylene disposal tubes. Once the samples were collected, they were placed in an ice-chest, transferred to the laboratory and kept in a –80^{o}C freezer until nucleic acid extraction.

Manure and drainage samples from Baker Farm (Clayton, MI) were collected during June and July 2008. Bovine manure was applied on the Baker Farm, and prior to this study, subsurface tile drainage had been installed to remove excess water from rain events. Three manure samples were collected when manure was first applied on the farm. Two drainage sampling events were conducted after heavy rainfall. 500 ml of water samples were collected and concentrated down to around 150 µl by the method described

in Haramoto *et al.* (2005) except Amicon 100K concentrator (Millipore, Billerica MA) was used to concentrate the NaOH eluent instead of Centriprep YM-50.

Twenty individual cattle feces samples were collected and tested. All of the fecal samples were collected from two different farms around East Lansing. These samples were collected during the months of March, October and November 2009.

BAdV qPCR assay description. The qPCR assays developed in this study were targeting the hexon gene of BAdV. A taqman assay (BAV4-8) was developed for targeting subgroup 2 (BAdV serotype 4-8) and a duplex fluorescence resonance energy transfer (FRET) qPCR assay (BAV1-2) was developed to detect two serotypes in subgroup 1 (BAdV 1 and 2). The nucleotide sequences of primers and probes for both real time PCR assays are summarized in Table 2.1.1.

In the BAdV4-8 taqman assay, each PCR reaction mix (total final volume of 20 µL) included 4 µL of 5X LightCycler TaqMan Master Mix, 1.0 µL of each 10 µM BAV48F and BAV48R primer (each final concentration = 500 nM), 0.5 µL of 10 µM BAV48P TaqMan probe (final conc. = 250 nM), 8.5 µL of PCR-grade water, and 5 µL of DNA sample or standard. The real-time PCR running program (all thermocycles were performed at a temperature transition rate of 20°C/s) was 95°C for 15 min; followed by 45 cycles at 95°C for 10 sec, 54°C for 30 sec, 72°C for 15 sec, and finally 30 sec at 40°C. The fluorescent signal was detected after each annealing cycle.

For the BAdV1-2 duplex-FRET, each 20-µL PCR reaction mix contained 2 µL of 10X LightCycler FastStart DNA Master HybProbe, 1 µL of 10 µM BAV1F, BAV2F, BAV1R, and BAV2R (each final conc. = 500 nM), 0.4 µL of 10 µM BAV1Pa (final conc. = 200 nM), 0.25 µL of 10 µM BAV2Pa (final conc. = 125 nM), 1 µL of 4 µM

BAV1Pb and BAV2Pb (each final conc. = 200 nM), 2.2 µL of 25 mM $MgCl_2$ (final conc. = 3.75 mM), 6.15 µL of PCR-grade water, and 5 µL of DNA sample or standard. The real-time PCR reaction program (all thermocycles were performed at a temperature transition rate of 20°C/s) was 10 min at 95° C; followed by 45 cycles at 95°C for 10 sec, 55°C for 15 sec, 72°C for 12 sec, fluorescence read; and finally 30 sec at 40°C. The fluorescent signal was detected after each extension cycle. Both real-time PCR assays were performed in a Roche LightCycler® 1.5 Instrument (Roche Applied Sciences, Indianapolis, IN).

Preparation of qPCR standards. For the taqman and duplex-FRET assays, a section of the hexon gene was PCR-amplified using primer sets specific for BAdV 1, 2 and 4 (Table 2.1.1). The amplicons were subsequently cloned into plasmid vector (i.e., pCR®4-TOPO®) based on the one-shot chemical transformation described in the manufacturer's instructions (TOPO TA Cloning® Kit for Sequencing, Invitrogen, Carlsbad, CA). Plasmid DNA carrying the cloned BAdV hexon gene was purified using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI) and quantified by Nanodrop to serve as stock genomic equivalent copies (GEC). Stock GEC was diluted to a desired range and used for creating standard curves.

Virus and nucleic acid extraction. Five BAdVs (type 1, 2, 4, 7 and 8) and seven HuAdVs (type 4, 6, 21, 31, 36, 40, 41) were obtained from American Type Culture Collection (ATCC). Nucleic acids of ATCC viruses and viruses in water samples were extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) based on the spin protocol listed in the manufacturer's handbook. For fecal samples, a stool extraction

kit (Qiagen, Valencia, CA) was used for DNA extraction. After extraction, all DNA samples were stored in a -20 ^{o}C freezer prior to PCR analysis.

Effect of environmental matrix on the sensitivity of qPCR assays. In order to determine how the environmental matrix would affect the sensitivity of the qPCR assays on the detection of BAdV, four different levels of standard solutions (plasmid DNA carrying the cloned BAdV hexon gene) were spiked into the negative bovine feces extract. After the spiking, it resulted in four different plasmid levels per PCR reaction (10, 25, 100 and 1000 copies). All reactions were run in triplicate and the sensitivity of each assay was determined by the consistent fluorescence signals, meaning all triplicate runs had positive signals.

Sequencing. Samples tested positive by the BAV4-8 taqman assay were amplified by a semi-nested PCR assay for sequencing, which targeted the hexon gene. The primer sequences are illustrated in Table 2.1.1. The PCR program was 95°C for 4 min; followed by 35 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and finally 7 mins at 72°C. The first and second round PCR programs were identical. The amplicon size is 30l bps. For BAdV1 and 2 positive samples, 5 μl of the original samples were amplified by the PCR program described above with the same forward and reverse primers as used in the qPCR assay. The PCR products were then analyzed by agarose gel electrophoresis to observe the target band before sequencing. If no target band was observed, 5 μl of the reaction mixture was used as a template for another 35 cycles of amplification. After observing the target band, the reaction mix was sent out for sequencing. All PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) prior to sequencing. Sequencing was performed by the Research

Technology Support Facility (RTSF) at Michigan State University. The sequencing results were blasted using the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/).

To study the diversity of subgroup 2 BAdVs identified in this study, the hexon gene from BAdV type 1, 2, 4, 5 6, 7, 8 and other reference adenovirus strains from ovine (OAdV 5), porcine (PAdV 3) and Human (HuAdV 40) obtained from the NCBI GenBank and nucleotide sequences from environmental samples were aligned using CLUSTAL X software. The phylogenetic trees were analyzed by MEGA 4.0 (www.megasoftware.net).

Comparison between qPCR Assay and previous published nested assay. The sensitivity of qPCR and nested PCR assays targeting the hexon gene developed by de Motes *et al.* (2004) were compared. ATCC strain of BAdV type 1, 2, 4, 7 and 8 were serially diluted from 10^4 to 10^0 copies/reaction levels. The qPCR and published nested PCR assays were run on five 10-fold diluted pure cultures to compare their sensitivities.

Results

The sensitivity and specificity of the qPCR assay. Figure 2.1.1 shows the standard curves of the taqman and duplex FRET assay developed in this study. The error bars represent the standard deviation of triplicate measurements. The slopes, regression coefficients and efficiencies of each assay are also illustrated in Figure 2.1.1. Both BAV4-8 and BAV2 assays showed a linear range from 10^1 to 10^7 genomic copies (GEC) per reaction. The BAV1 assay's linear range is from 2.5×10^1 to 10^7 GEC. The sensitivity of the assays are comparable with the bovine enteroviruses and human adenovirus assays

described in previous studies, where 11.6 RNA copies/reaction detection limit was reported by Jimenez-Clavero *et al.* (2005), 15 GEC/reaction by Heim *et al.* (2003), and 10 GEC/ reaction by He and Jiang (2005).

The sensitivity of BAV4-8 and BAV1 was not affected by the fecal matrix since BAV4-8 and BAV1 was able to detect down to 10 and 25 copies per reaction in all three triplicate runs, respectively. The sensitivity of BAV2 was affected slightly by the fecal matrix; it was able to detect down to 25 copies per reaction of all three triplicate runs. Only one run had a positive signal with 10 copies per reaction.

Five BAdV and seven HuAdV from ATCC were tested to determine the specificity of the qPCR assays. The concentration of viral genome tested was at least10^6 copies/reaction, and each specificity test was run in triplicate. The taqman assay was consistently able to amplify BAdV type 4, 7, 8 but neither BAdV 1 or 2. BAV1 and BAV2 did not amplify any other BAdVs except BAdV 1 and BAdV 2, respectively. Neither the taqman nor FRET duplex assay amplified any HuAdV. In order to test whether fecal material would be problematic to the reactions, such as unspecific binding, six swine fecal samples were tested with all three assays and results showed none of the assays would cross-react with the fecal material since all reactions had negative signals.

Occurrence of BAdV in environmental samples. Manure samples from two farms were tested with both qPCR assays (Table 2.1.2). All manure samples from Green Meadow Farm and Baker Farm were tested positive by the BAV4-8 assay. Only manure samples from Green Meadow Farm were tested positive for BAdV 2 (9/13) and only manure samples from Baker Farm were tested positive by BAdV 1 (3/3). BAdVs were present in both drainage samples from the Baker farm. Four cattle fecal samples tested

positive by the BAV4-8 (2/20) and BAV2 (2/20) assays. The lower occurrence of BAdV in fecal samples is most likely due to the fecal samples being collected from healthy cows, which were not infected by BAdV.

In both farm manure samples, the levels of BAdV measured by the BAV4-8 assay were between 10^3 to 10^4 copies/ml except for one sample from the Baker Farm, which was at 10^2 copies/ml level. The levels of BAdV 2 positive samples from Green Meadow Farm and the levels of BAdV 1 positive samples from Baker Farm were around 10^3 copies/ml. All of the manure samples were also analyzed for the occurrence of somatic coliphage by the EPA method 1602 (USEPA, 2006) and the mean levels of coliphage were at 10^3 pfu/ml. The comparison between the BAdV and coliphage concentrations in farm manure samples was illustrated in Figure 2.1.2. The results showed that the copy levels of BAdV and PFU levels of coliphage are comparable.

The total BAdV concentration (sum of BAdV detected by both qPCR assays) in the Baker Farm manure and drainage were compared. Results showed the BAdV concentration in manure was about 2 logs higher than the concentrations in the drainage. Higher concentrations in the manure were expected. Significant BAdV levels in the drainage samples (10^2 copies/ml) could indicate the potential of surface water pollution by drainage from manure applied farm fields.

The concentration of BAdV in positive feces samples was similar to the manure samples at 10^3 to 10^4 copies/gram of feces.

Sequence and phylogenetic analysis of BAdV hexon gene in environmental samples. Four randomly selected Meadow farm samples and all of the samples from Baker farm that tested positively by BAV1-2 were sequenced and blasted. All of the sequencing results were based on the hexon gene. Sequence homologies between the positive isolates identified by BAV1 and reference BAdV-1 (NC-006324), as well as, between the positive isolates identified by BAV2 and reference BAdV-2 (DQ630762) ranged from 94 to 100% (data not shown).

Eleven selected samples tested positive by BAV4-8 (8 from Meadow Farm, 3 from Baker Farm) were amplified by the semi-nested PCR assay described in the methods section. The 301 bp PCR products were also sequenced and compared with other adenovirus sequences available in GeneBank. The phylogenetic analysis (Figure 2.1.3) showed that most of the BAdV strains identified from Meadow Farm were closely related to BAdV4 (accession no. NC_002685). BAV4-8-MF7 was closely related to BAdV 8. One Baker farm sample was related to BAdV 4 and the other two samples were related to BAdV 5 and BAdV 8.

Comparison between qPCR Assay and previous published nested assay. Table 2.1.3 showed qPCR assays developed in this study have a greater sensitivity in detecting BAdV 1 and 2. The nested assays failed to detect BAdV 1 and were only able to detect BAdV 2 at a level equal to or above10^4 copies. However, BAV1-2 was able to detect BAdV 1 and 2 down to 10^1 copy level. The sensitivity of both qPCR and nested PCR assays on BAdV 4, 7 and 8 (subgroup 2) were comparable. It was also notice of that there were multiple bands appearing when the published nested assays were run (Figure 2.1.4). According to the author, the target band was supposed to be 430 bps, but two

other distinct bands between 400 and 500 bps and some vague bands around 550 and 350 bps were observed. The nested PCR assay developed in this study (BAV4-8n) was included in Figure 2.1.4 for comparison purpose and there is only one distinct band (301 bps).

Discussion

There are very limited studies on the presence of BAdV in environmental samples. Maluquer de Motes *et al.* (2004) detected the presence of BAdV in three out of four bovine fecal samples with a nested-PCR assay and Hundesa *et al.* (2006) found one out of twenty-two slaughterhouse sewage samples positive with BAdV. In this study, the presence of BAdV in all of the manure samples and tile drainage samples was detected, but only four individual cattle fecal samples were tested positive.

Bacteriophage is the most commonly used viral indicator for water monitoring; however, it may not be suitable to differentiate between human and animal fecal source. With the recent advances in molecular technology, many enteric viruses can be detected without extensive labor effort. The high abundance of BAdV in the manure samples proposed that BAdV could be used as an indicator for cattle fecal materials. Also, adenoviruses in general are highly UV resistant, allowing for high survival rates in natural water environments.

Subsurface draining systems (drain tiles) are installed to remove excess water and protect cropland and groundwater from contaminates in manure application sites. The significant levels of BAdV in drainage samples from this study show that drain tile systems could facilitate the transport of pathogens from land applied manure to surface

water systems during rain events, and in this case, viruses could be a more suitable agent for tracking fecal contamination in water samples since they could leach through soil and contaminate the groundwater whereas bacteria are more likely to retain in the overlying soil. Jimenez-Clavero *et al.* (2003) investigated the occurrence of swine teschovirus (PTV) using qPCR in an open duct from a slurry tank and in stream water samples. The samples had a similar nature to this study's manure and drainage samples. The levels of PTV in the open duct and stream samples ranged from 10^4 to 10^5 and 10^2 to 10^3 RNA copies/ ml, respectively, which is comparable to the findings in this study.

Based on the qPCR data and the sequencing analysis, the most prevalent BAdV in the environmental samples tested in this study were serotype 2 and 4. Interestingly, most of the BAdVs identified in previous studies were also related to serotype 2 (2/6 samples) and 4 (3/6 samples) (de Motes *et al.* 2004). The only sample tested BAdV positive in Hundesa *et al.* (2006) was 97% similar to BAdV type 6. None of the samples in this study were identified as closely related to this serotype. These results may indicate a high prevalence of BAdV 2 and 4 in the environment; however, more studies are needed in order to fully conclude this hypothesis.

Beside qPCR has higher sensitivity in detecting these two serotypes of BAdV over the published nested assay, it also shows high numbers of degenerate bases in PCR assay would greatly diminish the quality of the assay. Multiple bands of the PCR product from the published assay (Figure 2.1.4) are most likely due to the high number of degenerate bases in the primers (at least 4 degenerate bases in each primer) since the chance of non-specific binding increases as more degenerated bases were used in the primer set.

Conclusions

In conclusion, the levels of BAdV in manure, feces and drainage water were quantified by two qPCR assays. The high levels of BAdV in manure indicate it could be considered for use as a bovine fecal indicator. BAdV types 2 and 4 were the most dominant strains in the samples tested in this study but more studies are needed to determine the prevalence of different BAdV serotypes in the environment.

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Tables and Figures

Table 2.1.1. Nucleotide sequences of primers and probes for taqman and duplex FRET PCR assays.

Type of Assay	Serotype Specificity	Name (position)	Sequence (5' to 3')	Amplicon Size (bps)	Tm
Taqman	4,5,6,7 8	BAV4-8F (44-67)	CRAGGGAATAYYTGTCTGAAAATC	87	60.3
		BAV4-8R (105-130)	AAGGATCTCTAAATTTYTCTCCAAGA		59.1
		BAV4-8P (76-102)	FAM-TTCATCWCTGCCACWCAAAGCTTTTTT-BBQ1		61.7
Semi-nested PCR*	4,5,6,7 8	BAV4-8nF1 (20-42)	TYTTYCACATTGCGGGTAGAAAT	301	59.2
		BAV4-8nF2 (43-67)	GCRAGGGAATAYYTGTCTGAAAATC		62.1
		BAV4-8nR (320-343)	CWGTTCCTCCATAWGGYTTAAAAG		60.3
Duplex FRET	1	BAV1F (538-557)	GGAGAGGAATCTTGGTTGTC	158	59.9
		BAV1R (670-695)	ACTTGTATCAAATTGTTGTTAAGAGT		59.9
		BAV1Pa (617-638)	CCCTGCCATGTTACGGGTCTTA-Fluorescein		65.2
		BAV1Pb (641-664)	LC Red 640-CCGCTCCTACGAACATTGAGGGAG-Phosphate		77.7
	2	BAV2F (2227-2250)	GGTTACAAAGATAGGACATATTCG	188	59.6

Table 2.1.1 cont'd

		BAV2R (2369-2414)	GGCCAATTAGCTGGGTAAG		60.2
		BAV2Pa (2336-2363)	AGCACAATAATTCTGGCTTTACTGCTTT-Fluorescein		65.4
		BAV2Pb (2366-2390)	LC Red 705-CTAACGCTTCCCTGCCTAGAGAAGG-Phosphate		67.4
Regular PCR **	1	BAV1cF (277-295)	GTACTTGACATGGCGAGCA	523	60.1
		BAV1cR (780-799)	CTGGCACCTTGTACACTAAA		59.9
	2	BAV2cF (2104-2122)	ATTAAGCGAGCAGTAGACG	400	60.1
		BAV2cR (2486-2503)	TAGAGAATGGGATGCGCC		60.4
	4	BAV4cF (16-35)	GAATTTTTTCACATTGCGGG	505	59.8
		BAV4cR (497-520)	TTCTACCTTCTTGAGGATTAGGTT		60.1

* Semi-nested assay is used for sequencing the environmental samples.
** PCR assays are used for standard curve generation by cloning the target nucleotide sequences on the plasmid.
The position for BAV4-8 taqman and semi-nested assay is based on the hexon region of BAdV4 strain THT/62 (GeneBank accession no. AF036092).
The position for BAV1 assay is based on the hexon region of BAdV1 strain 10 (GeneBank accession no. DQ630761).
The position for BAV2 assay is based on the hexon region of BAdV2 (GenBank accession no. DQ630762).

Table 2.1.2. The occurrence of BAdV in environmental samples.

	Assay Type		
Environmental Samples	BAV1	BAV2	BAV4-8
Meadow Farm Manure	0/13	9/13	13/13
Baker Farm Manure	3/3	0/3	3/3
Drainage	2/2	0/2	2/2
Cattle Feces	0/20	2/20	2/20

Table 2.1.3. Comparison the sensitivity between qPCR assay and previously published nested assay.

copies/rxn	BAV1	Nested Assay	BAV2	Nested Assay	BAV4-8	Nested Assay	BAV4-8	Nested Assay	BAV4-8	Nested Assay
	BAdV-1		BAdV-2		BAdV-4		BAdV-7		BAdV-8	
10^4	+	-	+	+	+	+	+	+	+	+
10^3	+	-	+	-	+	+	+	+	+	+
10^2	+	-	+	-	+	+	+	+	+	+
10^1	+	-	+	-	+	+	+	+	+	+
10^0	-	-	-	-	-	-	-	-	-	-

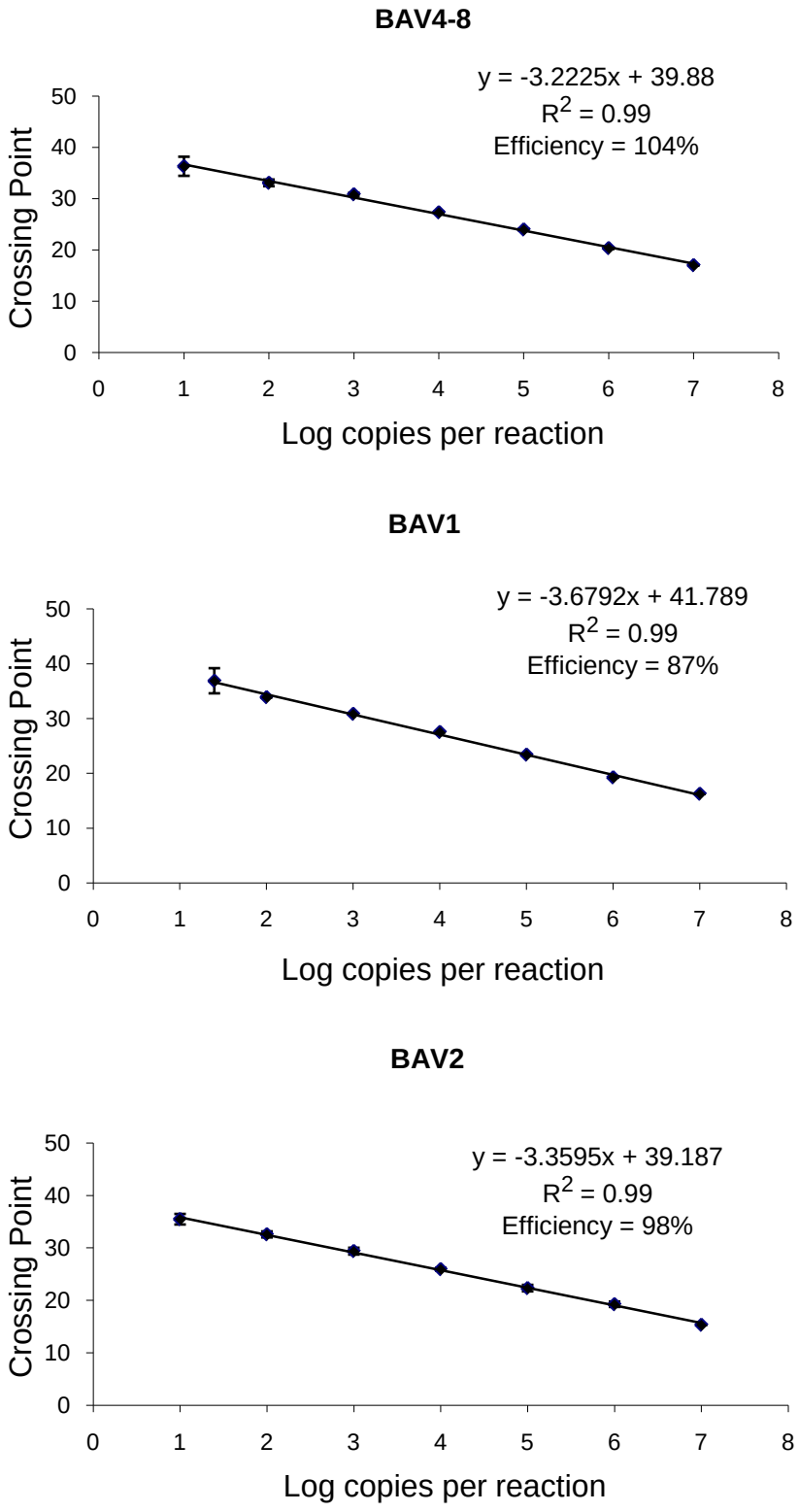

Figure 2.1.1. Standard Curves for BAV4-8, BAV1, and BAV2.

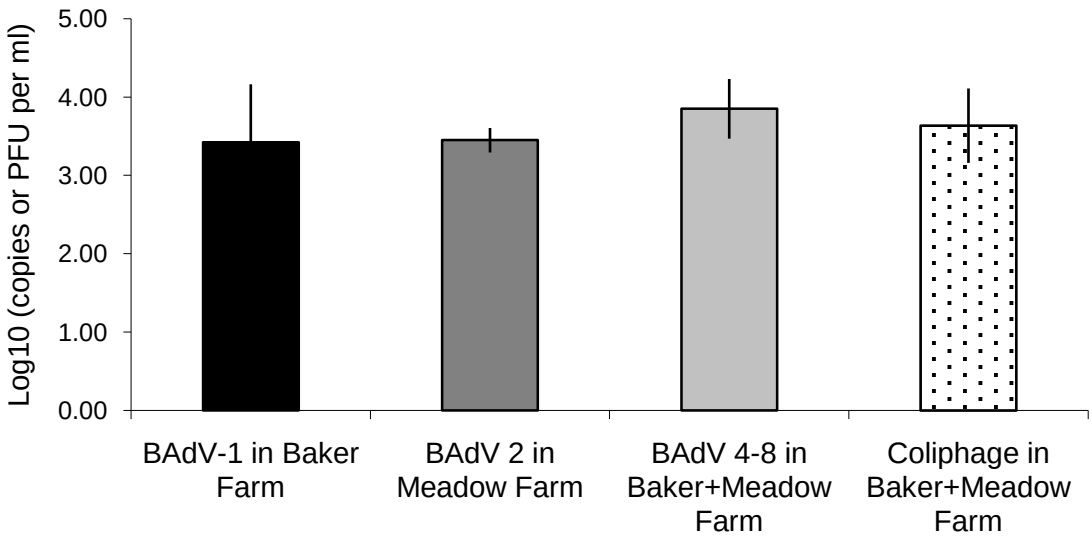

Figure 2.1.2. Comparison between the BAdV and coliphage concentrations in farm manure samples.

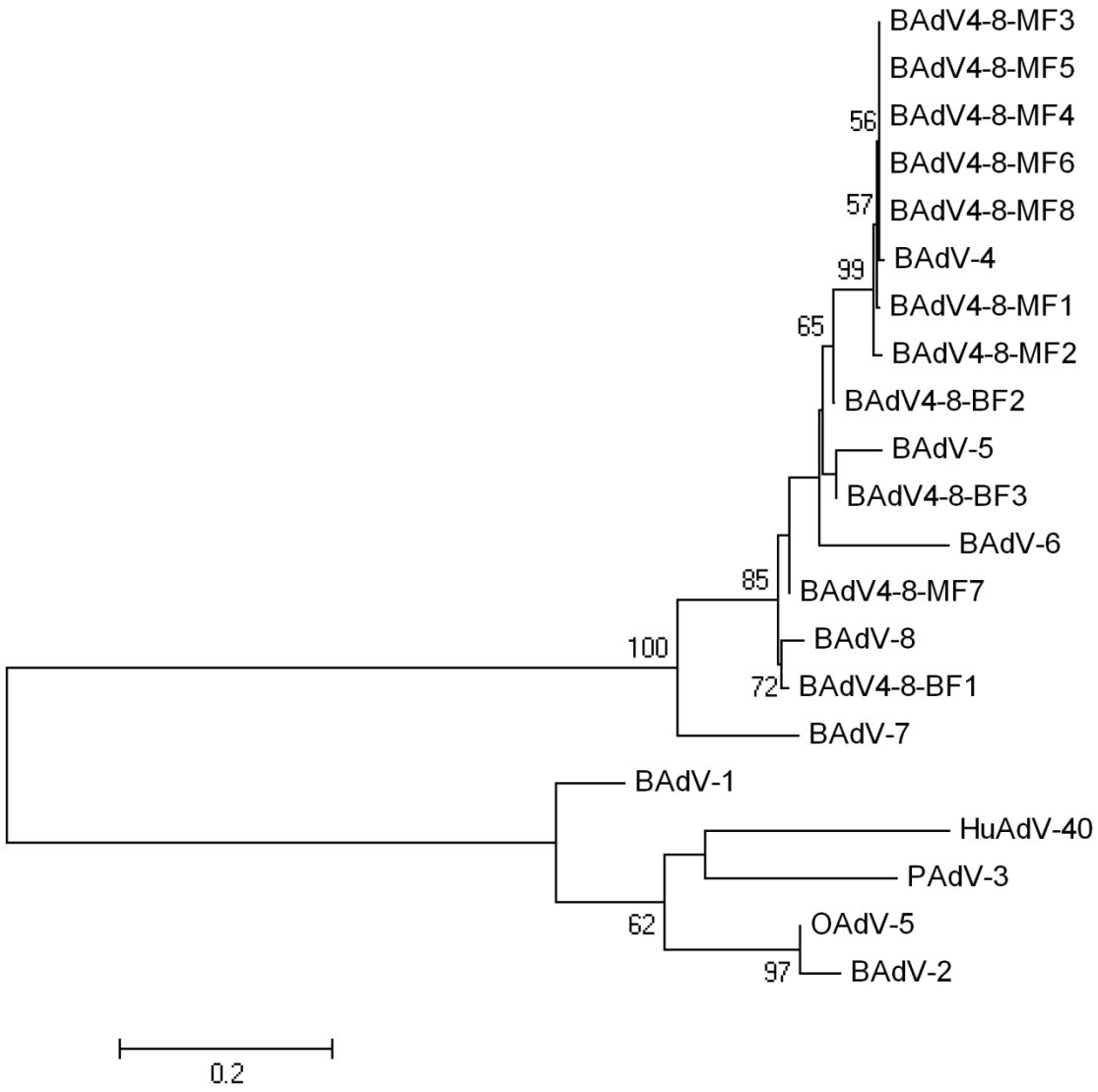

Figure 2.1.3. Neighbor Joining tree of group 2 BAdV identified in Meadow Farm (BAdV4-8-MF1 to BAdV4-8-MF8), Baker Farm (BAdV4-8-BF1 to BAdV4-8-BF3). Hexon gene from bovine adenoviruses, other animal adenoviruses, and human adenoviruses (see methods for detail descriptions) were included in the tree. The numbers on the tree nodes represent 500 replicates of bootstrap values higher than 50% and the scale bar represents maximum likelihood distance.

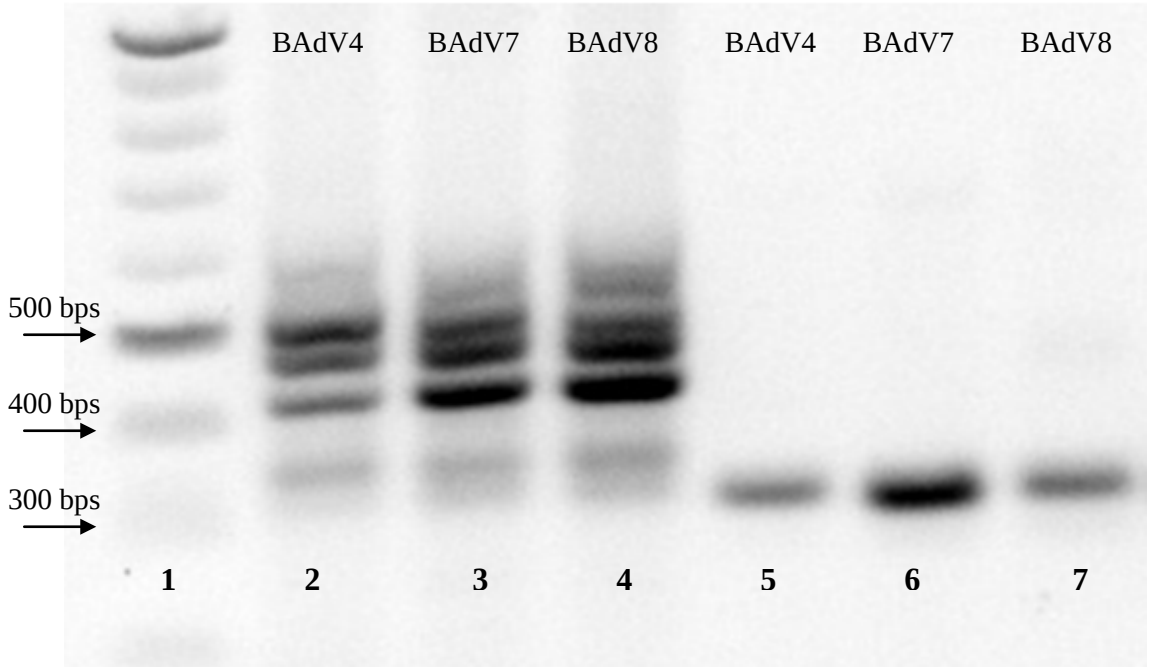

Figure 2.1.4. Image of agarose gel of nested PCR products from previous published assay and assay developed in this study. Lane 1 (DNA ladder); Lane 2 to 4 (published nested PCR product of ATCC BAdVs); Lane 5 to 7 (BAV4-8n product of ATCC BAdVs).

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CHAPTER 2.2

EVALUATION OF BOVINE ADENOVIRUS AND POLYOMAVIRUS AS BOVINE FAECAL INDICATORS

Submitted for consideration to Journal of Applied Microbiology (Wiley-Blackwell Publications)

Abstract

Aim: Bovine adenovirus (BAdV) and polyomavirus (BPyV) have been proposed for bovine faecal indication. However, their relative prevalence and concentration in manure and bovine faeces is still unclear. This study evaluated and compared the occurrences and concentrations of BPyV and BAdV in manure and faecal samples. The comparability between the concentration/prevalence of these viruses and bacterial faecal indicators (cow-associated *Bacteroidetes*, *Escherichia coli* (*E. coli*) and enterococci) in manure and faecal samples was also determined.

Methods and Results: A total of 26 dairy manure samples, 5 beef manure samples and 18 individual dairy cow faeces were tested. The results showed the mean concentration of BAdV in all of dairy manure samples was at least 1 log lower than BPyV ($p \leq 0.005$). All of the dairy manure samples tested positive with BPyV but not BAdV. However, results indicate BAdV could be more concentrated and prevalent than BPyV in beef manure. After combining all of the dairy manure measurements, bacterial indicators had 0.3-0.7 logs ($p \leq 0.05$) and 1.8-2.2 logs ($p \leq 0.005$) higher concentration than BPyV and BAdV, respectively. Only four and one out of eighteen faecal samples tested positive with BAdV and BPyV, respectively.

Conclusion: This study showed that BPyV were more concentrated and prevalent than BAdV in manure samples, which indicates BPyV could be a better indicator than BAdV for bovine faecal pollution at manure application sites. Also, the concentration of BPyV in manure was more comparable than BAdV to the concentration of bacterial faecal indicators. Low occurrence of these viruses in faeces samples indicates that the major source of these viruses could be from urine.

Significance and Impact of the Study: The results of this study provide a better understanding of comparability between the concentrations of BPyV and BAdV, and between the concentrations of BPyV/BAdV and bacterial faecal indicator in manure and faecal samples.

Keyword: bovine polyomavirus, bovine adenovirus, quantitative PCR, faecal indicator

Introduction

Faecal coliforms, *E. coli*, enterococci, and *Clostridium perfringens* (*C. perfringes*) have been the gold standard for faecal pollution indication. Yates (2007) summarized the criteria for ideal faecal indicators: high abundance in faecal materials, simple analytical procedure and high survival rates are some of the factors for choosing these traditional indicators for faecal pollution indication. However, it is difficult to differentiate human and animal faecal source by using the traditional indicator culture methods. With the recent advancement of molecular science and technology, many faecal associated pathogens could be identified and quantified directly.

Bovine adenovirus (BAdV) and polyomavirus (BPyV) have been proposed for bovine faecal indication (de Motes *et al.* 2004; Hundesa *et al.* 2006; Hundesa *et al.* 2010; Wong and Xagoraraki 2010). One of the advantages of using these viruses for faecal indication is that they are generally more host specific than bacteria and do not grow in the environment. Also, study showed both human adenovirus and polyomavirus was highly stabilized in urban sewage (Boill-Mas *et al.* 2006). Thus, it is reasonable to assume that BAdV and BPyV are also stable in the environment. We previously reported the concentrations of BAdV in environmental samples (Wong and Xagoraraki 2010) and the concentrations of BPyV have only been reported in one recent study (Hundesa *et al.* 2010). It would be useful to know which of these two viruses have the higher prevalence and concentration since the ideal faecal indicator should have high abundance in faecal materials. Also, the main disadvantage of using enteric virus for microbial source tracking is their low concentration in the environment (Scott *et al*. 2002), which may result in levels of virus below the detection limit of the assay. Therefore, virus with

higher levels in the faecal source would be likely to have higher chance being detected in water environment. However, the quantitative data of these two viruses are still limited and it is difficult to determine and compare the results from two different previous studies (Hundesa *et al.* 2010; Wong and Xagoraraki 2010) since the samples and sample processing methods varied.

Therefore, the main objective of this work was to evaluate BAdV and BPyV as bovine faecal indicator focusing on the quantitative and prevalence perspectives. The occurrences and concentrations of BAdV, BPyV as well as other bacterial indicators (*Bacteroidetes*, *E. coli* and enterococci) in manure and faecal samples (individual cow faeces) were determined by qPCR assays and the results were compared by statistical analysis to determine which of these viruses is more prevalent and concentrated in manure and faecal samples. Sequence analysis of BPyV from different environmental isolates was performed and the results were compared with previous BAdV sequencing results (Wong and Xagoraraki 2010) to determine which virus is more suitable for bovine faecal indication by using the molecular approach.

Material and Methods

Manure and faeces sampling. A total of 26 dairy manure samples were collected from Green Meadow Farm (Elsie, MI), Baker Farm (Clayton, MI), Michigan State University (MSU) dairy lagoon (East Lansing, MI) and a total of 5 beef manure samples were collected from the MSU beef lagoon (East Lansing, MI). Eighteen individual diary cow faeces were collected. The manure samples collected from Green Meadow and Baker Farm and individual cow faeces were the same samples described in our previous

study (Wong and Xagoraraki 2010) but not all Green Meadow Farm and cow faeces samples from the previous study were analyzed due to insufficient quantities of some of those samples. Both MSU dairy and beef manure samples were collected during May 2009. All of the samples were collected in 25 ml sterilized polyethylene disposal tubes. Once the samples were collected, they were placed in an ice-chest and transferred to the laboratory immediately and stored in a -80 °C freezer prior to nucleic acid extraction.

Wastewater samples for BPyV qPCR specificity testing. Sixteen domestic raw sewage samples (sewage prior to the entrance into the the primary clarifiers) were collected from the municipal wastewater treatment plant (WWTP) at East Lansing (n=2), Imlay City (n=2), Lansing (n=2), Romeo (n=2) and Traverse City (n=8) to test whether the BPyV qPCR assay would react with human faecal material. 1MDS filter was used and the filtering volume was between 20 to 25 L. Virus elution and further concentration was carried out by organic flocculation as described in the US EPA Virus Monitoring Protocol for the Information Collection Requirements Rule (ICR). Briefly, the filters were backwashed twice with 0.5 L of beef extract solution (1.5% w/v beef extract, 0.05 M glycine, pH 9.0–9.5) to elute absorbed viral particles. Subsequently, the eluates were flocculated by adding ferric chloride to a final concentration of 2.5 mM and by lowering the solution pH to 3.5. The flocs were collected by centrifugation at 2,500×g for 15 min and re-suspended in 30 mL of 0.15 M sodium phosphate (final pH 9.0). The re-dissolved precipitates were centrifuged at 10,000×g for 10 min. Finally, the supernatants (approx. 30 ml) were collected (pellet was discarded), neutralized (pH 7.0-7.5) with 1 M HCl, supplemented with 100 units of penicillin, 100 µg of streptomycin, and 0.25 µg of fungizone and stored in aliquots at –80 °C.

Nucleic acid extraction. *E. coli* (15597), enterococci (19433), two human polyomavirus (JCPyV (VR-1583) and BKPyV (VR-837)) were obtained from American Type Culture Collection (ATCC). *E. coli* and enterococci cultures were for generating standard curves, and JCPyV and BKPyV cultures were for testing the specificity of BPyV qPCR assay. The nucleic acids of these microorganisms were extracted by the MagNA pure automatic extraction machine (Roche) and the extraction kits used were MagNA Pure Compact Nucleic Acid Isolation Kit-Large Volume (Roche). A volume of 500 µL of the sample was extracted and the final elution volume was 100 µL. For faecal and manure samples, a stool extraction kit (Qiagen, Valencia, CA) was used for DNA extraction. A volume of 200 µL and a mass of 200 µg was extracted for each manure and faecal sample, respectively. The final elution volume was 200 µL for both types of sample. After extraction, all DNA samples were stored in a -20 °C freezer prior to qPCR analysis.

Development of BPyV qPCR assay. This qPCR assay was designed to target the VP1 gene of BPyV. The primers and probe were designed using primer express 2.0 (Applied Biosystem Inc, CA). The forward and reverse primer sequence is 5′-TGGCTTTCTGACTCAGCCAAA-3′ (BPV-F) and 5′-TCTCTTCCTGAGAGTCACAGACATG-3′ (BPV-R), respectively. The probe sequence is FAM-5′-ACCAACAGCAATTTAGAGGCCTTCCCAG-3′-TAMRA (BPV-P). The amplicon size is 79 base pairs (bp). Each qPCR reaction mix (total final volume of 20 µL) included 4 µL of 5X LightCycler TaqMan Master Mix, 1.0 µL of each 10 µM BPV-F and BPV-R primer (each final concentration = 500 nM), 0.5 µL of 10 µM BPV-P TaqMan probe (final conc. = 250 nM), 8.5 µL of PCR-grade water, and 5 µL of DNA

sample or standard. The real-time PCR running program was 95 °C for 15 min; followed by 45 cycles at 95 °C for 10 sec, 55 °C for 30 sec, 72 °C for 15 sec, and finally 30 sec at 40 °C, and all thermocycles were performed at a temperature transition rate of 20 °C/s. The fluorescent signal was detected after each annealing cycle.

To prepare the standard, a section of the VP1 gene from a bovine manure sample was PCR-amplified by the primers (VP1F and VP1R) which were previously published (Hundesa et al, 2006). The amplicon was subsequently cloned into plasmid vectors (pCR®4-TOPO®, Invitrogen). Plasmid DNA carrying the cloned BPyV VP1 gene was purified using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI) and quantified by Nanodrop™ to serve as a stock genomic equivalent copies (GEC). Stock GEC was diluted in a range from 10^7 to 10^1 copies/reaction and used for creating standard curves.

Effect of environmental matrix on the sensitivity of BPyV qPCR assays. Four different levels of standard solutions (plasmid DNA carrying the cloned BPyV VP1 gene) were spiked into the negative bovine faeces extract to determine how the environmental matrix would affect the sensitivity of the qPCR assay on the detection of BPyV. Spiking resulted in four different plasmid levels per PCR reaction (10, 25, 100 and 1000 copies). All reactions were run in triplicate and the sensitivity of BPyV assay was determined by the consistent fluorescence signals.

***E. coli*, enterococci, *Bacteroidetes* and BAdV qPCR assay.** The BAdV qPCR assays developed previously (Wong and Xagoraraki 2010) were used to quantify BAdV. The assays developed by Chen *et al.* (2006) , Haugland *et al.* (2005) and Layton *et al.* (2006) were used to quantify the *E. coli*, enterococci, and cow-associated *Bacteroidetes*,

respectively. We followed the exact reaction condition, amplification cycle, nucleotide sequence of primer/probe, and primer/probe concentration as described in the literature.

Phylogenetic analysis of BPyV. Two BPyV qPCR amplicons from each farm and lagoon were selected randomly and sent for sequencing. The amplicons were cloned into TOPO plasmid vectors. The clones were sent for sequencing at the Research Technology Support Facility (RTSF) at Michigan State University. The sequencing results were blasted using the National Center for Biotechnology Information (NCBI) website. To study the diversity of BPyV identified in this study, the nucleotide sequences of BPyV, BKPyV, JCPyV and BAdV-D obtained from the NCBI GenBank and nucleotide sequences from environmental samples were aligned using CLUSTAL X software. The phylogenetic trees were analyzed by MEGA 4.0 (www.megasoftware.net).

Data analysis. To determine significant differences, the concentrations of viruses and indicators were first transformed to $\log_{10}$ copies per ml or gram, and then analysis of variance (ANOVA) single tests were performed using Microsoft Excel™ program. A p-value of less than 0.05 indicates a significant difference.

Results

Sensitivity and specificity of BPyV qPCR assay. BPyV assay showed a linear range from 10^1 to 10^7 genomic copies (GEC) per reaction. The linear correlation coefficient (R^2), slope of the standard curve and amplification efficiency of this assay were 0.9996, -3.6891 and 90%, respectively. The amplicon was sequenced and the result showed that it is 100% identical to the complete BPyV genome sequence (accession no. D13942.1). The sensitivity of BPyV was also not affected by the faecal matrix since the

assay was able to detect down to 10 copies per reaction in negative faecal extract for all three triplicate runs.

None of the sixteen sewage samples or human polyomaviruses showed signals with the qPCR assay. Also, the primers and probe were checked with BLAST search engine and the E-values of forward primer, reverse primer and probe to bovine polyomavirus DNA, complete genome (accession no. D13942.1) was $2e^{-02}$, $1e^{-04}$, and $1e^{-06}$, respectively with no matching results from human and/or other animal polyomaviruses. Based on these results, this assay would not react with any human faecal source.

In order to test whether faecal material would be problematic to the reactions, such as unspecific binding, ten swine faecal samples were tested with the assay and results showed none of the assays would cross-react with the faecal materials since all reactions had negative signals.

The specificity of the bovine adenovirus (BAdV) assay was discussed in Wong and Xagoraraki (2010). Ten copies per qPCR reaction was the method detection limit for BAdV, *E. coli,* enterococci, and *Bacteroidetes* assay.

Occurrences and concentrations of enteric viruses and indicators in manure samples. The results showed the mean concentration of BAdV in all three sets of dairy manure samples was at least 1 log lower than BPyV ($p \leq 0.005$) (Figure 2.2.1). Also, BPyV was detected in all of the dairy manure samples but not all dairy manure samples tested positive for BAdV (22/26) (Table 2.2.1). However, the concentrations of BAdV in beef manure samples were significantly higher than the concentrations of BPyV by 1.1

log ($p \leq 0.05$) (Figure 2.2.1), and all five beef manure samples tested positive with BAdV but not with BPyV (3/5).

The concentrations of BAdV and BPyV were compared with the concentrations of *E. coli*, enterococci and cow associated *Bacteroidetes* in all of manure and faecal samples. For *E. coli*, enterococci and cow associated *Bacteroidetes*, one, four and six copies of genes are equal to one cell, respectively (Chen *et al.* 2006; Haugland *et al.* 2005; and Okabe *et al.* 2007). For BPyV and BAdV, one copy of gene is equal to one virus. The concentrations of BAdV were significantly lower than bacterial indicators in all of the manure samples ($p \leq 0.05$). However, there were no significant differences between the concentrations of BPyV and bacterial indicators in both Meadow Farm and Baker Farm samples ($P \geq 0.05$), except for enterococci which had slightly higher concentrations in the Baker Farm samples ($p \leq 0.05$). The concentrations of BPyV were 0.7 to1.3 and 1.6 to 2.7 log lower than bacterial indicators in MSU dairy and beef manure samples, respectively ($p \leq 0.005$). All three bacterial indicators were detected in all of the manure samples.

Figure 2.2.2 illustrates the measurement results of the BPyV, BAdV and indicators for all of the dairy manure samples (n=26). Results showed that the concentration of *Bacteroidetes* was 1.3-1.4 logs higher than the concentrations of *E. coli* and enterococci (≤0.005). No significant difference between the concentration of *E. coli* and enterococci was observed (>0.05). Bacterial indicators had 0.3-0.7 logs (≤0.05) and 1.8-2.2 logs (≤0.005) higher concentration than BPyV and BAdV, respectively. The concentration of BPyV was 1.4 logs higher than the concentration of BAdV (≤0.005).

Occurrences and concentrations of enteric viruses and indicators in faecal samples. All of the faecal samples tested positive with *E. coli*, enterococci and cow-associated *Bacteroidetes*. Only one faecal sample (1/18) tested positive with BPyV and the concentration was 9.8×10^4 copies per gram. As reported previously (Wong and Xagoraraki 2010), four faecal samples (4/18) tested positive with BAdV and the concentration ranged from 10^3 to 10^4 copies per gram. While the faecal samples had such a low occurrence of BPyV and BAdV, the majority of the manure samples from MSU Dairy Farm tested positive with BPyV and BAdV. The concentrations of *E. coli*, enterococci, *Bacteroidetes*, BPyV and BAdV in manure and bovine faecal samples from MSU Dairy Farm are illustrated in Figure 2.2.3. For the samples which tested negative with either BPyV or BAdV, the concentrations of BPyV and BAdV were calculated by using the method detection limit, which is 10 copies per qPCR reaction. Also, the density of manure was assumed to be one mL per gram. Interestingly, while *E. coli* and *Bacteroidetes* had at least one log reduction from faecal to manure samples, BPyV and BAdV showed a significant increase in concentration and occurrence. The concentration of enterococci remained the same between manure and faecal samples.

BPyV sequence diversity. The phylogenetic analysis (Figure 2.2.4) shows that all the dairy manure isolates are 100% identical to each other and to the complete BPyV genome sequence (accession no. D13942.1). Both of the beef manure isolates are 98.7% identical to the dairy manure isolates. All the sequences were deposited to the gene bank and the accession number of each sequence is illustrated in Figure 2.2.4.

Discussions

Previous studies have indicated BPyV could be more prevalent than BAdV (Hundesa *et al.* 2006; Wong *et al.* 2009). Hundesa *et al.* (2006) tested slaughterhouse wastewater and river water, and found only one sample was BAdV positive but twenty-two samples were BPyV positive. Wong *et al.* (2009) also found none of the membrane bioreactor (MBR) effluent samples treating bovine manure was BAdV positive but three out of eight samples were tested positive with BPyV. The quantitative results from this study showed that BPyV had higher concentrations and prevalence than BAdV in all the dairy manure samples, and these results can provide an explanation of the previous observations. However, results may also indicate BAdV could be more prevalent and concentrated than BPyV in beef manure. Since the samples tested in this study were all from State of Michigan, more studies on the manure samples from in other geographical region are needed to make a definite conclusion.

In addition to high abundance, the sequencing results also showed the high homology of BPyV genetic sequences between different sources of environmental samples and these observations were also found by other studies (Hundesa *et al.* 2006; Hundesa *et al.* 2010). The sequence analysis from our previous study showed that BAdV had high sequence diversity between different samples (Wong and Xagoraraki 2010). High homology of genetic sequences could give a significant advantage to the design of the molecular assay for BPyV compared to BAdV since we found that numbers of degenerate bases in primers/probe and multiple assays are needed to target all serotypes of BAdV from our previous study.

Based on the MSU manure and faecal sample results, high occurrence of BPyV and BAdV was observed in manure but not in faecal samples. Even though it is possible that all of faecal samples were collected from cows not infected by these viruses, we do not think this is the major cause. Manure samples are mixtures of the faeces with the presence and absence of these viruses; therefore, faecal samples would have had high occurrence and concentration in order to have such a high prevalent results in manure samples. Also, these viruses do not grow in the environment and should have some degree of decay in manure samples stored in the lagoon. Therefore, we think the more plausible explanation of these observations is that most of these viruses attributed to the manure samples originated from urine instead of faeces. This explanation agrees with the results from the recent study showing that BPyV was significantly more prevalent in urine than faecal samples (Hundesa *et al.* 2010). In that study, all of the faecal samples (n=10) tested negative with BPyV but eight out of twenty six bovine urine samples tested positive. Another study also reported human polyomavirus can be excreted by urine (McQuaig *et al.* 2009). Not many studies have reported the presence of adenovirus in urine but one study did show adenovirus was present in urine of healthy and human immunodeficiency virus-infected individuals (Echavarria *et al.* 1998). Therefore, the high concentrations of these viruses in manure were possibly attributed by the mixing of urine and faeces, which leads to high numbers of these viruses adsorbed to the faecal materials. More studies should investigate whether urine are the main source of these viruses especially for BAdV.

Conclusions

In conclusion, this study showed that BPyV had higher concentration and prevalence than BAdV in dairy manure samples. BPyV concentration in manure was also more comparable to the concentration of traditional faecal indicators. This suggests that BPyV could be a better indicator than BAdV for bovine faecal pollution. Even though the concentration of BAdV and BPyV was comparable to the concentration of bacterial indicator in some samples, screening the concentration of enteric virus at the possible source and/or using multiple bovine faecal markers (both bacterial and enteric virus) are recommended when doing the faecal pollution tracking at the field since the concentration of bacterial indicator in the manure samples collected from different locations was significantly more consistent than the concentration of BAdV and BPyV. Finally, low occurrence of these viruses in faecal samples indicates that the major source of these viruses could be from urine instead of faeces shedding.

Tables and Figures

Table 2.2.1. The quantitative levels and occurrences of BPyV and BAdV in manure samples.

Sampling Location	BPyV		BAdV	
	Fraction of positive sample	Mean concentration (log_{10} copies/ml)	Fraction of positive sample	Mean concentration (log_{10} copies/ml)
Meadow farm	8/8	6.33 ± 0.61	8/8	4.15 ± 0.25
Baker farm	3/3	6.44 ± 0.13	3/3	4.59 ± 0.32
MSU dairy lagoon	15/15	5.11 ± 0.21	11/15	4.05 ± 0.89
MSU beef lagoon	3/5	3.86 ± 0.80	5/5	4.92 ± 0.34

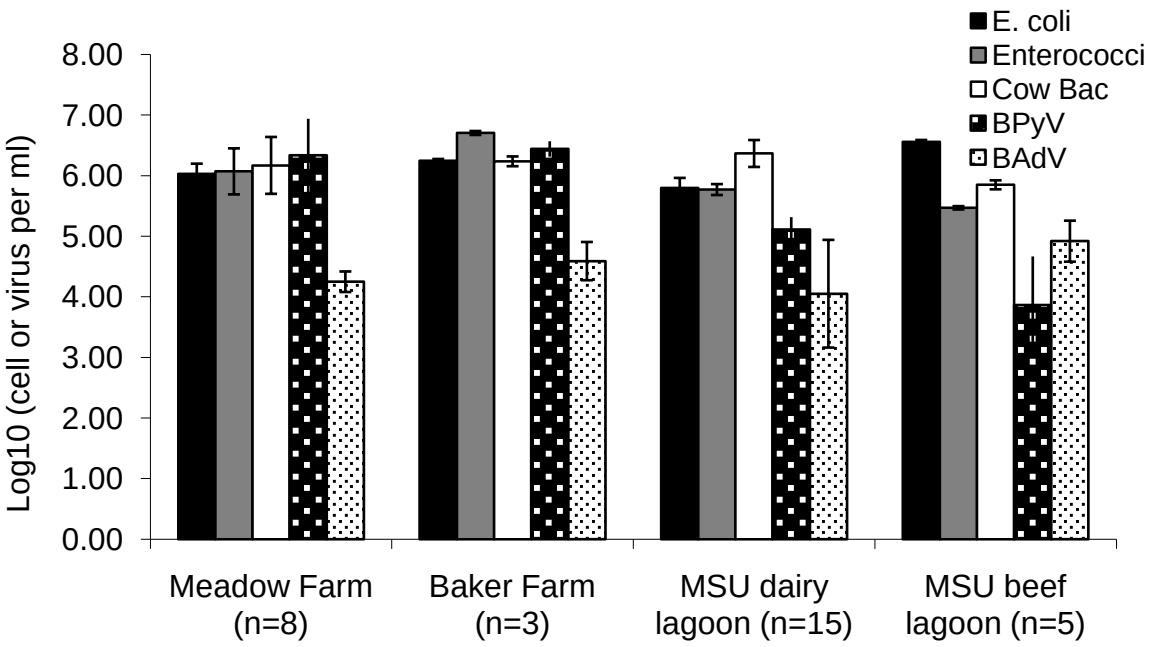

Figure 2.2.1. Comparison between the concentration of BPyV, BAdV and bacterial indicators in manure samples at different sampling locations.

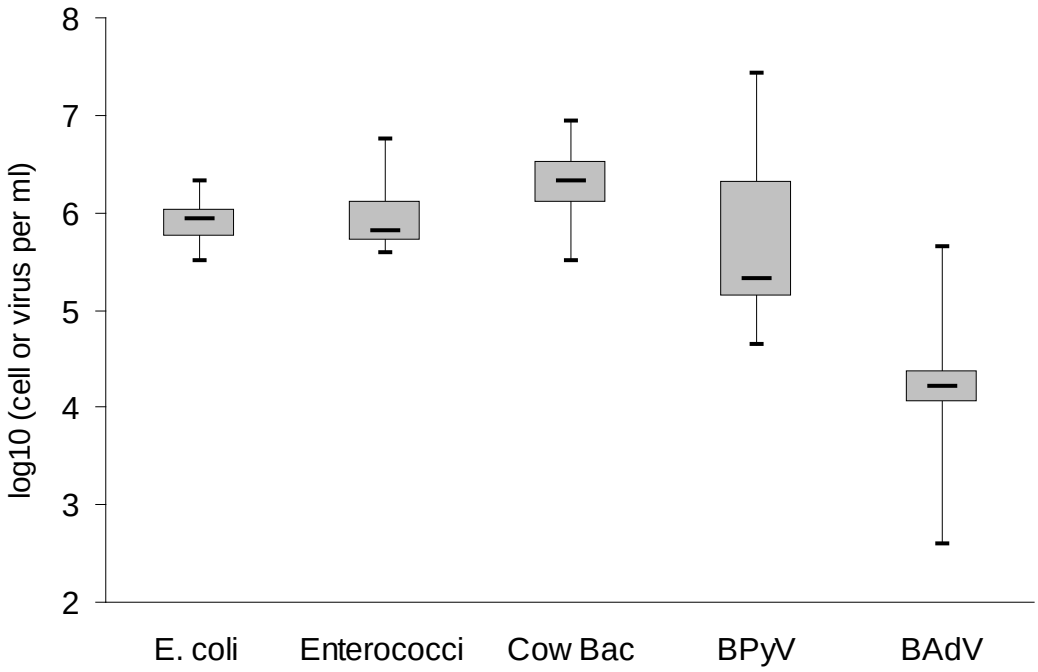

Figure 2.2.2. Box-and-whisker plots of the concentration of BPyV, BAdV and bacterial indicators in dairy manure samples (n=26). The inner box lines represent the geometric medians and the outer box lines represent the 25^{th} and 75^{th} data percentiles. The whiskers extend to minimum and maximum of the data.

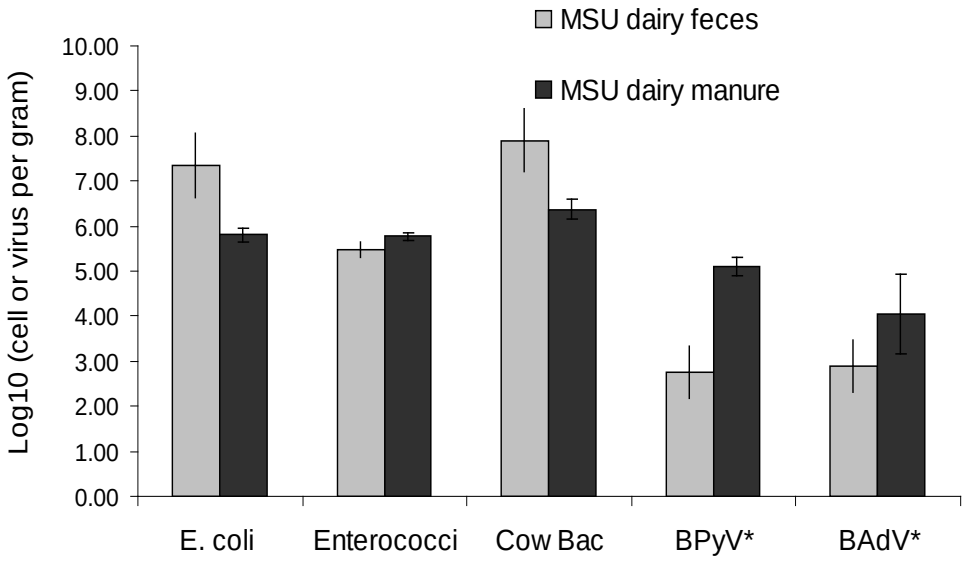

Figure 2.2.3. Comparison between the concentrations of viruses/indicators in manure and faecal samples from MSU Dairy Farm.

* For the samples tested negative with either BPyV or BAdV, the concentrations of BPyV and BAdV were calculated by using method detection limit, which is 10 copies per PCR reaction.

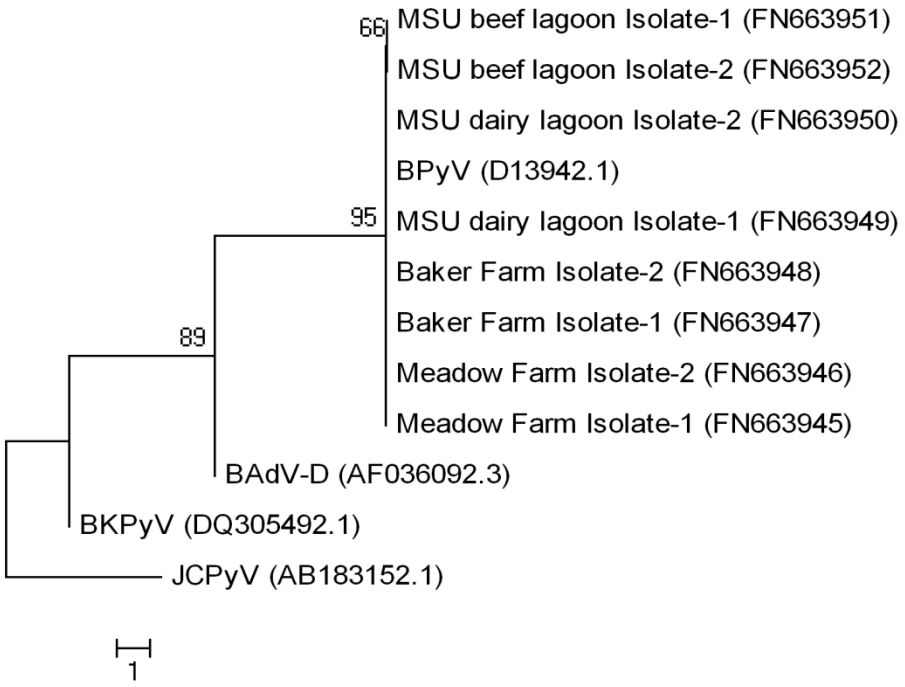

Figure 2.2.4. Neighbor joining tree of BPyV identified in the dairy and beef manure samples. BPyV, BAdV serotype D, and two human polyomaviruses (BKPyV and JCPyV) were included in the tree. The letters/numbers inside the parenthesis are the accession numbers. The numbers on the tree nodes represent 500 replicates of bootstrap values higher than 50% and the scale bar represents maximum likelihood distance.

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CHAPTER THREE

Wong, K., B. Onan, and I. Xagoraraki. (2010) Quantification of enteric viruses, indicators and salmonella in class B anaerobic digested biosolids by culture and molecular methods. Applied and Environmental Microbiology. doi:10.1128/AEM.02685-09

QUANTIFICATION OF ENTERIC VIRUSES, INDICATORS AND SALMONELLA IN CLASS B ANAEROBIC DIGESTED BIOSOLIDS BY CULTURE AND MOLECULAR METHODS

Abstract

The most common class B biosolids in the United States are generated by mesophilic anaerobic digestion (MAD) and MAD biosolids have been used for land application. However, the pathogen levels in MAD biosolids are still unclear especially with the respect to enteric viruses. In this study, we determined the occurrence as well as the quantitative levels of enteric viruses and indicators in 12 MAD biosolids samples and of *Salmonella enterica* in 6 MAD biosolids samples. Three dewatered biosolid samples were also included in this study for comparison purposes. Adenoviruses (HAdV) had the highest gene levels and were detected more frequently compared to other enteric viruses. The gene levels of noroviruses (NV) reported were comparable to enteroviruses (EV) and polyomaviruses (HPyV). The occurrence percentages of HAdV, HAdV-F, EV, NV-GI, NV-GII and HPyV in MAD samples were 83, 83, 42, 50, 75, and 58% respectively. No hepatitis A virus was detected. Infectious HAdV was detected more frequently than infectious EV and all infectious HAdV were detected when samples were propagated in A549 cells. Based on the MPN number, A549 cells were more susceptible to biosolid-associated viruses than BGM cells. All indicator levels of MAD biosolids were approximately 10^4 MPN or PFU per gram (dry) and the dewatered biosolids had significantly higher indicator levels than the MAD biosolids. Only two MAD samples tested positive for *Salmonella enterica*, where the concentration was below 1.0 MPN/4g. This study provided a broad comparison on the prevalence of different enteric viruses in

MAD biosolids and reported the first detection of noroviruses in class B biosolids. The observed high quantitative and infectivity levels of adenoviruses in MAD biosolids indicate that adenovirus is a good indicator for the evaluation of sludge treatment efficiency.

Keyword: biosolids, mesophilic anaerobic digestion, enteric virus, cell culture

Introduction

Over the last decade, thousands of people in the United States have been infected with waterborne diseases, a large number of whom were hospitalized. Most of the waterborne disease outbreaks in the US that occurred between 1991 and 2004 were related to microbial agents, i.e. viruses, bacteria, and parasites (Blackburn *et al*. 2004; Liang *et al*. 2006). The majority of the outbreaks involved un-identified agents and the Environmental Protection Agency (EPA) suspects that many of the outbreaks due to unidentified sources were caused by enteric viruses (USEPA 2006c). Indeed, viruses have a high potential for groundwater pollution due to their small size and low die-off rates. The occurrence of enteric viruses in groundwater has been reported (Abbaszadegan *et al*. 2003; Borchardt *et al*. 2003; Davis and Witt 1998; Fout *et al*. 2003). In the US, approximately 5.6 million dry tons of biosolids are generated annually and 60 percent of the biosolids are land applied (National Research Council 2002).

Several studies have reported the occurrence of enteric viruses in biosolids (Gallagher *et al*. 2007; Monpoeho *et al*. 2001; Viau and Peccia 2009); however, information on quantity and infectivity of enteric viruses in biosolids is still limited and most studies focused solely on enteroviruses (Sidhu and Toze 2009). Few studies have reported the levels of adenoviruses in biosolids (Bofill-Mas *et al*. 2006; Viau and Peccia 2009) and no quantitative results have yet been reported on some of the emerging viruses, such as hepatitis A and noroviruses. Also, only one or two types of enteric viruses were quantified in the previous studies; therefore, it is hard to determine and compare the prevalence of different types of enteric virus in biosolids since the samples and sample processing methods varied from study to study. A few studies focused on the viral

infectivity of biosolids and results showed infectious astrovirus and enteroviruses were still presented in the treated biosolids (Chapron *et al*. 2000; Gallagher *et al*. 2007; Soares *et al*. 1994). However, no results on the occurrence of adenoviruses in biosolids have been reported.

Polymerase chain reactions (PCR) techniques have been used in most of the recent environmental virology studies. Comparing these techniques to cell culture, the main advantages of PCR methods for virus detection are fast result, less intensive labor, high specificity and sensitivity, and the capability of detecting difficult-to-culture or non-culturable viruses (for examples, human noroviruses and adenovirus 40/41). Real time PCR (qPCR), which is considered the latest advancement in PCR technology, can provide both qualitative and quantitative results. However, PCR results may not reflect the infectivity of the samples since PCR only detects the gene of the pathogens; therefore, integrated cell culture-PCR (ICC-PCR) was developed to identify the specific infectious enteric viruses. ICC-PCR has been used to detect infectious enteric viruses in river water, tap water, beach water, and wastewater effluent samples (Lee *et al*. 2004; Lee *et al*. 2005; Rodriguez *et al*. 2008; Xagoraraki *et al*. 2007). However, Buffalo Green Monkey (BGM) cell culture, currently recommended by the EPA, has been compared with other cell lines such as A549 and PLC/PRC/5 (Lee *et al*. 2004; Rodriguez *et al*. 2008) and the results showed enteric viruses were propagated better with these cell lines than with BGM.

The main objective of this work was to investigate the occurrence as well as the quantitative levels of the enteric viruses in class B mesophilic anaerobic digested (MAD) biosolid samples by molecular and cell culture methods. These results can be used for risk assessment at biosolid application sites. Also, enteric virus has been suggested as

fecal source tracking indicators (Harwood *et al*. 2009; McQuaig *et al*. 2009) and the levels of enteric virus in biosolids reported in this study would be useful for the determination of which enteric virus is a better fecal source tracking indicator at biosolid application sites. MAD biosolids were chosen since it is the most common class B biosolid produced in the US (Viau and Peccia 2009). Three dewatered biosolid samples were also included for comparison purposes. The levels of adenovirus (HAdV), adenovirus type 40/41 (HAdV 40/41), enterovirus (EV), norovirus GI and GII (NV-GI and NV-GII), polyomavirus (HPyV) and hepatitis A (HAV) were quantified by qPCR methods. BGM and A549 cell lines were used to quantify the infectious viruses in the biosolids and the effectiveness of these two cell lines ability to propagate infectious viruses was compared. The occurrence of infectious EV and HAdV in biosolids was determined by ICC-PCR and the serotype of the infectious adenoviruses propagated on A549 was further determined. The levels of pathogen indicators and *Salmonella enterica* were also presented in this study.

Materials and Methods

Sampling. Biosolid samples were collected from five different wastewater treatment plants in Michigan (US) with three different sampling events at each plant. Four of the plants produced class B, mesophilic anaerobic digested (MAD) biosolids, and one of the plants produced non-digested (dewatered) biosolids. A total of twelve MAD and three dewatered biosolid samples were collected from December 2008 to September 2009. Approximately one or two samples were collected each month during the study period. The class B biosolids are land applied in agricultural plots and the dewatered

biosolids are disposed to the local landfill. Table 3.1 displays the stabilization temperatures, solid retention time, and dewatering processes by each plant for producing biosolids. Two liter grab samples of each of the anaerobically digested samples were collected from the post digestion holding tanks and then transferred or shipped to the laboratory on ice overnight. The dewatered samples were collected from the exiting conveyor belt in the loading bay. Upon acquisition of the sample, all indicator tests, *Salmonella enterica* tests, gravimetric analysis for determination of solids content and viral elution/concentration of each sample were performed immediately.

Virus elution process. Figure 3.1 shows a flow chart describing the methodology applied to virus determination. The virus elution and concentration were performed according to ASTM D 4994-89 (ASTM 2002). Briefly, beef extract was added to 10 to 20 g (dry) biosolids and stirred for 30 minutes to elute the viruses. Then, the solids were spun down by centrifugation and the supernatant was kept for further concentration. The supernatant was flocculated by adjusting the pH to 3.5 and spun again to form a pellet. The pellet was then dissolved in phosphate buffer saline (PBS) and 0.22-μm filtered. The final eluent was kept in a -80^{o}C freezer for further analysis.

Indicators and *Salmonella enterica* analysis. All of the samples were analyzed immediately after they were delivered to the laboratory. The indicators included in this study were fecal coliform (FC), *E. coli*, enterococci, and somatic phage. FC was analyzed according to the U.S. EPA method 1680 (USEPA 2006a). *E. coli* and enterococci were analyzed by IDEXX methods (APHA 2000; ASTM 2005). Somatic phage was analyzed by the double layer agar method (USEPA 2001). Each of the indicator measurements were run in triplicate for each sample. *Salmonella enterica* was measured according to

the EPA method 1682 (USEPA 2006b). All dilutions were made with sterilized phosphate buffer water (PBW).

Nucleic acid extraction. The virus eluent and cell culture supernatant was extracted by the MagNA pure automatic extraction machine (Roche) and the extraction kits used were MagNA Pure Compact Nucleic Acid Isolation Kit-Large Volume (Roche). 1 mL of the sample was extracted and the final elution volume was 100 µL. The nucleic acid eluents were stored in a -80 ^{o}C freezer for molecular analysis.

Nucleic acid (NA) extraction efficiency was evaluated and the method used was adapted from previous studies (Rajal *et al*. 2007; Viau and Peccia 2009). NA extraction efficiencies for dewatered and MAD bisolids were evaluated for all the biosolid samples. 6.2×10^{2} plaque forming unit (PFU) of bovine enteroviruses (BEV) (ATCC VR-754) were spiked into the biosolid eluent and NA-free water. After extraction, the levels of BEV were determined by qPCR. The extraction efficiency was calculated by dividing the BEV RNA recovered from the biosolid matrix by the BEV RNA recovered from the NA-free water. BEV was chosen since no samples tested positive with BEV. The extraction efficiency was incorporated into the calculation of virus concentration in the biosolid samples.

qPCR standards. Concentration of the target pathogens in the biosolid samples were quantified by using the standard curves generated from ATCC viruses or environmental isolates. Human Adenovirus 40 (ATCC VR-930), Coxsackie virus B5 (ATCC VR-1036AS/MK), hepatitis A HM175 (ATCC VR-1402), JC polyomavirus (ATCC VR-1583), *Salmonella enterica* (ATCC 14028), and norovirus isolates provided by Ingham County Health Department were used to generate the standard curves.

PCR amplicons of the target gene from HAdV, HPyV, *Salmonella enterica* and cDNA of the target gene from EV, BEV, NV, and HAV were cloned into plasmid vectors according to the one-shot chemical transformation described in the manufacturer's instructions (TOPO TA Cloning® Kit for Sequencing, Invitrogen, Carlsbad, CA). Plasmids carrying the cloned target gene were purified using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI) and sent for sequencing at the Research Technology Support Facility at Michigan State University. The target gene sequences were compared with those published in the National Center for Biotechnology Information (NCBI) database by using the program of Basic Local Alignment Search Tool (BLAST).

The gene equivalent copies (GEC) of the standard stocks were quantified using the NanoDrop® 1000 spectrophotometer and then 10-fold serially diluted. The dilutions ranged from 10^1 to 10^8 GEC/real-time PCR (qPCR) reaction and used to calibrate the concentration of the target gene detected in the qPCR assays. The efficiency for each standard curve is illustrated in Table 3.2.

qPCR assays. All of qPCR assays were performed in a Roche LightCycler® 1.5 Instrument (Roche Applied Sciences, Indianapolis, IN). Each target in each sample was run in triplicate qPCR reactions for determination. All PCR runs included a negative control reaction (PCR-grade H_2O without template) and a positive control reaction. The crossing point (Cp) of each PCR reaction was automatically determined by the LightCycler® Software 4.0 and used to calculate the genomic copies. All of the primer and probe sequences are summarized in Table 3.2. Each qPCR reaction mix included 10 µL of 2X LightCycler 480 TaqMan Master Mix, appropriate volume of primers and

probes to obtained the concentration described in Table 3.2, 5 µL of DNA or cDNA sample, and appropriate volume of PCR-grade water to make up a final volume of 20 µL reaction mix. The real-time PCR running program (all thermocycles were performed at a temperature transition rate of 20°C/s) was 95°C for 15 min; followed by 45 cycles of denaturation, annealing, extension (temperature and time are listed in Table 3.2). All reactions ended with a final cooling step at 40°C fo 30 sec.

Reverse transcription was required before qPCR on EV, BEV, HAV and NV. Each reverse transcription reaction mix included 2.5 µL of 10 µM reverse primer, 1 µL of reverse transcriptase (Promega), 4 µL of 5X transcriptor reaction buffer (Roche), 20U of protector Rnase inhibitor (Roche), and 2 µL of 10 mM deoxynucleotide (Roche). The reaction conditions for all three RNA viruses were the same; the reaction mix was incubated at 55 ^{o}C for 30 minutes and then 85 ^{o}C for 5 minutes to inactivate the enzyme.

To evaluate the presence of inhibition in the biosolid extracts, 10^4 copies of BEV RNA were spiked into each biosolid NA extracts at different dilutions (no dilution, 1:5 dilution, and blank control consisting of NA-free water). The BEV threshold cycle values of each biosolid NA extract with no dilution and 1:5 dilution, were compared with the one in blank control. If the threshold cycle value of blank control was 5% lower than the one in the biosolid NA extract with no dilution, it indicated that inhibition was present and dilution of the biosolid NA extract would be performed until no inhibition was observed.

Limit of quantification (A_{LOD}) was defined previously as the lowest concentration of the target gene remaining within the linear range of quantification (Rajal *et al*. 2007). Ten copies of virus gene was the A_{LOD} for all qPCR assays used in this

study. Even though < 10 copies of virus gene were detected in some assays, the signals were not consistent. Therefore, we set 10 copies as our qPCR detection limit and the gene levels of the samples tested negative were calculated using 10 copies per qPCR reaction.

Cell culture and ICC-PCR assay. Cell culture was performed on ten biosolid samples (two samples from each WWTP) to determine their infectivity levels. Two cell lines, BGM (passage 140 to 170, obtained from Dr. Shay Fout at EPA) and A549 (passage 100 to 130, obtained from ATCC (CCL 185)), were used to culture the viruses in biosolids. Briefly, the cells were grown in flasks until reaching at least 80-90% confluence. Virus eluents with different serial 10 fold dilutions were added to multiple culture flasks at each dilution and incubated at 36.5±1ºC for one hour with rocking every 15 minutes to ensure complete contact between the cells and viral particles. Cells were maintained with minimum essential media (MEM) supplemented with L-glutamine, Earle's salts, and 2% fetal bovine serum. Cytopathic effects (CPE, indicative of a viral infection) in the cell cultures were monitored for up to 14 days. All of flasks that displayed CPE were frozen at -80 ºC for confirmation (second passage). The confirmation was done by aliquots of 1ml of the supernatant to new 80-90% confluent flasks and incubate for 7 days at 36.5±1ºC. CPE of each flask was recorded and the mean viral concentration of the samples was estimated by the free most-probable-number (MPN) software downloaded from (http://www.i2workout.com/mcuriale/mpn/index.html). The results were expressed as MPN/ 4g (dry) and only one MPN value was obtained for each sample due to the high intensive laboring for the replication of MPN cell culture experiments. The positive

flasks were then frozen at -80 ºC for ICC-PCR assay. The primers/probes of Total HAdV and EV assays described in Table 3.2 were used for the ICC-PCR assay to determine the occurrence of infectious HAdV and EV in the biosolid samples. ICC-PCR assays were carried out on all flasks for the samples that had no flasks displaying CPE after first passage.

PCR primers developed by Xu *et al*. (2000) were used to further classify the infectious HAdV species in A549 positive samples. The PCR amplification conditions were 94°C for 4 *min.*; followed by 30 cycles at 94°C for 60 sec, 54°C for 45 sec, 72°C for 2 *min.,* and finally 5 *min.* at 72°C. A total of 50 μL of reaction mix consisted of 1μL of each 10 μM primers, 25 μL of Promega2X Master Mix (Promega, WI), 18 μL of molecular graded water and 5 μL of DNA sample.

Statistical analysis. All microbial data was $\log_{10}$ transformed before the statistic analysis since it was determined by the Anderson Darling test that the data was lognormally distributed. To determine significant differences between the concentrations of indicators and enteric viruses, analysis of variance (ANOVA) single test was performed using SPSS version 17.0. P-values less than 0.05 indicate a significant difference.

Results

Indicators and *Salmonella enterica* levels in biosolids. Figure 3.2 illustrates the indicator levels in biosolids. All of the indicator levels in the MAD biosolids were around 10^4 MPN or PFU per gram and no significant differences between each indicator was observed ($P \geq 0.05$). Only 1 out of 12 MAD samples exceeded the US EPA regulatory

limits for class B biosolids (2×10^6 CFU/g). The dewatered biosolids had significantly higher levels of all four indicators than the MAD biosolids ($P \leq 0.05$). The log differences between dewatered and MAD biosolid samples were 3.56, 3.06, 1.74 and 0.81 for FC, *E. coli*, enterococci, and somatic phage, respectively.

Six MAD and one dewatered samples were tested for *Salmonella enterica*. The only two MAD samples tested positive were from the St. Clair WWTP and had concentrations of only 0.487 and 0.954 MPN/4g. However, the dewatered sample had much higher level of *Salmonella enterica*, which was 976 MPN/4g. All seven samples were also tested by qPCR but none of the samples tested positive.

Nucleic acid extraction efficiency and inhibition control. No inhibition was observed in any of the 15 biosolid extracts since the difference between the threshold cycle values of the blank control and the biosolid NA extract with no dilution, as well as the 1:5 dilution were less than 5%. The NA extraction efficiency for dewatered and MAD biosolids was 31.0± 10.4% and 80.0± 35.2%, respectively.

Gene levels of enteric viruses in biosolids. Figure 3.3 and Table 3.3 illustrate the average gene levels of enteric viruses and the percentage of qPCR and cell culture positive samples in both types of biosolid samples. The levels of HAdV were significantly higher than other enteric viruses ($P \leq 0.005$) and they were detected more frequently than other enteric viruses. The levels of HAdV were at least 0.71 and 2.0 logs higher than the levels of other enteric viruses in the MAD and dewatered biosolids, respectively. The average levels, and percentage of positive samples of HAdV in the MAD biosolids were 7.5×10^5 copies/g and 83%, respectively. No significant differences were observed among the levels of EV, NV and HPyV ($P \geq 0.05$) in both types of

biosolids. No HAV was detected in any of the samples. The levels of NV-GI and NV-GII were 5.0×10^4 and 1.5×10^5 copies/g in the MAD biosolids, which is comparable to the levels of EV. NV was detected more frequently than EV in the MAD samples. Both HPyV and HAdV are double stranded DNA viruses but the levels of HPyV (7.4×10^4 and 2.5×10^5 copies/g for the MAD and dewatered samples, respectively) were lower than HAdV and HPyV was detected less frequently. Only the levels of HAdV and EV in dewatered samples were significantly higher than the ones in MAD samples ($P \leq 0.05$).

Infectivity of enteric viruses. Figure 3.4 illustrates the average MPN levels of biosolids determined by both cell lines. Results showed that the MPN levels using A549 were significantly higher than the levels determined using BGM ($P \leq 0.005$). The mean levels of infectious viruses in MAD biosolids were 2.9 and 480 MPN/4g on BGM and A549, respectively. The mean levels in dewatered biosolids were 67 and 2210 MPN/4g on BGM and A549, respectively. CPE was observed in all samples on A549 but CPE was observed in only 50% of the MAD samples on BGM (Table 3.3). With the exception of one dewatered sample, all nine samples had higher MPN counts when propagated on A549 cells. There was no significant difference between MPN values of dewatered and MAD samples propagated on both types of cell lines.

Table 3.4 illustrates the ICC-PCR results for HAdV and EV on BGM and A549 flasks. Infectious HAdV and EV was detected in 70% and 10% of the A549 propagated samples, respectively. No infectious HAdV was found in any of the BGM propagated samples but infectious EV was found in 30% of the BGM propagated samples. Infectious HAdV and EV were found in both dewatered samples. 75% and 12.5% of the MAD

samples were positive for infectious HAdV and EV, respectively. Interestingly, both HAdV qPCR negative samples were also ICC-PCR HAdV negative in both cell lines (Table 3.4). The other ICC-PCR HAdV negative sample on 549 (Plainwell 8/4/2009) also had relatively lower HAdV gene concentration. All of the flasks with no CPE were tested negative by both HAdV and EV ICC-PCR.

Species of infectious HAdV detected in positive A549 flasks were illustrated in Table 3.5. The HAdV species detected were A (*f*=4), B (*f*=3), C (*f*=3) and D (*f*=2) (*f* is frequency of detection). No species E and F were found.

Discussion

Land application of biosolids has been increasingly practiced worldwide since it has the benefit of reducing the environmental contamination by reuse of the biosolids and provides biosolids as an additional source of nutrients to agricultural field (Sidhu and Toze 2009). However, there is a growing concern over whether land-applied biosolids would pose a risk of groundwater and/or surface water contamination. Microorganisms generally tend to attach to solid surfaces (Maier *et al*. 2008). Therefore, the majority of viruses and other pathogens in wastewater utilities are likely associated with sludge particles and are expected to end up in wasted sludge. The most common class B sludge treatment in the U.S. is mesophilic anaerobic digestion (MAD). Previous study suggested viruses are resistant to MAD treatment (Viau and Peccia 2009). The information provided in this study provides a better understanding on the quantity and infectivity levels of several of the most critical emerging viruses in MAD biosolids measured by both molecular and cell culture methods.

The higher indicator levels in dewatered verse MAD biosolids were expected since the only treatment on dewatered biosolids is to lower the moisture content. The log reduction of FC between dewatered and MAD samples was greater than the reduction of somatic phage. This observation was similar to previous findings, where FC had a greater reduction than male-specific phage between class A and B biosolids (Viau and Peccia 2009). A similar trend was also observed in our previous study of an anaerobic membrane bioreactor that treats animal waste (Villar *et al*. 2007). The log reduction of *E. coli* by anaerobic digestion was 1.5, but only 0.5 for somatic phage. Also, no significant difference was observed between the enteric virus levels in dewatered and MAD samples by both molecular and cell culture measurements (except for total HAdV and EV by qPCR). These findings showed anaerobic digestion may be effective for the removal of bacterial indicators but not viruses. Lower levels of *Salmonella enterica* in the MAD biosolids were expected since previous studies also found *Salmonella enterica* concentrations in the MAD biosolids were several orders of magnitude lower than the indicator concentrations (Dahab and Surampalli 2002; Gantzer *et al*. 2001). Low occurrences of *Salmonella enterica* in the MAD biosolids were also observed in the previous study, where Gantzer *et al*. (2001) found only 55% of the MAD samples positive for *Salmonella enterica*.

The levels and occurrence of HAdV measured in this study were comparable to 5.0×10^{5} copies/g and 88% reported by Viau and Peccia (2009). The average HAdV levels of MAD biosolids reported by Bofill-Mas *et al*. (2006) were 10^{3} copies/g, which was approximately 2 logs lower than the HAdV levels observed in this study. The higher levels of HAdV over other enteric viruses in biosolids could be due to its high resistant to

treatment processes and high concentrations in wastewater. Enriquez *et al*. (1995) conducted a survival study of HAdV40/41 in tap, sea, and wastewater and concluded that HAdV40/41 is more stable in tap water and wastewater than poliovirus. Irving and Smith (1981) reported that HAdV are more likely to survive the conventional sewage treatment than EV. Katayama *et al*. (2008) found that HAdV had the highest levels during a one year survey of NV, EV and HAdV in six WWTP.

The average levels of EV in the MAD biosolids were 1.9×10^4 copies/g, which is also comparable to 1.2×10^4 copies/g reported by Monpoeho *et al*. (2004); however, EV were detected in all of their MAD samples but only 42% of the samples were detected positive in this study. The qPCR assay used in this study was adapted from Dierseen *et al*. (2008) and it is different than the one used in the previous study where their assay was adapted from Monpoeho *et al*. (2000). However, we think the difference in occurrence frequency is likely due to the lower levels of EV in our MAD biosolids samples rather than the use of a different qPCR assay. A comparison study between these two assays that was run in our laboratory for selected samples indicated that the assay developed by Dierseen *et al*. (2008) resulted in higher virus quantities and more frequent detection than the one by Monpoeho *et al*. (2000).

NV is an emerging virus and is one of the causes of gastroenteritis disease worldwide. NV has been detected and quantified in raw sewage and treated effluent (da Silva *et al*. 2007; Haramoto *et al*. 2006; Katayama *et al*. 2008; Laverick *et al*. 2004). However, no quantitative results of NV in biosolids have been reported prior to this study. Even though the levels of NV were not as high as HAdV, they were comparable to EV and HPyV. The occurrence levels were also significant, where at least 50% of the

MAD samples were detected with either NV-GI or NV-GII. NV-GII had about half a log higher concentration and 25% more positive samples than NV-GI in the MAD biosolids. This observation is similar to previous studies where NV-GII was found more abundantly than NV-GI in raw sewage (da Silva *et al*. 2007; Haramoto *et al*. 2006; Katayama *et al*. 2008).

The HPyV levels of the MAD biosolids reported by Bofill-Mas *et al*. (2006) were between 10^3 to 10^4 copies/g (dry) and all of their biosolid samples were positive for HPyV. The mean HPyV level of our MAD biosolids was 5.91×10^4 copies/g, which is comparable to the previous findings. However, only 58% of our biosolid samples were positive for HPyV. The difference in occurrence frequency between these two studies may be due to our MAD biosolids being collected from several different treatment plants since HPyV was detected in all of the samples from the St. Clair and Romeo WWTPs but only one sample was positive in all of the Plainwell and Traverse City samples. The positive samples in this study however had a higher concentration ranging from 2.41×10^5 to 1.18×10^6 copies/g. This may be due to the nature of our samples or it could be due to the qPCR assay used in this study, which could target two main types of HPyV (JCPyV and BKPyV), whereas the assay used in the previous study mainly targets the JCPyV.

Even though some studies have reported the presence of HAV in environmental water media (Brooks *et al*. 2005; De Paula *et al*. 2007; Rose *et al*. 2006; Villar *et al*. 2007), no HAV was detected in any of our biosolid samples. The occurrence of HAV indicates that the low risk of transporting HAV from land applied biosolid to the natural environment is minimal.

The cell culture MPN results indicated that A549 cells were more susceptible to biosolid-associated viruses than BGM cells. Interestingly, the ICC-PCR results showed that infectious HAdV was presented in more samples than infectious EV and the infectious HAdV was only found in A549 propagated samples. Also, qPCR results showed a higher quantitative level and occurrence frequency of HAdV than EV. Previous studies compared BGM with A549 and showed BGM was not effective in propagating HAdV (30). We have also tested the effectiveness of propagating ATCC HAdV (serotype 4, 6, 21, 31, 36, 40, 41) between BGM and A549 cells (data not shown). Results showed all seven serotypes of HAdV propagated using A549 cells but only serotype-6 propagated using BGM cells. Based on these facts, we believe infectious HAdV levels are higher than the infectious EV in the biosolids samples and BGM not being able to propagate the infectious HAdV effectively resulted in a lower infectious unit. Most of the previous studies used BGM to evaluate the viral infectivity in biosolids (Monpoeho *et al*. 2000; Monpoeho *et al*. 2001; Monpoeho *et al*. 2004; Sidhu and Toze 2009). However, most of the recent findings have shown HAdV are more prevalent than EV in environmental samples. Therefore, HAdV could be a more suitable enteric virus for the use as an indicator of human fecal pollution at biosolid application sites.

HAdV species A, B, C, and D were detected in the positive ICC-PCR samples. In a previous study (Lee *et al*. 2004), the infectious HAdV species in river water detected using A549 cells were C (*f*=7), D (*f*=5), A (*f*=4) and F (*f*=1). No species B or E was detected. HAdV-A, C and D were detected in the previous study as well as this one. The HAdV-F (type 40 and 41) was detected by qPCR in biosolids and the levels were relatively high compared to other enteric viruses. As mentioned in the previous

paragraph, we have done a study on propagation of different ATCC HAdV species in A549 cells and species F (both 40 and 41) had the least increase in concentration compared to the other HAdV species after one week of incubation. Therefore, we think that it is likely to have infectious HAdV-F in the biosolids but other HAdV species may have out-grown the HAdV-F, which resulted in no detection of HAdV-F during ICC-PCR.

Conclusions

Currently, monitoring the occurrence of EV in biosolids is suggested by the EPA. However, the results from this study showed HAdV had the highest gene levels compare to other enteric viruses in biosolids. Infectious HAdV was detected more frequently than infectious EV. Therefore, more studies on inactivation of HAdV by different sludge treatment processes should be investigated since high levels of HAdV still remained in the MAD biosolids. More cell lines which are susceptible to HAdV should also be investigated. This study provided the quantitative levels of NV in biosolids, which has never been reported in the published literature. There is a need to conduct more studies on the occurrence of NV in different types of biosolids since significant levels of NV were found. Finally, low levels of HAV and *Salmonella enterica* in biosolids may suggest the risk of water contamination by these pathogens from biosolid application sites would be minimal.

Acknowledgements

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Tables and Figures

Table 3.1. Summary of operation parameters and biosolid characteristics.

	East Lansing	Traverse City	Romeo	Plainwell	St. Clair
Wastewater Treatment Process	Activated sludge	Activated Sludge + MBR	Trickling filter/ Rotating biological contactors	Rotating biological contactors	Trickling filter
Capacity	18.8 MGD	17 MGD	2.2 MGD	1.3MGD	4.2 MGD
Average Flow	13.4 MGD	8.5 MGD	0.8 MGD	0.5MGD	1.3 MGD
Sludge Treatment	Dewatering	MAD	MAD	MAD	MAD
Dewatering Process	Bell Press	Gravity Thickened	Gravity Thickened	Gravity Thickened	Gravity Thickened
MAD temperature	NA	35^{o}C	35^{o}C	37^{o}C	37^{o}C
Solid Retention Time	NA	~ 45 days	~ 7 days	~7.8 days	~ 7 days
Percent Solids	18.30±1.57	5.04±1.28	7.95±3.63	5.19±2.56	6.47±1.24
Disposal of Biosolids	Landfill	Agricultural Land	Agricultural Land	Agricultural Land	Agricultural Land

NA= Not applicable; MGD=million gallons per day

Table 3.2. Primers and probes for enteric virus detection.

Virus Type	Primers /Probe	Conc (μM) per rxn	Sequence (5' to 3')	Reaction Condition (temp ^{o}C, time)	Amplification Efficiency (%)	Reference
Total HAdV	Forward	0.9	C(AT)TACATGCACATC(GT)C(CG)GG	95, 15s - denaturation	95.4	16
	Reverse	0.9	C(AG)CGGGC(GA)AA(CT)TGCACCAG	60, 60s - annealing		
	Probe-1	0.45	CCGGGCTCAGGTACTCCGAGGCGTCCT			
	Probe-2	0.45	CCGGACTCAGGTACTCCGAAGCATCCT			
HAdV40/41	Forward	0.4	ACCCACGATGTAACCACAGAC	95, 10s - denaturation	107.2	50
	Reverse-1	0.2	ACTTTGTAAGAGTAGGCGGTTTC	60, 30s - annealing		
	Reverse-2	0.2	CACTTTGTAAGAATAAGCGGTGTC	72, 12s - extension		
	Probe	0.3	CGACKGGCACGAAKCGCAGCGT			
EV	Forward	1.0	ACATGGTGTGAAGAGTCTATTGAGCT	95, 15s - denaturation	112.8	14
	Reverse	1.0	CCAAAGTAGTCGGTTCCGC	60, 60s - annealing		
	Probe	0.6	TCCGGCCCCTGAATGCGGCTAAT			
NV-GI	Forward	0.2	CGCTGGATGCGNTTCCAT	95, 15s - denaturation	93.9	10
	Reverse	0.2	CCTTAGACGCCATCATCATTTAC	60, 60s - annealing		
	Probe	0.2	TGGACAGGAGAYCGCRATCT			

Table 3.2 cont'd

NV-GII	Forward	0.4	CARGARBCNATGTTYAGRTGGATGAG	95, 15s - denaturation	98.2	25
	Reverse	0.4	TCGACGCCATCTTCATTCACA	56, 60s - annealing		
	Probe	0.25	TGGGAGGGCGATCGCAATCT			
HPyV	Forward	0.5	AGTCTTTAGGGTCTTCTACCT TT	95, 15s - denaturation	96.6	32
	Reverse	0.5	GGTGC AACCTATGGAACAG	55, 15s - annealing		
	Probe	0.15	TCATCA CTGGCA AACAT	60, 60s - extension		
HAV	Forward	0.25	GGTAGGCTACGGGTGAAAC	95, 10s - denaturation	92.3	24
	Reverse	0.25	AACAACTCACCAATATCCGC	55, 20s - annealing		
	Probe	0.15	CTTAGGCTAATACTTCTATGAAGAGATGC	72, 15s - extension		
BEV	Forward	0.5	GCCGTGAATGCTGCTAATCC	95, 15s - denaturation	99.1	23
	Reverse	0.5	GTAGTCTGTTCCGCCTCCACCT	60, 60s - annealing		
	Probe	0.25	CGCACAATCCAGTGTTGCTACGTCGTAAC			
Salmonella	Forward	0.25	GCGTTCTGAACCTTTGGTAATAA	95, 15s - denaturation	94.5	37
	Reverse	0.25	CGTTCGGGCAATTCGTTA	62, 60s - annealing		
	Probe	0.4	TGGCGGTGGGTTTTGTTGTCTTCT	72, 10s - extension		

Table 3.3. Percentage of qPCR and cell culture positive samples.

qPCR assay / cell line	% of qPCR positive sample		% of cell culture positive sample	
	dewatered (n=3)	MAD (n=12)	dewatered (n=2)	MAD (n=8)
Total HAdV	100	83	NA	NA
HAdV 40/41	100	83	NA	NA
EV	100	42	NA	NA
NV-GI	67	50	NA	NA
NV-GII	67	75	NA	NA
HPyV	100	58	NA	NA
HAV	0	0	NA	NA
BGM	NA	NA	100	50
A549	NA	NA	100	100

NA= Not applicable

Table 3.4. Occurrence of HAdV and EV by qPCR and ICC-PCR.

	qPCR		ICC-PCR (A549)		ICC-PCR (BGM)	
	HAdV copies/g	EV copies/g	HAdV	EV	HAdV	EV
East Lansing (12/1/2008)	9.4×10^6 (3.3×10^5)	2.2×10^5 (6.0×10^4)	+	+	-	+
East Lansing (6/29/2009)	3.9×10^8 (7.5×10^6)	2.4×10^5 (3.1×10^4)	+	-	-	+
St. Clair (2/10/2009)	4.3×10^6 (8.5×10^4)	2.6×10^4 (1.3×10^3)	+	-	-	-
St. Clair (5/4/2009)	6.9×10^6 (4.2×10^5)	2.9×10^4 (2.5×10^4)	+	-	-	-
Plainwell (4/22/2009)	1.1×10^6 (2.9×10^5)	ND	+	-	-	-
Plainwell (8/4/2009)	1.5×10^5 (5.8×10^4)	ND	-	-	-	-
Romeo (5/29/2009)	4.1×10^5 (1.4×10^5)	4.4×10^4 (1.5×10^4)	+	-	-	-
Romeo (6/23/2009)	9.1×10^4 (1.4×10^5)	7.6×10^4 (7.6×10^3)	+	-	-	-
Traverse City (7/16/09)	ND	ND	-	-	-	+
Traverse City (8/2/09)	ND	ND	-	-	-	-

East Lansing-dewatered samples; St. Clair, Plainwell, Romeo, Traverse City-MAD samples.

Numbers inside the parenthesis represent the standard deviation of the triplicate qPCR measurement values.

ND = None Detected

Table 3.5. Species of infectious HAdV detected in A549 positive flasks.

	Species of infectious HAdV
East Lansing (12/1/2008)	B
East Lansing (6/29/2009)	B,C,D
St. Clair (2/10/2009)	A,C
St. Clair (5/4/2009)	A,C
Plainwell (4/22/2009)	B
Romeo (5/29/2009)	A,
Romeo (6/23/2009)	A,D

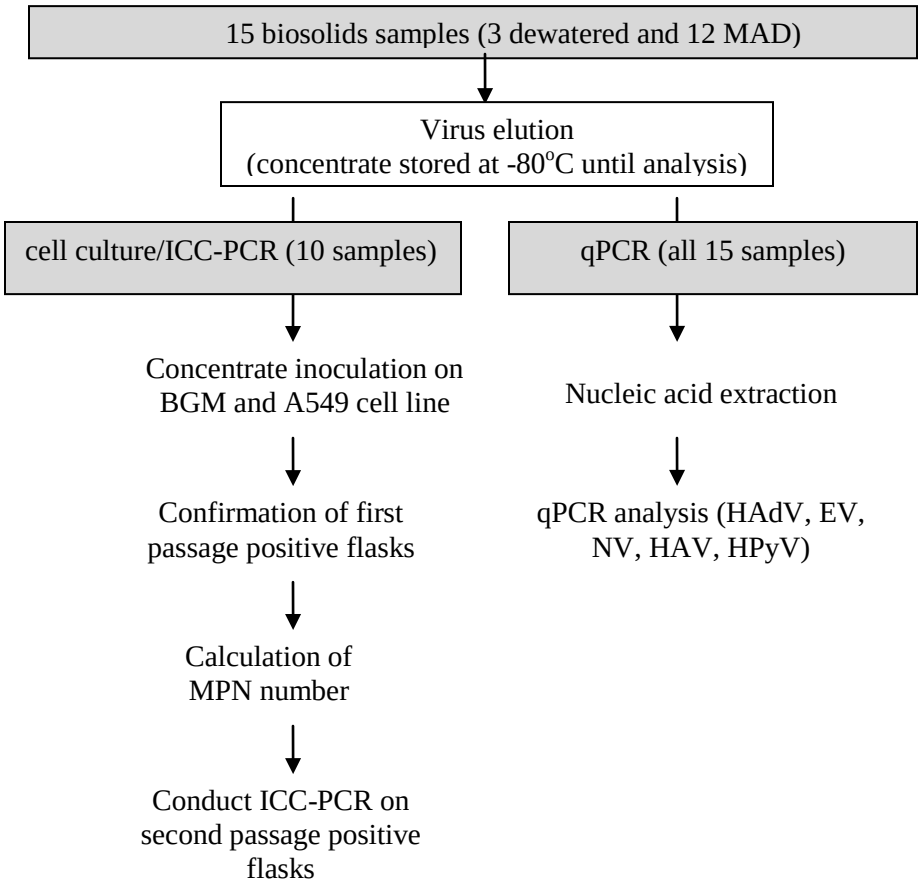

Figure 3.1. The methodology of enteric virus determination by cell culture, ICC-PCR and qPCR.

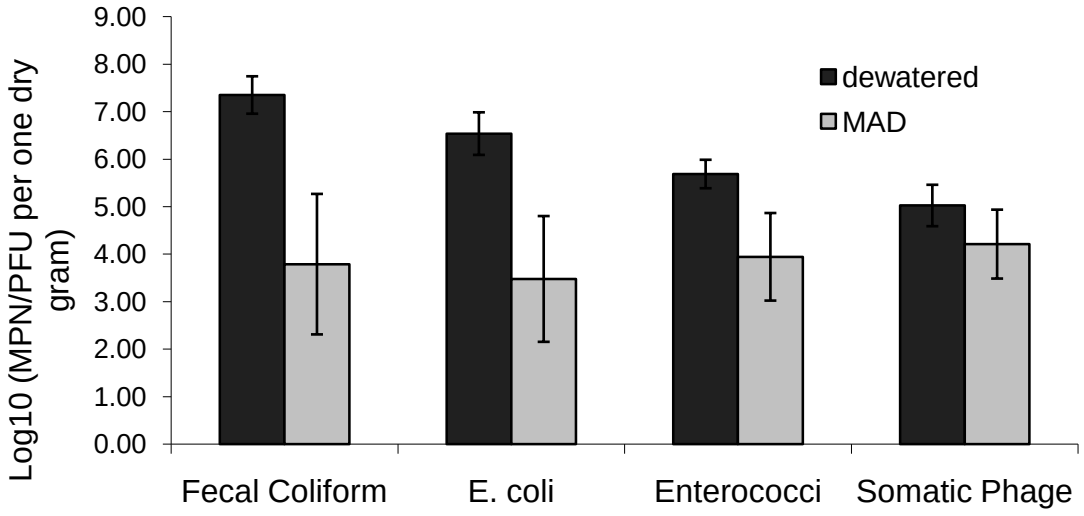

Figure 3.2. Indicator levels in biosolids samples (n for dewatered=3; n for MAD=12). Error bars represent the standard deviations of measurement values of samples collected from different sampling events.

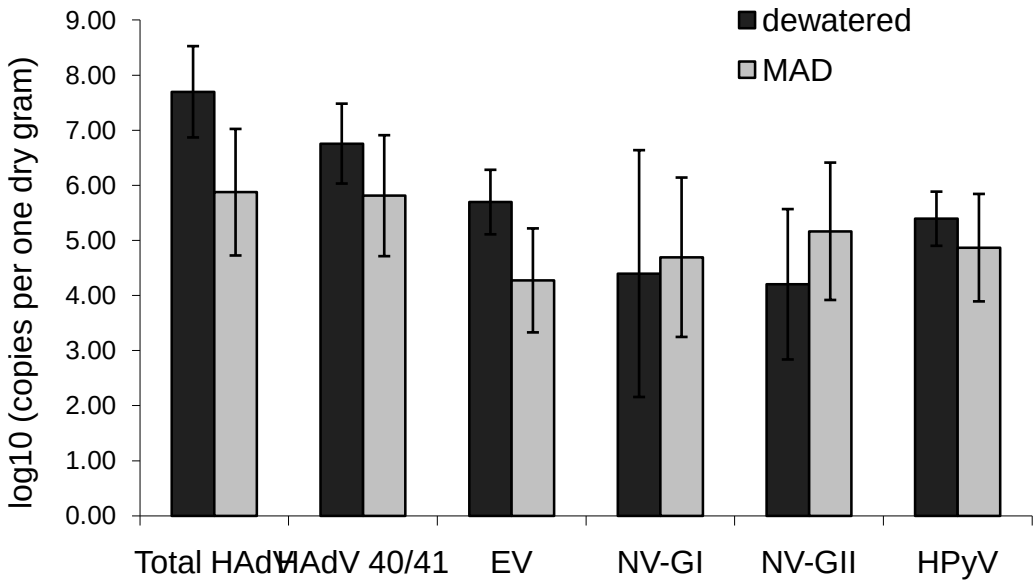

Figure 3.3. Enteric virus levels in biosolids samples (n for dewatered=3; n for MAD=12). Error bars represent the standard deviations of measurement values of samples collected from different sampling events.

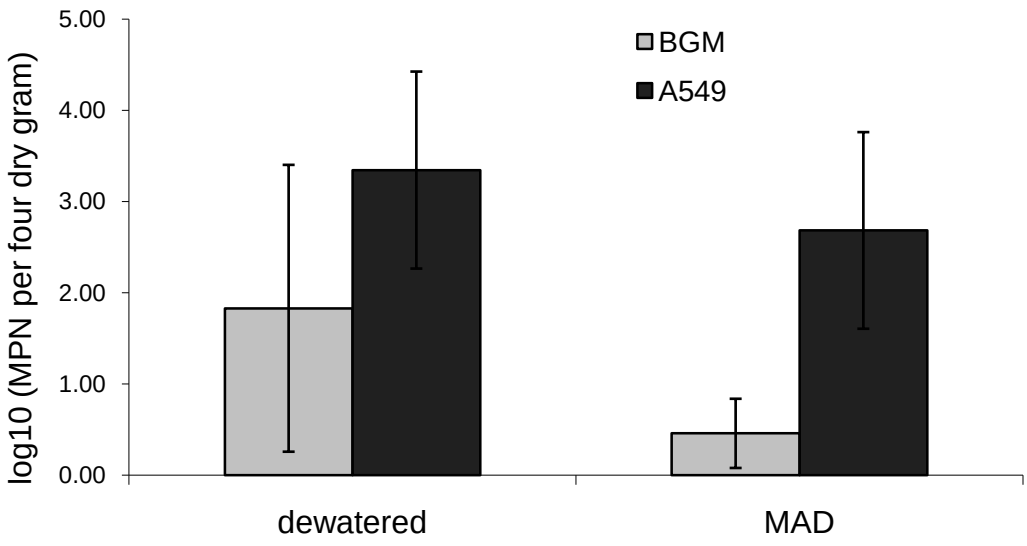

Figure 3.4. Enteric virus MPN levels propagated by BGM and A549 cells (n for dewatered=2; n for MAD=8). Error bars represent the standard deviations of measurement values of samples collected from different sampling events.

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CHARTER 4

EVALUATION OF LEACHING AND PONDING OF VIRAL CONTAMINANTS FOLLOWING LAND APPLICATION OF BIOSOLIDS ON SANDY-LOAM SOIL

Submitted for consideration to Journal of Environmental Quality (American Society of Agronomy Publications)

Abstract

Much of the land available for land application of biosolids is farm ground near residential areas. Biosolids are often applied on hay or grassland during the growing season or on corn ground before planting or after harvest in the fall. In this study, mesophilic anaerobic digested (MAD) biosolids were applied at 56,000 L/ha on a sandy-loam soil over large containment lysimeters seeded to perennial covers of orchardgrass (*Dactylis glomerata* L.), switchgrass (*Panicum virgatum*) or planted annually to maize (*Zea mays* L.). Portable rainfall simulators were used to evaluate the transport of viral contaminants under nearly saturated (90%, volumetric basis) conditions. Lysimeter leachate and surface ponded water samples were collected and analyzed for somatic phage, adenoviruses, anionic (chloride) and microbial (P-22 bacteriophage) tracers. Neither adenovirus nor somatic phage was recovered from the leachate samples. The P-22 was found in leachate from three lysimeters (removal rates ranged from 1.81 to 3.2 $\log_{10}$/m). Although the peak of the anionic tracer breakthrough occurred at a similar pore volume in each lysimeter (around 0.3 pore volume), the peak of P-22 breakthrough varied between lysimeters (<0.1, 0.3 and 0.7 pore volume). The early and variable time to peak breakthrough of anionic and microbial tracers indicated preferential flow paths,

presumably from soil cracks, root channels, worm holes or other natural phenomena. The concentration of microbial contaminants collected in ponded surface water ranged from 1 to 10% of the initial concentration in the applied biosolids. The die off of somatic phage and P-22 in the surface water was fit to a first order decay model and somatic phage reached background levels at about day ten. Microbial pollution from runoff following significant rainfall events is likely when biosolids remain on the soil surface.

Keyword: Containment lysimeter, biosolids, land application, rainfall simulation, microbial pollution, viruses, subsurface transport, ponding, leaching

Introduction

Waterborne disease statistics estimate a growing global burden of infectious diseases from contaminated drinking water. It has been reported that 1.5-12 million people die per year from waterborne diseases (Gleick, 2002). Most of the waterborne disease outbreaks in the US between 1991 and 2004 were related to microbial agents, i.e. viruses, bacteria, and parasites (Blackburn et al., 2004; Liang et al., 2006). Approximately 5.6 million dry tons of biosolids are generated annually in the U.S. and 60 percent are applied on land (National Research Council, 2002). Because significant levels of pathogens are present in treated biosolids at the time of application (Sidhu and Toze, 2009; Viau and Peccia, 2009), pathogens released from land applied biosolids can be transported by infiltration and runoff during rain events.

Column experiments have been used in laboratory studies to evaluate the effect of organic matter, water saturation, ionic strength, pH, soil texture and other factors on pathogen transport in the soil environment (Cheng et al., 2007; Chu et al., 2001; Powelson et al., 1991; Jin et al., 1997; Jin et al., 2000; Zhang et al., 2003; Chetochine et al., 2006). Because laboratory soil columns are screened and packed to a uniform density they lack the variability of undisturbed soil and may not always be representative of field conditions. For example, even though clay particles are small with great filtering and adsorption capacity, undisturbed clay soil was associated with lower microbial removal than sandy and silt soils in some lysimeter studies (Aislabie et al., 2001; McLeod et al., 2001; Carlander et al., 2000). Carlander et al. (2000) and Pang (2009) pointed out that the formation of macropores and preferential flow paths from shrinking and cracking of clay soils is the reason for inefficient microbial removal. Pang (2009) compared microbial

removal reported in soil column and field studies and reported column experiments had a one to three log greater microbial removal than field studies.

Large-scale, undisturbed soil lysimeters have been used to investigate manure associated pathogens and microbial tracer transport in soil systems (Aislabie et al., 2001; Carlander et al., 2000; McLeod et al., 2001, 2003, 2004; Pang et al., 2008; Jiang et al., 2008). Soil depth in these studies ranged from 40 to 100 cm with diameters ranging from 30 to 50 cm (volume ranged from 6.6 to 11.6 m^3). Most studies were performed indoors except for the work reported by Jiang et al. (2008) and Carlander et al. (2000). The focus of their work was on the transport of fecal coliforms, *E. coli* and salmonella phage from dairy manure or pure cultures. Pang (2009) concluded the removal of indicators in these studies was influenced by macropore flow largely through soil cracks. Preferential flow pathways have the potential to move contaminants very rapidly to shallow groundwater and possibly deeper groundwater at land application sites.

Land applied biosolids with a high pathogen load can contaminate surface water during rain events by runoff and the occurrence of enteric viruses in surface water has been reported in previous studies (Castignolles et al., 1998; Chapron et al., 2000; Jiang et al., 2001; Xagoraraki et al., 2007). Muirhead et al. (2005) used an artificial rainfall simulator to evaluate *E. coli* runoff from manure application. There is a need to evaluate the movement of viruses and indicator organisms from biosolids applications on undisturbed soil.

In this study, we applied MAD biosolids and simulated an extended rainfall event over large scale lysimeters (n=5) at an outdoor location. The specific objectives of this work were to: 1) evaluate the movement of indigenous viruses (somatic phage and

adenovirus), and anionic (chloride) and microbial (P-22 bacteriophage) tracers through the soil under nearly saturated conditions, and 2) evaluate the viral concentration of ponded surface water. To the best of our knowledge no research has evaluated the leaching of biosolid-associated pathogens through undisturbed soil in large-scale lysimeters with soil depths greater than 100 cm. The results obtained from the freely drained lysimeters in this study would be particularly applicable to land with tile drain installed. Subsurface draining systems (drain tiles) are installed to remove excess water and protect cropland and groundwater from contaminates in land application sites. However, biosolids-associated pathogens can potentially contaminate the surface water through draining systems. Significant levels of BAdV in drainage samples at the manure application sites were reported previously (Wong and Xagoraraki, 2010).

Methods and Materials

Lysimeters. Six stainless steel containment lysimeters enclosing a monolith of undisturbed soil were installed in large experimental plots (600 m^2) on a *Kalamazoo fine-loamy, mixed mesic Typic Hapludalfs* soil at the Kellogg Biological Station of Michigan State University (MSU) in southwest Michigan. The lysimeters were used from 1994 to 1999 in a study of nitrate movement to groundwater in a rotation of corn (*Zea mays* L.) and alfalfa (*Medicago sativa* L.) with various treatments of compost, manure or inorganic fertilizer (Basso and Ritchie, 2005). They have not been used for experimental work since that time. From 2000 to 2003, continuous corn was grown on all plots and the lysimeter leachate was evacuated annually. No manure or compost has been applied to the plots since 1999.

Figure 4.1 illustrates the cylindrical stainless steel lysimeters over a 0.6 m funnel-shaped extension filled with sand and pea gravel. The dimension of the lysimeters is 1.5 m wide and 2.1 m deep. The open tops are about 30 cm below the soil surface to allow normal tillage and planting operations. A drainage tube extends from the bottom of each lysimeter to the plot edge to avoid unnecessary disturbance of the cropped area. The bulk density of the soil ranged from 1.5-1.6 g/cm^3 (Basso and Ritchie, 2005) and the porosity was approximately 40% on a volumetric basis (based on a particle density of 2.65g/cm^3), which convert to a pore volume of 1.48m^3.

Lysimeter experiments. Biosolids (56,100 L/ha) from the Plainwell (2008) and St. Clair (2009) wastewater treatment (WWTP) plants (Michigan, US) were applied to the surface of the lysimeters during the growing season. The biosolids were received from the WWTP within 24 hr of application and stored at 4^oC. Immediately before application the biosolids were spiked with P-22 bacteriophage to a concentration of 3.00×10^{11} and 1.25×10^{10} PFU/100ml in 2008 and 2009, respectively (Table 4.1). Subsamples were analyzed within 24 hr for concentrations of somatic phage (2009), adenovirus, P-22, and percent total solids (Table 4.1). One mole of anionic tracer (potassium chloride) was mixed in 4L of water followed by 6.4 mm simulated rainfall on the day before biosolids application. The average ambient temperature during the 2008 and 2009 study was 13.3 ^oC (ranging from 7.2 to 19.4^oC) and 15.0 ^oC (ranging from 8.3 to 20 ^oC), respectively. The average surface soil temperature during the 2008 and 2009 study was 10.0 ^oC (range from 5.0 to 16.8^oC) and 11.3 ^oC (ranging from 5.9 to 19.2 ^oC), respectively. Three sensors (5TM Soil Moisture and Temperature Sensor, Decagon

Devices Inc.) were installed in each lysimeter at the depth of 10 cm, 30 cm and 100 cm to monitor soil moisture. The water saturation in each lysimeter soil was about 90% (volumetric basis) during the study period.

Six lysimeters were used: lysimeter numbers one to three (L1 to L3; 2008) and four to six (L4 to L6; 2009). Biosolids were applied on all lysimeters except L3, which was a control. Leachate samples were collected in 2008 and 2009 study; surface water and core soil samples were collected in 2009 study. Leachate and surface samples were monitored for somatic phage (only 2009 study), P-22, adenovirus. The anionic tracer was only monitored in leachate samples.

The biosolids were applied uniformly (9.9 L/lysimeter) to the soil surface and allowed to remain undisturbed for 12 hours prior to simulated rainfall. The uniformity of the application was controlled by using small containers to evenly distribute the biosolids on the lysimeter surface. The rainfall simulator applied water at a rate of 5 cm/h but on a semi-continuous (on and off) basis to minimize surface ponding. A rain gauge was used to monitor the amount of water applied on each lysimeter and water application rate was approximately 6 to 8 cm per day. Irrigation and water sampling continued on a daily basis for about 12 h/day until about 1.7 pore volumes of leachate was collected from each lysimeter. In 2009, leachate samples were collected once a month for three consecutive months after completion of 1.7 pore volumes sampling. Leachate samples (3.9L) were drawn from the bottom of the lysimeters with a peristaltic pump every 0.1 pore volumes and stored in sterilized containers, placed on ice and transported to the laboratory for analysis each day. The leachate collected between each 0.1 pore volume sample was

discharged at least 5 m from the lysimeters. The leachate volume was recorded by a graduated five gallon bucket to monitor the pore volume pass through lysimeter. Approximately 0.1 pore volume of water leached through the lysimeter for every 24 to 36h.

A circular, galvanized steel ring (137 cm by 30cm) was fixed in the soil at a depth of 5 cm on L4, L5 and L6 to retain ponded surface water. Ponded surface water samples were collected daily during the sampling period. Samples (3.9L) were collected in sterilized containers, stored on ice and transported to the laboratory for analysis each day.

Chloride analysis. Chloride concentration in the leachate samples was analyzed by an ion selective electrode (model no. 27502-13, Cole Parmer). The chloride ion instrument is calibrated by plotting the millivoltage reading versus three standard chloride solutions on a semi-log scale. The equation of the calibration curve was used to determine the chloride concentration in the leachate samples.

P-22 propagation and phage analysis. *Salmonella* phage (P-22) was used as a microbial tracer. The P-22 was propagated by infecting its host strain *S. typhimurium* overnight in Tryptic Soy Broth (TSB) (Difco) at 37 ^{o}C and isolated by filtering through 0.45 μm cellulose ester-based membrane (Millipore, MA) to remove cell debris. Somatic phage and P-22 were analyzed by the double layer agar method (USEPA method 1602). The host cell for somatic phage was *E. coli* CN13. All dilutions were made with sterilized phosphate buffer water (PBW).

Adenovirus analysis. Water samples were concentrated to achieve a larger equivalent volume during the qPCR reaction for adenovirus detection. The concentration method developed by Haramoto et al. (2005) was used with the following modification:

Amicon Ultra (Millipore, Billerica MA) rather than Centriprep YM-50 was used to concentrate the NaOH eluent. The filtered volume for surface and groundwater was 100 ml and 2 L, respectively. The final volume of concentrated eluent was around 140 µL and it was stored at -80 ^{o}C for DNA extraction. The primers and probe were adopted from Heim et al. (2003). Each qPCR reaction mix included 10 µL of 2X LightCycler 480 TaqMan Master Mix; 1.0 µL of each forward and reverse primer (each final concentration was 500 nM); 0.6 µL of 10 µM TaqMan probe (final conc. = 300 nM); 2.7 µL of PCR-grade water and 5 µL of DNA sample or standard. The real-time PCR running program (all thermocycles were performed at a temperature transition rate of 20°C/s) was 95°C for 15 min followed by 45 cycles at 95°C for 3 sec; 55°C for 10 sec; 65°C for 60 sec and 30 sec at 40°C. The fluorescent signal was detected after each extension cycle.

Infiltration rates. The *in situ* water infiltration rate was measured with a double-ring infiltrometer (ASTM D3385 – 09) upon completion of the experiment and before the soil cores were removed from the lysimeters. The infiltration rate measured was in upper soil layer and only single measurement was taken due to the time required for each measurement (10 hours per each measurement).

Soil characterization and viral analysis of soil samples. Intact soil cores (3.8 cm diameter) were extracted to a depth of about 90 cm after completion of the rainfall simulation experiments in 2009. Soil probe was only able to extract the soils above the 90 cm depth since the soils below that depth were very sandy and the soil probe was not unable to retain those sandy soils. The soil cores were divided into different layers based on identifiable changes in soil color or texture. A compost of duplicate extracted soil

samples was analyzed for physical and chemical properties (all lysimeter except L3) and residual virus concentrations (L4 to L6). No virus analyses were performed on L1 and L2 soil samples because the core soil samples were taken more than one year after the completion of 2008 experiments. Analysis of the soil physical and chemical properties was done by Soil and Plant Nutrient Laboratory at MSU.

Viruses were eluted from the soil samples by stirring 50 grams of soil in 50ml of 10% beef extract for 30 minutes (Williamson et al., 2003). The solid phase of the mixture was spun down by centrifugation at 10,000 x g for 30 minutes at 4^{o} C and the supernatant was retained for virus analysis.

Recovery of tracer from leachate samples and removal rate of P-22 by the lysimeter. Mass recovery of anionic and microbial tracer was calculated by using trapezoidal rule, where the area under the plot of the effluent concentration (virus/L) versus pore volume (0.1 pore volume = 148L) was measured and then normalized with the initial mass input into the system.

Pang (2009) described the removal rate (λ), the measure of the relative log-reduction in microbial concentration achieved per unit of distance traveled, as the following equation:

$$\lambda = -\frac{Ln\left(mass\ reovery\right)}{x} \quad [1]$$

where *x* is the depth of the lysimeter. The removal rate of P-22 was calculated using equation [1] and the unit is log/m.

Recovery of P-22 from lysimeter soils. The recovery of P-22 from the extracted soil cores (down to depth of 76 to 91 cm) was calculated as the sum of P-22 recovered in

each layer of lysimeter soils normalized with the initial mass of P-22 applied on the lysimeter surface. The P-22 recovered (M_{soil}) in each layer of soil (PFU) was calculated as:

$$M_{soil} = \frac{\left(A \times D \times (1-\theta) \times B \times C_{soil}\right)}{26\%} \quad [2]$$

Where A is the surface area of the lysimeter (cm^2), D is the thickness of each layer of core soil samples (cm), Θ is the porosity (0.40 $V_{pore}/V_{lysimeter}$), B is the bulk density ($1.6 g/cm^3$), C_{soil} is P-22 concentration in each layer of core soil samples (PFU/g) and 26% is the recovery of virus from soils by using the beef extract (Williamson et al., 2003). A homogenous distribution of P-22 in the soils within each layer is assumed, and porosity and soil bulk density are based the previous reported values (Basso et al., 2005).

Decay analysis. First order decay model [eq. 3] was fitted into the phage concentration in the ponded surface water:

$$Ln\left(C_t\right) = -Kt + Ln\left(C_0\right) \quad [3]$$

where C_t is the microorganism concentration (pfu per 100ml) at time t (days), C_o is the phage concentration (pfu per 100ml) at time zero, and k is the decay coefficient (d^{-1}).

Results

Soil characterization. The physical and chemical properties of the soil in each lysimeter are listed in Tables 4.2. The common name of the soil series is Kalamazoo

Loam (Kalamazoo fine-loamy, mixed mesic Typic Hapludalfs). In general, the soil texture was classified as sandy loam although most of the lysimeters had a sandy-clay-loam layer at depths ranging from 20 to 40 cm. The sand content generally increased at greater depth.

The Natural Resource Conservation Service (NRCS) drainage classification was 'poor' with infiltration rates ranging from 3.6 to 8 mm/h for the lysimeters containing orchardgrass or switchgrass (Table 4.3) and the infiltration rates for the lysimeters containing corn was very slow (1.0 mm/h), which is even below the "poor" classification.

Lysimeter effluent. The flow characteristics of each lysimeter varied based on the BTC of the anionic tracer (Figure 4.2). This is reasonable given the variability in soil texture and vegetative covers on the surface. However, the peak concentration occurred around 0.3 PV for all lysimeter. The recovery between lysimeters also varied (Table 4.4) and beside variability in soil physical property, chloride naturally presented in soils and biosolids may also contribute to the recovery differences between each lysimeter.

P-22 was recovered from L2 (orchardgrass), L5 (switchgrass) and L6 (continuous corn) leachate, but no P-22 was detected in L1 or L4. The P-22 breakthrough curves of L2, L5 and L6 are shown in Figure 3. The peak breakthrough in L2, L5 and L6 occurred at 0.7, 0.3 and <0.1 pore volumes (PV), respectively (Figure 4.3). The rapid breakthrough in each of the lysimeters demonstrates transport through preferential flow paths. There was a three to four log reduction of P-22 from the initial concentration in the spiked biosolids to the peak concentration in the leachate. The recoveries of P-22 in L2, L5 and L6 leachate were 0.59, 0.12 and 2.14% of the initial concentration (Table 4.3),

respectively. The P-22 removal rate (λ) for L2, L5 and L6 was 2.4, 3.2 and 1.8 log/m (equation 1).

Viral levels in soil samples. The soil cores extracted from (L4, L5 and L6) at the completion of the simulated rainfall events were evaluated for P-22, somatic phage and adenovirus. No somatic phage or adenovirus was detected, but P-22 was detected in all samples. Interestingly, no P-22 was observed in L4 leachate but the concentration of P-22 in the L4 soil samples was higher than the concentrations in L5 and L6 soils at almost every depth (Figure 4.4). P-22 recovered from top half of L4 soils was significantly higher than the recoveries by L5 and L6 soils (Table 4.4). This may indicate greater sorption to soil particles or possible interactions with the root system.

Surface water. The somatic phage and P-22 concentration in the ponded surface water is shown in Figure 4.5. Somatic phage reached the non-detectable levels around day ten. Adenoviruses were detected in all surface water samples but no decay trend was observed. The average concentration of adenovirus in the surface water by the end of the study was $7.92 \pm 4.56 \times 10^3$ copies/100ml, which was about 4 logs lower than the concentration in biosolids. It took longer for P-22 to completely decay (>21 days), this is likely because the initial concentration of P-22 was several orders higher than the concentration of somatic phage. The greatest viral concentration in the ponded water was at the start of the simulated rainfall events and the samples with the greatest concentration were about 1 to 10% of the initial concentration in the spiked biosolids. The reduction of viruses in the ponded water samples over time fit the first order decay model (Figure 4.6). The decay coefficients of somatic phage and P-22 were similar (slightly less than 0.40/day).

Discussion

Based on the P-22 results, the viral removals in three of our lysimeters (1.83 to 3.21 log/m) were similar to that reported by Jiang et al. (2008) (1.92 to 2.80 log/m) and Carlander et al. (2000) (3.76 log/m); however, manure-associated indicators were detected in the leachate samples from the study by Jiang et al. (2008) but no biosolids-associated viruses were detected in our study. Perhaps the reason no biosolids-associated viruses were detected was because the lysimeters were 2.1 m in depth compared to depths of 0.4 to 1.0 m reported in earlier work. The results of our work indicate that sandy loam soil can be an effective filter for removing enteric viruses for groundwater protection, but the depth to the water table is an important consideration.

The low quantity of P-22 recovered from leachate and core soils (<2.3% of recovery in each lysimeters; Table 4.4) indicates that the majority of P-22 and indigenous virus are sorbed to soil particles and eventually decay. Also, there was a three to four log reduction in the P-22 concentration from the initial concentration in the spiked biosolids to the peak concentration in leachate. Because the concentration of somatic phage in the biosolids was only 8.00×10^{2} PFU /ml (or 8.00×10^{4} PFU /100ml) and the volume of leachate samples analyzed was only 2 ml, an inability to detect somatic phage is reasonable.

The adenovirus genome concentration in the biosolids was about 3 logs greater than the somatic phage and the equivalent volume of each qPCR reaction was about 50 ml but adenoviruses were not detected. There is evidence that an indigenous virus can have a strong attachment to sludge materials. Chetochine et al. (2006) reported that a majority of the indigenous phage remained in the solid pellet after a series of extractions.

Gerba et al. (1980) reported that enteroviruses formed strong attachment to sludge particles and were difficult to elute. Sano et al. (2004) reported that the virus-binding proteins (VBPs) in a bacterial culture from activated sludge play a key role in attaching indigenous viruses to sludge particles. Based on reports, it is likely that adenovirus formed a stronger attachment to the sludge solids than the P-22 did and was more easily sorbed or filtered and retained in the soil. There is a need for additional work investigating the transport of indigenous viruses in the natural environment. Additionally, the low recovery of adenovirus may be related to the sampling process. Based on recent published work (Fong et al., 2010), the recovery of adenovirus in MilliQ water and a river water matrix ranged from 0.17 to 6.98 percent, much lower than the 30 to 74 percent recovery reported earlier (Haramoto et al., 2005). Perhaps 1MDS filter would have been a better option for this work because it has greater recovery (around 30%) and is able to accommodate a larger sample volume.

The rapid breakthrough of the anionic and microbial tracers (<1PV) revealed the action of preferential flow pathways in all of the lysimeters. Preferential flow was also reported in earlier lysimeter studies (Aislabie et al., 2001; McLeod et al., 2001, 2003, 2004; Pang et al., 2008; Jiang at al., 2008). Most column studies simulate piston flow in a homogenous soil matrix with a peak breakthrough about 1 PV (Cheng et al., 2007; Chu et al., 2001; Powelson et al., 1991; Jin et al., 1997; Jin et al., 2000; Chetochine et al., 2006). Because of the presence of preferential flow pathways in the natural environment, the transport rate measured in laboratory scale experiments may be considerably less than the transport rate in the natural environment. Additionally, a greater microbial removal rate would likely occur in a repacked soil column compared to actual soil conditions,

because the preferential flow pathways allow the contaminants to bypass the soil matrix. Beside natural crack of soils, vegetative covers could also improve infiltration by providing root channels for preferential water movement. Annual tillage reduces soil aggregation and buries crop residue, which could reduces the macropores and thus prevent pathogens transport through the soil.

We attempted to correlate the P-22 recovery and breakthrough characteristic with the chloride recovery and BTC, infiltration rate, root system, and the existence and depth of sandy clay loam; however, no correlation was observed. High concentration of P-22 in L4 soils could indicate more P-22 got trapped in L4 soils, which can explain the result of no breakthrough in the leachate samples. However, we are not sure why L4 soils trapped more P-22 since L4 soils were similar to the other lysimeters.

Because rain drops have velocity and break down soil particles upon impact there was mixing of water, soil and biosolids at the soil surface during the simulated rainfall. The ponded water samples included viruses attached to soil particles, waste or slurry particles, and unattached cells or clumps. Each of these are sources of contaminants that can be transported in overland flow (Tyrrel and Quinton 2003; Muirhead et al., 2005). Based on the results of our work, the virus concentration in ponded surface water can be as great as 1% to 10% of the initial virus concentration in the biosolids. These virus concentrations represent a considerable threat to water quality from surface runoff if biosolids are allowed to remain on the soil surface after application. The biosolid-associated pathogens could potentially exists for several days under wet conditions based on the decay rate reported in this study.

In previous study, no occurrence of enteroviruses in sludge contaminated soil was observed after 14 days of application (Pourcher et al., 2007) but adenovirus was still detected after 20 days in our study; the difference in the observations may due to the strong UV resistance of adenoviruses.

Conclusions

Based on the results from this study, preferential flow plays a critical role in terms of virus transport in the subsurface. The salmonella phage removal rates obtained in this study ranged from 1.8 log/m to complete removal. The viral levels in the runoff could be as much as 1 to 10% of the microbial levels in the biosolids. More studies should investigate the transport of microorganism in subsurface by the experiments designed with more natural system simulations.

The results of this work and related works with the application of manure on artificially drained land will be used to develop best management practices for the land application of manure and biosolids on drained land. Some of the management practices that will protect water quality are: 1) pre-tillage to disrupt the continuity of macro-pores, 2) controlled (low) application rates, and 3) timing manure application rates to avoid application on wet ground, when tiles are flowing, or when there is a chance of significant rainfall (> 0.5 inches) within the next few days.

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Tables and Figures

Table 4.1. Initial somatic phage, P-22 and adenovirus concentration in biosolids.

Year of study	% solid	Somatic phage (PFU/100ml)	P-22 (PFU/100ml)	Adenovirus (copies/100ml)
2008	5.0	NA	3.00×10^{11}	4.20×10^{8}
2009	6.0	8.00×10^{4}	1.25×10^{10}	3.30×10^{7}

NA = not available

Table 4.2. Physical and chemical characteristics for each lysimeter applied with biosolids.

Lysimeter and depth (cm)	pH	CEC (meq/100g)	OM %	Sand %	Silt %	Clay %	Soil classification	P ppm	K ppm	Mg ppm	Ca ppm
L1											
0-25	6.4	7.7	1.8	55.2	35	9.8	Sandy loam	54	131	165	1196
25-46	7.1	7.7	1.4	53.2	35	11.8	Sandy loam	54	69	157	1235
46-61	7.3	9.1	1.1	56.4	28.8	14.8	Sandy loam	52	95	180	1474
61-91	7.1	8.7	0.6	71.8	9.4	19.8	Sandy loam	28	18.1	79.1	1372
L2											
0-25	6.9	8.2	1.1	62.4	23.8	13.8	Sandy loam	28	95	196	1273
25-43	7.1	8.8	1.2	54.8	29.4	15.8	Sandy loam	36	83	210	1368
43-76	6.9	11	0.9	67.8	9.4	22.8	Sandy Clay Loam	27	120	238	1735
L4											
0-23	7.2	9.1	2.1	52.4	36.8	10.8	Sandy loam	66	111	218	1408
23-41	7.2	11.6	1.1	52.8	25.4	21.8	Sandy Clay Loam	28	115	249	1843
41-51	7.2	10.5	0.9	68.8	12.8	18.4	Sandy loam	29	106	228	1971
51-76	7.2	3.5	0.5	86.9	3.2	9.9	Loamy Sand	35	54	98	519
L5											
0-20	6.8	8	2.2	53.8	35.8	10.4	Sandy Loam	64	126	201	1210
20-36	7	9	1.2	43.8	32.8	23.4	Loam	40	76	216	1393
36-51	6.9	11.7	1.1	47.8	25.4	26.8	Sandy Clay loam	29	127	303	1769
51-66	6.9	8.6	0.8	73.9	7.7	18.4	Sandy loam	30	77	166	1399
66-84	7	4.5	0.5	88.1	2.5	9.4	Loamy sand	37	38	92	733
L6											
0-15	6.6	7	2	51.2	36	12.8	Sandy loam	50	104	171	1062
15-25	6.9	6.6	1.3	51.4	33.8	14.8	Sandy loam	56	57	162	1016
25-46	7.1	9.3	1.2	52.4	32.8	14.8	Sandy loam	64	90	242	1415
46-61	7	7.7	0.8	54.8	21.4	23.8	Sandy Clay Loam	41	67	200	1164

Table 4.2 cont'd

61-74	7.1	8	0.6	69.8	11.4	18.8	Sandy loam	40	75	210	1205
74-91	7.1	7.5	0.6	63.8	16.4	19.8	Sandy loam	39	68	188	1155

CEC = Cation exchange capacity; measured by ammonium acetate method (Thomas 1982)
OM = Organic matter; measured by Loss-on-ignition method (Brown et al., 1998)
P = Phosphorus; measured by ascorbic acid method (Brown et al., 1998)
K = Potassium; Ca=Calcium; measured by flame emission spectrophotometry (Brown et al., 1998)
Mg = Magnesium; measured by colorimetrical method (Brown et al., 1998)
Sand/Clay/Slit percentage measured by Bouyoucos Hydrometer (Bouyoucos, 1962)

Table 4.3. Infiltration rates, drainage classification, root system of each lysimeters.

Lysimeter	Infiltration rate (mm/hr)	Drainage class	Crop
L1	5.9	poor	orchard grass
L2	3.6	poor	orchard grass
L3 (control)	8.0	poor	orchard grass
L4	7.7	poor	switch grass
L5	4.3	poor	switch grass
L6	1.0	poor	corn

Table 4.4. Recovery percentage of chloride and P-22 from leachate and top half of lysimeter soils.

lysimeter	leachate		soil[a]
	Chloride	P-22	P22
L1	71.3	ND	NA
L2	74.2	0.59	NA
L3	32.6	NA	NA
L4	99.3	ND	0.19
L5	74.2	0.12	0.011
L6	51.2	2.14	0.012

ND = not detected; NA = not available
a = no chloride analysis on soil samples

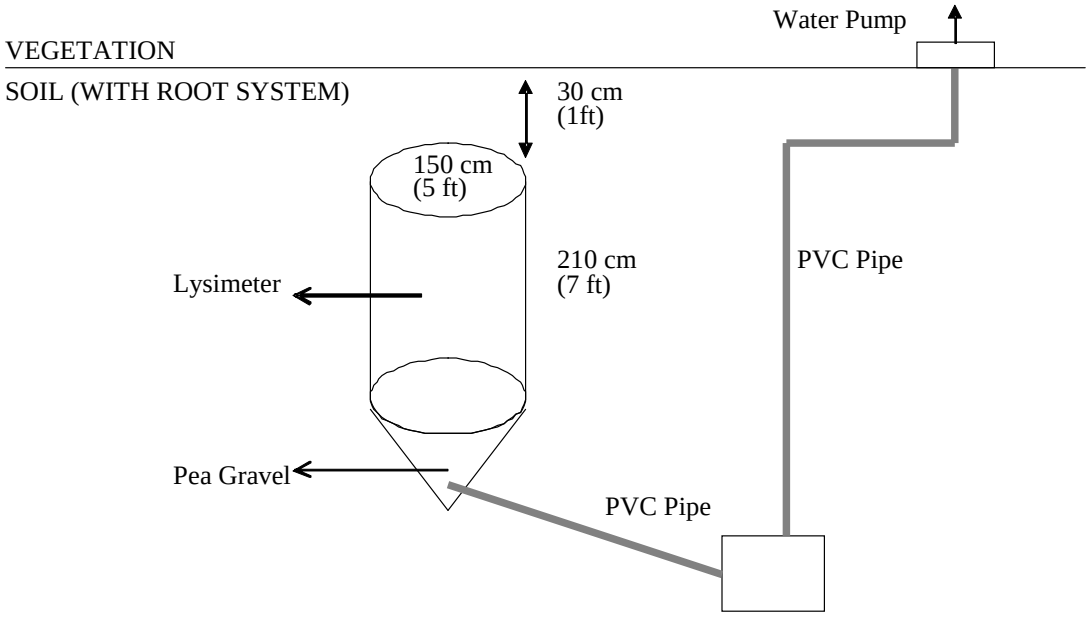


Figure 4.1. Diagram of the field site lysimeter.

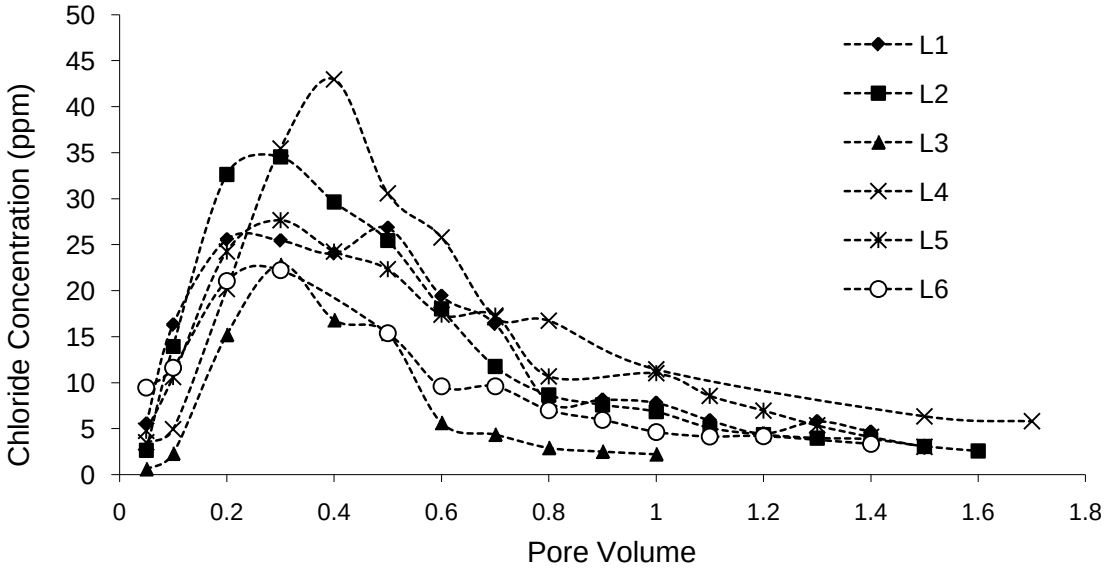


Figure 4.2. Breakthrough curves of chloride in each lysimeter.

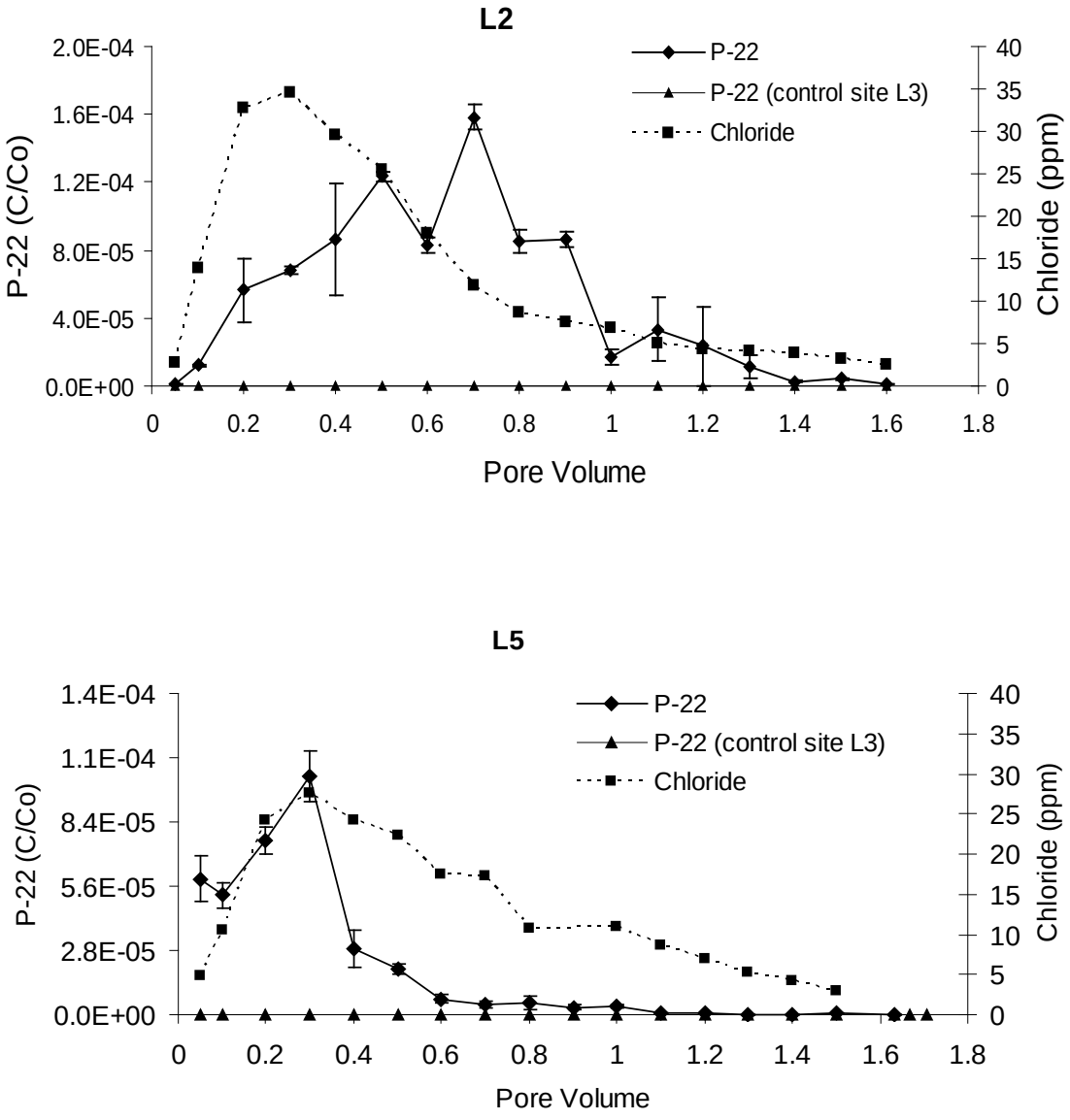
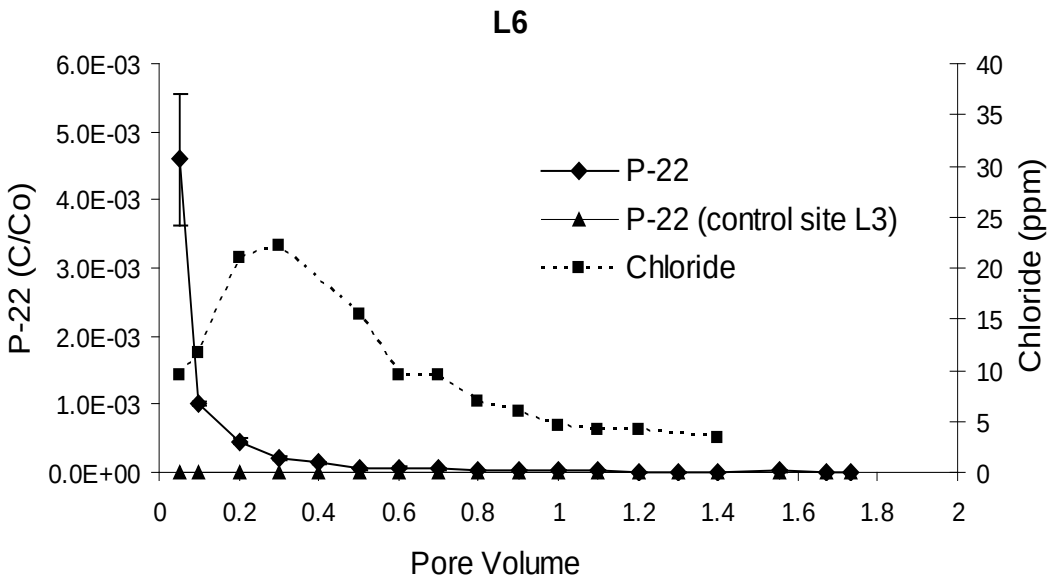


Figure 4.3. BTC of P-22 in L2, L5 and L6 leachate samples. Error bars represent the standard deviation of the duplicate measurements from each sample.

Figure 4.3 cont'd



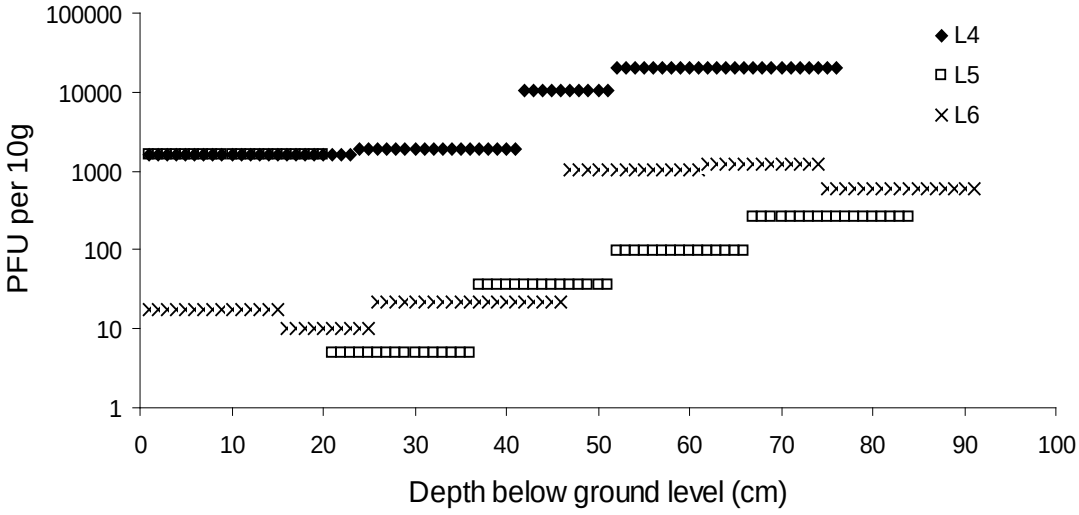


Figure 4.4. P-22 concentrations in soils with different depth below the surface; No somatic phage nor adenovirus was detected in soil samples.

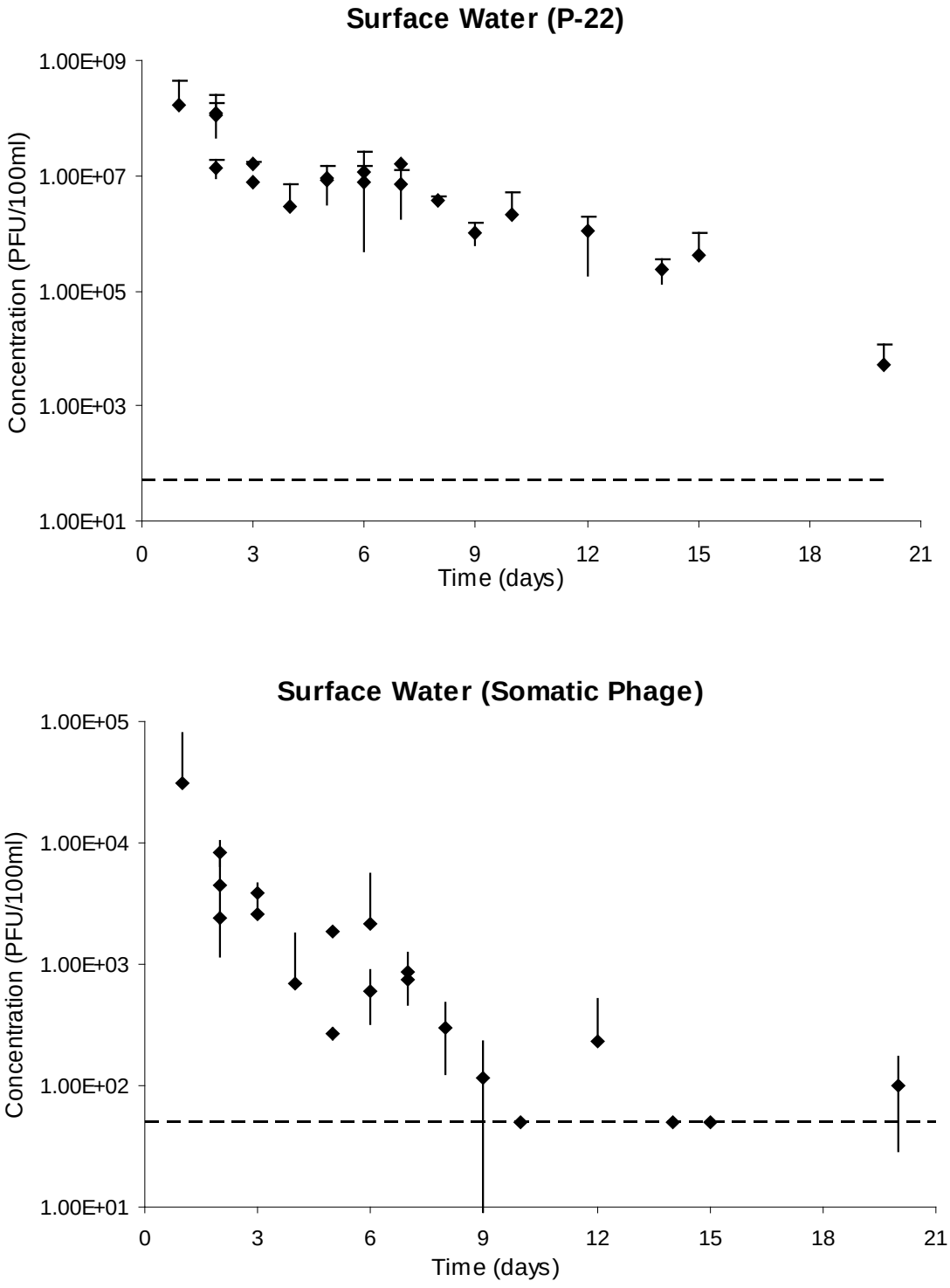


Figure 4.5. P-22 and somatic phage levels in surface water samples over the course of study. Dot-line represents the detection limit. Error bars represent the standard deviation of the measurements from each lysimeter (L4, L5 and L6).

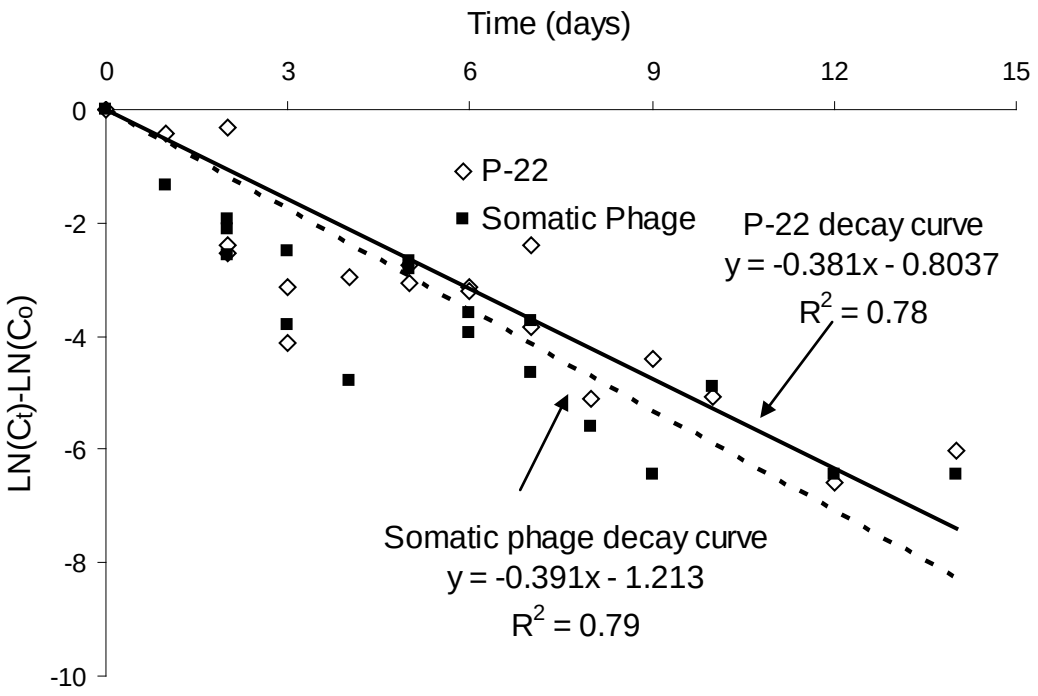


Figure 4.6. Decay curves of P-22 and somatic phage in surface water samples from L4, L5, and L6.

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CHAPTER 5

THE EFFECT OF ORGANIC MATTER ON ADENOVIRUS SORPTION TO SOLIDS: SOIL PARTICLES AND POLYETHYLENE SURFACES

Abstract

Human adenovirus (HAdV) is considered the most prevalent enteric virus in human fecal material and environmental media, but its sorption characteristics have not been reported in the literature. We investigated the sorption characteristic of HAdV to two solid materials, soil and polyethylene (PE), with the main focus on the effect of organic matter (OM). Sorption isotherm and sequential extraction experiments were performed to determine the effect of OM on sorption and desorption of HAdV, respectively. The hypothesis of sorption causing the loss of HAdV tumbled in PE vials was also investigated. Soils with 2%OM had a sorption capacity for HAdV that was 4 times higher than 8%OM soils. The percentage of HAdV desorbed from 8%OM soils was almost 8 times higher than the one from 2%OM soils. Dissolved organic matter (DOM) in the liquid phase also significantly inhibited the sorption of HAdV to both soils and PE vials. The results from virus recovery and DNA stability experiment provided evidences that the loss of HAdV tumbled in PE vial was due to sorption rather than inactivation. This study suggested that OM plays an important role on sorption and desorption of HAdV.

Keywords: Adenovirus, sorption, desorption, organic matter, soil, polyethylene.

Introduction

Sorption to soil is one of the most important factors contributing to the removal and transport of virus and other water-transmitted pathogens in the environment (Schijven and Hassanizadeh, 2000). Batch experiments have been used to investigate factors affecting the virus-soil sorption behavior. With the exception of studies on enteroviruses (Jin and Flury, 2002), most viral agents selected for previous sorption experiments were bacteriophage which are viral indicators and have a simple analytical procedure. However, many enteric viruses can be quantified directly without extensive labor by recently developed molecular methods such as quantitative polymerase chain reaction (PCR).

Human adenovirus (HAdV) is an enteric virus which has received a lot attention recently. HAdV has been included in the Environmental Protection Agency's contaminant candidate list (CCL) one, two and three. HAdV is a common cause of gastroenteritis, upper and lower respiratory system infections, and conjunctivitis (Jiang 2006). HAdV has been found to have the highest concentration in both sewage and biosolids when compared to other enteric viruses (Katayama et al 2008; Wong et al 2010). Therefore, the potential of transmission of HAdV through water should not be overlooked. Jin and Flury (2002) summarized virus-soils sorption batch studies done in last 20 years. Most of these studies have focused on the effect of pH and ionic strength of the solution, presence of compounds that compete for binding sites on sorbents (e.g. organic matter), and properties of the sorbent. However, no study has investigated these factors on the sorption characteristic of HAdV. This knowledge gap should be addressed since not all viruses have the same responses to these factors. For example,

dissolved humic acid significantly promoted the transport of MS2 but little effect on the transport of ΦX174 was observed (Zhuang and Jin 2003a).

The presence of organic matter (OM) is a major factor responsible for the uncertainty associated with predicting virus transport in soils and groundwater. However, there is controversial discussion on the effect of bonded-OM on virus sorption. Bales *et al.* (1991) and Kinoshita *et al.* (1993) reported OM coated on grain surface could enhance the virus sorption by increasing the hydrophobicity of the solid surfaces. However, a decrease of virus sorption or increase of virus transport was observed in soils with higher OM from other studies (Fuhs *et al.* 1985; Moore *et al.* 1981; Zhuang and Yin 2003a). The results from these studies create some ambiguity and hinder our ability to draw conclusions about the effect of bonded-OM on virus sorption.

Previous studies have shown that tumbling virus suspensions in polypropylene vessels resulted in significant loss of virus (Thompson et al 1998; Thompson and Yates 1999). The authors concluded that the loss of virus in the suspension was due to the force at the triple-phase boundary (TPB) damaging the virus protein capsids and thus inactivating the virus (Thompson et al 1998). This observation of inactivation occurred with MS2 but not ΦX174. The authors explained that since ΦX174 is more hydrophilic than MS2, ΦX174 becomes more resistant to forces at the hydrophobic TPB and does not partition at the air-water interface (AWI) to the same extent as MS2. However, Zhuang and Jin (2003b) suggested that virus with high hydrophobicity (such as MS2) increases sorption at the AWI and the sorption become irreversible. Zhao et al (2008) stated that the discussion on the effect of soil water content on virus

adsorption/inactivation remains largely speculative and that mechanistic understanding can only be achieved through further experiments that can provide direct evidence.

In this study, we investigated the sorption characteristic of HAdV to two solid materials, soils and polyethylene (PE) surface, with the main focus on the effect of organic matter (OM). Isotherm sorption and sequential extraction experiments with soils at two different levels of natural OM (2 and 8%) were used to investigate the effect of natural bonded-OM on adsorption and desorption of HAdV, respectively. The effect of dissolved organic matter (DOM) in liquid solution was also included in this study. We attempted to provide evidence that the loss of HAdV in the suspension tumbled in PE vial was due to sorption rather than inactivation by performing a virus recovery and DNA stability experiment. The effect of DOM and solution ionic strength on sorption of HAdV to PE surface was also determined.

Methods and Materials

Propagation of adenovirus. The adenovirus (HAdV) serotype 2 was selected for this study and it was obtained from American Type Culture Collection (ATCC) (VR-846). HAdV was propagated in A549 cell lines (passage 108, obtained from ATCC (CCL 185)). The propagation procedure is briefly described as the following; the A549 cells were grown in flasks until reaching at least 80-90% confluence and then 100 μl of stock ATCC virus culture were added to culture flask. Cells were maintained with minimum essential media (MEM) supplemented with L-glutamine, Earle's salts, and 2% fetal bovine serum. Cytopathic effects (CPE, indicative of a viral infection) in almost the entire cell cultures developed two days after infection. The flasks were

frozen and thawed three times, and the virus culture was transferred to a 50 ml centrifuge tube and centrifuged at 10,000x g. After centrifugation, the supernatant was filtered through 0.22 μm membrane to remove the cell debris. Filtrate was aliquoted into cryogenic vials and stored in a -80 °C freezer immediately. This filtrate served as the HAdV stock for the entire study.

To determine the infectious HAdV concentration of the stock, different serial 10- fold dilutions of stock were added to multiple cell culture flasks and the CPE was monitored in each flask. The concentration of infectious HAdV was estimated by the free most-probable-number (MPN) software downloaded from (http://www.i2workout.com/mcuriale/mpn/index.html) and the MPN values for the stock was 2.4×10^{9} MPN/ml.

Nucleic acid extraction. The DNA of HAdV was extracted by the MagNA pure automatic extraction machine (Roche), using MagNA Pure Compact Nucleic Acid Isolation Kits (Roche). 400 μL of the sample was extracted and the final elution volume was 100 μL. The nucleic acid eluents were stored in a -80 ^{o}C freezer prior to molecular analysis.

For the recovery of sorbed HAdV from PE vials, HAdV-DNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) based on the spin protocol listed in the manufacturer's handbook.

Quantitative PCR assay and reaction condition. Quantitative PCR (qPCR) was used to determine the concentration of HAdV in this study. The primers and probe for quantification of HAdV were adopted from Heim et al (2003). Each qPCR reaction mix included 10 μL of 2X LightCycler 480 TaqMan Master Mix; 1.0 μL of each

forward and reverse primer (each final concentration was 500 nM); 0.6 µL of 10 µM TaqMan probe (final concentration 300 nM); 2.7 µL of PCR-grade water and 5 µL of DNA sample or standard. The real-time PCR running program was 95°C for 15 min followed by 45 cycles at 95°C for 3 sec; 55°C for 10 sec; 65°C for 60 sec and 30 sec at 40°C. All thermocycles were performed at a temperature transition rate of 20°C/s and the fluorescent signal was detected after each extension cycle.

Calculation of virus concentration in the solution. The following formula illustrates the calculation of the concentration of virus in the liquid phase for all the experiments performed in this study:

$$C_{liquid} = M_{qPCR} \times \frac{V_{DNA}}{V_{qPCR}} \times \frac{1}{V_{extract}} \times \frac{1000\,\mu l}{1 ml} \qquad [1]$$

Where C_{liquid} is virus concentration in the liquid phase (virus per ml), M_{qPCR} is the copy number of HAdV-DNA quantity detected in the PCR reaction (copies) and one copy of DNA is equivalent to one HAdV, V_{qPCR} is the qPCR reaction volume (µL), V_{DNA} is the volume of extracted DNA (µL), $V_{extract}$ is the volume of solution used for DNA extraction (µL).

Soils. Soils with two different organic matter (OM) content were used in this study and The OM content, dissolved organic matter (DOM), clay/silt/sand percentage, pH, cation exchange capacity (CEC), bulk and particle density of the soil are illustrated in Table 5.1.

Tumbling condition for sorption experiments. The tube and vials were tumbled by a tube rotator (Cole Palmer). The tumbling speed for all of the experiments

performed in this study was 20 rpm. The tumbling period for all sorption and desorption experiments was 24 hours and 5 hours, respectively. The vials and tubes were tumbled in a 4 ^{o}C incubator to minimize the inactivation.

Sorption of HAdV to polyethylene vials. PE vials used in this study were cryogenic vials (Corning, NY) with a volume capacity of approximately 2.5mL. Suspensions with four different virus concentrations (10^8, 10^7, 10^6, 10^5 viruses/ml) were prepared and the initial concentration of HAdV (C_o) was quantified immediately by qPCR. The five different suspension media used in this part of study were phosphate buffer saline (PBS), soil/PBS, soil extracted solution (SES), MilliQ water and humic acid (HA) solution. Soil/PBS vials were prepared by mixing 2 ml of PBS with 200μg of 8%OM soils (soil-liquid ratio=1:10 by weight). To prepare SES, 8%OM soil-PBS suspension (1:10 soil-PBS ratio) was tumbled for 24 hours and then centrifuged at 2,000xg for 5 minutes. The supernatant after centrifugation was the SES. HA solution was prepared by dissolving appropriate amount of Elliott Soil Humic Acid Standard (International Humic Substances Society, MN) into PBS. This is a natural humic acid derived from soil. The total organic carbon (TOC) concentration of HA solution was 30ppm. After the tumbling, 400 μL of the suspension was withdrawn and the concentration of HAdV (C_{SORB}) in the suspension was measured and the fraction of HAdV remained in the suspensions tubes was calculated ($C_{SORB/}$ Co). For soil/PBS vials, the suspensions were transferred to 2 ml PE centrifuge tubes and centrifuged at 2,000xg for 5 minutes. Then, the supernatants were withdrawn and analyzed for HAdV. All steps were performed in duplicate.

Recovery of HAdV from the polyethylene vials. As mentioned above, four different virus concentrations (10^8, 10^7, 10^6, 10^5 viruses/mL) suspended in PBS were prepared and the initial concentration of HAdV (C_O) was quantified immediately by qPCR. Then 2 mL of suspension was added to PE tube and tumbled. Following tumbling, the concentration of HAdV (C_{SORB}) in the suspensions was quantified and the fraction of HAdV that remained in the suspension was calculated (C_{SORB} / C_O). Then the vials were emptied and two approaches were used to recover the HAdV sorbed to the PE tube. The first approach used 10% beef extract (BE) pH 9.5 to elute the HAdV sorbed to the PE tube. The second approach used virus lysis buffer (AVL) (Qiagen, CA) to lyse the protein capsid of the sorbed HAdV, which would result in releasing the HAdV-DNA into lysis buffer. For the BE approach, vials were filled with 2 mL of BE and tumbled for 24 hours at 4^{o}C. For the lysis buffer approach, vials were filled with 2 mL of AVL and tumbled for 20 minutes at room temperature. The concentrations of HAdV in BE (C_{BE}) and AVL (C_{AVL}) were determined by qPCR as described above and the recovery of HAdV (C_{BE}/C_O and C_{AVL}/C_O) was calculated. All steps were performed in duplicate.

Stability of HAdV-DNA in the polyethylene vials during the tumbling process. To investigate the fate of capsid-free HAdV-DNA after the tumbling process, the DNA of HAdV pure culture was extracted and spiked into the PBS solution and then tumbled. The concentrations of HAdV-DNA were quantified by qPCR and the recovery was calculated. Experiments were performed with four different concentrations of DNA suspensions, where the concentrations of the DNA were similar

to the HAdV concentrations used in the experiments described in the polyethylene vial sorption and recovery of HAdV from the polyethylene vials experiments. All steps were performed in duplicate.

Sorption of HAdV with glass centrifuge tubes. The sorption of HAdV to glass centrifuge tubes was evaluated. The procedure was similar to the procedure used for the PE vial sorption experiments except only suspensions in PBS and SES matrices (2%OM soils and PBS) were tested. Briefly, fourteen mL of HAdV suspension was added to15 mL Kimble glass centrifuge tubes (Vineland, NJ) and tumbled at 4 ^{o}C for 24 hours. After the tumbling, 400 μL of the suspension was withdrawn and the concentration of HAdV (C_{SORB}) in the suspension was measured by qPCR and the fraction of HAdV that remained in the suspensions tubes was calculated ($C_{SORB/}$ C_{O}). All steps were performed in triplicate.

Sorption isotherm experiments. Three isotherm experiments were conducted in glass centrifuge tubes using the following three soil/suspension conditions: (1) 2%OM soils and PBS suspension, (2) 8%OM soils and PBS suspension, and (3) 2%OM soils and 150ppm HA suspension. HAdV suspension for isotherm experiments was prepared by diluting HAdV stock in PBS or HA solution to the desired concentration (10^3 to 10^6 virus/ml). Fourteen mL of suspension were added to glass centrifuge tubes containing 1.4 gram of soils (soil-liquid ratio=1:10) and then tumbled. The tumbling period of 24 hours was selected based on a preliminary experiment showing that sorption equilibrium was reached after 24 hours of equilibration (data not shown). Then tubes were centrifuged at 2,000xg and 400μl of the supernatant was collected for virus assay.

Virus inactivation and sorption to the tube was monitored by control tubes. Controls tubes were filled with 14 mL of SES suspension. The procedure for preparing SES was similar to the procedure described in the "polyethylene vial sorption experiments' section except three types of SES were prepared using the 3 soil/suspension condition described above. SES was chosen as the liquid matrix for the controls because soils may release enzyme that could cause inactivation of virus (Nasser et al 2003) and DOM may cause PCR inhibition (Wilson 1997). Therefore, we believe that SES is a better suspension than PBS or HA solution to correct for other factors (beside sorption) that might cause losses of HAdV and/or decrease in quantification values. Control tubes received only virus solution and were treated in the same manner as the experimental tubes. All steps of this experiment were performed in triplicate.

The mass balance equation for computing virus sorbed on solid is described by the following equation:

$$C_S = \left[C_I - C_L\right] / M \quad [2]$$

where C_I, C_L, and C_S are, respectively, the concentrations of virus in the control liquid phase (virus/ml), in the experimental liquid phase (virus/ml), and sorbed to the solid (virus/ml) and M is the total mass of solid per unit volume of virus suspension (grams per ml) used in each batch experiment.

The sorption data was fitted to the logarithmic form of the Freundlich equation:

$$\log C_S = \log K_F + N \log C_L \quad [3]$$

Where K_F (ml/g) is the Freundlich constant, N is the slope of the plot of log C_S vs. log C_L. K_F is roughly related to sorbent capacity and N relates to the intensity of sorption (Burge and Enkiri 1978).

Sequential desorption of HAdV from soils. To evaluate the effect of OM on desorption of HAdV from soils, sequential extraction experiments were conducted after the completion of the sorption experiments. Sequential desorption experiments were only performed on soils sorbed with the highest initial spiked HAdV suspension (10^8 virus/mL). After the sorption experiment, the remaining supernatant was decanted and replaced by the fresh PBS solution and tumbled for 5 hours. After tumbling, the tubes were centrifuged and an aliquot of the supernatant was taken out for virus analysis and the remaining supernatant was decanted. The new PBS was then added to the tubes again and the same desorption procedure was repeated seven times. All steps of this experiment were performed in triplicate.

The accumulated percentage of HAdV desorbed from the sequential extraction was calculated by the following formula:

$$\%_{DESORBED} = \sum_{i=1}^{n} \left(\frac{M_{DESORBED}}{M_{SORB}} \right) \times 100\% \quad [4]$$

Where $M_{DESORBED}$ is the quantity of virus desorbed at each sequential extraction (numbers of virus), M_{SORB} is the quantity of virus sorbed to soils after the sorption experiment (numbers of virus) and N is numbers of extraction.

Statistical analysis. To determine significant differences, analysis of variance (ANOVA) single test was performed using SPSS version 17.0. P-values less than 0.05 indicate a significant difference.

Results and Discussion

Recovery of virus from polyethylene tubes. Figure 5.1 illustrates the recovery of HAdV sorbed to the polyethylene (PE) vials. The recovery by BE was significantly lower than the recovery by AVL for all four suspensions ($P \leq 0.05$). The recovery by AVL for vials tumbled with 10^7, 10^6 and 10^5 virus/ml suspension was 1.62, 0.30 and 0.99, respectively. Lower recovery by AVL for vials tumbled with 10^8 virus/ml suspension was reasonable since high fraction (0.63) of HAdV remained in this suspension after tumbling.

Because of the low recovery results using beef extract/Tween 80/glycine, Thompson et al (1998) concluded that the loss of MS2 in suspensions in polypropylene vessel was not due to adsorption. However, the recovery results by lysis method presented in this study give strong evidence that the loss of HAdV in the suspensions was by sorption. Even though these sorbed HAdV could have been inactivated before or after the sorption, it is clear that they did sorb to PE surface by the end of the tumbling period. Low recovery results by BE elution method were similar to previous findings (Thompson et al 1998). The inability to elute the virus is not unexpected since both reversible and irreversible sorption take place when virus sorbed to solids (Schijven and Hassanizadeh, 2000). Jin and Flury (2002) suggested that protein sorption is similar to virus sorption since the virus are composed of RNA and DNA that is surrounded by a

protein capsid. Yuan et al (2000) described irreversible protein sorption as the adhesion between the charged particle and the surface with opposite charge is very strong and it would maintain in a stable position at the surface. Also, desorption process could be an extremely slow rate process following an initial fast phase (Pavlostathls and Mathavan 1992) and a long period of time may be required to desorb a majority of the virus from the solid surface.

Furthermore, Thompson et al (1998) described the loss of virus by first-order decay. The first-order decay rate is not concentration dependent; therefore, the fraction of HAdV loss in PE vials with different concentration of suspension should have been similar if the loss of HAdV was due to inactivation. However, our data showed significantly more loss of HAdV in lower concentration vials, and in our opinion, this observation could be explained as HAdV completely saturated the sorption sites of PE surface in the vials with high virus concentration, and therefore the fraction of virus losses in those vials became relatively insignificant.

Stability of HAdV-DNA in the polyethylene vials during the tumbling process. Unlike the culture technique used in previous sorption studies, qPCR measures both viable and inactivated HAdV; therefore, the decrease of HAdV in the suspension measured by qPCR should not be affected by the virus being inactivated by the TPB force. If the TPB force was strong enough to destroy the virus protein capsid, it may result in three scenarios involving the capsid-free HAdV-DNA; (1) TPB force could also destroy the DNA (2) DNA could adsorb to PE surface or (3) DNA could degrade after the tumbling process. All of these scenarios could lead to decrease of measurement values by qPCR. However, results showed the concentration of HAdV-DNA in the PBS

suspension remained the same after 24 hours of tumbling. The mean fraction of DNA remaining in the suspensions was 1.03±0.05 (range from 0.96 to 1.10). This result clearly showed that the capsid-free HAdV-DNA would not degrade or sorb to the PE vials during the tumbling period.

Effect of ionic strength on sorption of HAdV to polyethylene vials. Figure 5.2 illustrates the effect of ionic strength, the present of soils, soil extracted solution and humic acid (HA) to the sorption of HAdV to PE vials. For 10^7, 10^6 and 10^5 virus/ml suspension, the loss of virus in PBS suspension was greater than the loss of virus in milliQ water. There was no significant loss for 10^8 virus/ml suspension in either water or PBS matrix. There was no loss of HAdV for water suspension until the concentration reached 10^6 and 10^5 virus/ml. Increasing ionic strength shrinks the thickness of the electrical-double-layer surrounding viruses and solids, which would result in a closer proximity between the surface of virus and solid, and therefore enhance the sorption (Jin and Flury 2002). This could explain the larger HAdV loss in PBS solution than in water since the ionic strength of PBS is higher than MilliQ water.

Effect of dissolved organic matter on sorption of HAdV to polyethylene vials. The soil-extracted solution (SES) had DOM concentration of 108 ppm. No loss of HAdV was observed with SES suspension (Figure 5.2). Also, the loss of HAdV was minimal with HA suspension. The pH values for PBS, SES and HA virus suspensions were 7.5, 7.1, and 7.5, respectively. Therefore, we do not think pH is the controlling factor of the virus sorption in this case. We believe the DOM was the major factor in preventing virus sorption to the PE surface. Previous virus transport works have

indicated that DOM enhances the transportation of virus (Powelson et al., 1991; Zhuang and Yin 2003a; Bradford et al., 2006). In these studies, the mechanism of how DOM enhances virus transport is usually explained as competition between DOM and viruses for sorption sites and thus DOM inhibits the sorption of viruses to soil particles. DOM could therefore have a similar effect on virus sorption to PE surface. There were some losses of HAdV in the vials with the presence of soils, which is likely due to sorption of viruses to soils.

Sorption of HAdV with glass centrifuge tubes. After HAdV suspension tumbled for 24 hours in glass centrifuge tubes, the mean recovery of four different concentrations of HAdV suspensions in PBS were 0.83±0.08 (range from 0.78 to 0.95) and in SES were 1.00±0.21 (range from 0.72 to 1.24). Based on this result, there was still some loss of HAdV in glass tube during tumbling but it was significantly better than PE vials ($P \leq 0.05$). The loss of HAdV could be due to sorption of HAdV to the Teflon lining on the cap. Since the loss of virus was minimal and significantly lower than the loss of virus in PE tube, virus-soils sorption isotherm and desorption experiments were performed in glass tubes.

Effect of organic matter on HAdV sorption to soils. Figure 5.3 illustrates the sorption isotherms determined in this study. The $\log_{10} K_F$ values for 2% and 8% OM soils are 3.30 and 2.65 ($\log_{10}$ ml/g), respectively (Figure 5.3a). After the antilog calculation the K_F values for 2% and 8% OM soils are 1.9×10^3 and 4.3×10^2 (ml/g), respectively. The sorption capacity for 2% OM soils was about 4 times higher than 8% OM soils. The pH values were 7.3 and 7.1 in the liquid phase of 2% and 8% OM soils

tubes, respectively, which is very similar. The physical and chemical characteristics of both soils (Table 5.1) are very similar except for the OM content and cation exchange capacity (CEC). Higher CEC is expected in higher OM soils since OM is well known to hold cations. The isotherm results provide strong evidence that OM content in soils was the factor causing the difference in HAdV sorption between these two soils. The mechanism could be bonded-OM blocking the favor sorption site for HAdV (Zhuang and Jin 2003a). A study showed octadecyltrichlorosilane-bonded silica could enhance the MS2 sorption by increasing the hydrophobicity of the solid surfaces (Bales et al 1991). However, our data suggested OM originally present in the soil did not react strongly with HAdV through hydrophobic interactions.

Our results also showed that DOM in the suspension inhibit the HAdV sorption since the K_F value of virus suspension in 150ppm HA suspension was $10^{2.46}$ or 2.9×10^{2} (ml/g), which is about a factor of 1/7 of K_F value of the isotherm of 2% OM soils and PBS (Figure 5.3b). As discussed earlier in this paper, previous works have indicated that DOM enhances the virus transport by inhibiting the sorption of virus to soil particles. The current study showed that both natural bonded-OM and DOM would inhibit the sorption of HAdV to soils.

Zhuang and Jin (2003a) compared the effect of OM on the transport and sorption of MS2 and ΦX174, and results showed OM significantly promoted the transport of MS2 but not ΦX174. The authors explained the difference between the transport/sorption behavior of these two viruses based on the fact that MS2 is a more hydrophobic virus than ΦX174 (Shields and Farrah, 1987). Since OM had a similar effect on the sorption of HAdV and MS2, the hydrophobicity of these two viruses are

probably more similar than the hydrophobicity of ΦX174 and HAdV. More evidence would be needed to support this hypothesis. A comprehensive study on the hydrophobicity of enteric virus would definitely be beneficial to selection of the most representative bacteriophage surrogate for each enteric virus in terms of sorption characteristics.

To the best of our knowledge, this is the first study using qPCR as the measurement technique in this kind of soils-virus sorption study. The R^2 values for all three isotherm curves were almost equal to 1.0 and the standard deviations of each point on the isotherm curve were very small. These results showed that qPCR could be considered as the measurement technique for the isotherm experiment and the main advantage is that the actual pathogen of interest can be quantified without extensive labor on laboratory analysis.

Effect of organic matter on HAdV desorption from soils. After seven series of sequential extraction, 3.5% and 26.7% of sorbed HAdV were desorbed from 2% and 8% OM soils, respectively (Figure 5.4). The average percentage of HAdV desorbed from each extraction is 0.50±0.10% and 3.82±1.20% for 2% and 8% OM soils, respectively. This result showed soils with higher natural OM could also enhance desorption of HAdV from soils. As discussed previously, the OM could block the favored sorption site on soil surface, which leads to virus sorbing to other sites where bonding is weaker. This could be the reason why higher natural OM on soils could lead to more desorption. More investigation on how the physical and chemical characteristic of soils affecting virus desorption can be done since most of the previous virus sorption studies were focused on adsorption but desorption is rarely studied.

Conclusions

Based on the results presented in this study, both natural bonded-OM and DOM inhibit the sorption of HAdV to soils. Also, results showed soils with higher natural OM could also enhance desorption of HAdV from soils. Therefore, the practice of biosolids land application on soils with lower OM content is recommended to minimize the risk of groundwater contamination with HAdV. Also, sorption experiments between virus suspensions and containers should be performed to determine the extent of virus sorption to container surface before proceeding to soil-virus sorption experiments. For HAdV suspensions, glass containers are definitely preferable to containers made with plastic materials like polyethylene.

Acknowledgements

We would like to thank Dr. Alvin J. M. Smucker at Crops and Soils Science department, Michigan State University for providing the soils for this study.

Tables and Figures

Table 5.1. Physical and chemical characteristic of soils.

Parameter	Low OM soil	High OM soil
Organic matter (%)[a]	2.3	7.6
Dissolved organic matter (ppm)[b]	37.5	107.5
Clay (%)[a]	36.7	35.9
Silt (%)[a]	47.0	45.9
Sand (%)[a]	16.3	18.2
Bulk density (g cm^{-3}) [a]	1.60	1.84
Particle density (g cm^{-3})[c]	2.64	2.44
pH [d]	6.8	6.7
CEC (meq/100g)[e]	21.0	42.9

[a] data obtained from Park and Smucker (2005a)

[b] dissolved organic carbon: water extractable (1:10 soil to water)

[c] data obtained from Park and Smucker (2005b)

[d] 1:2 soil to water suspension.

[e] CEC – cation exchange capacity: ammonium acetate method (Thomas, 1982)

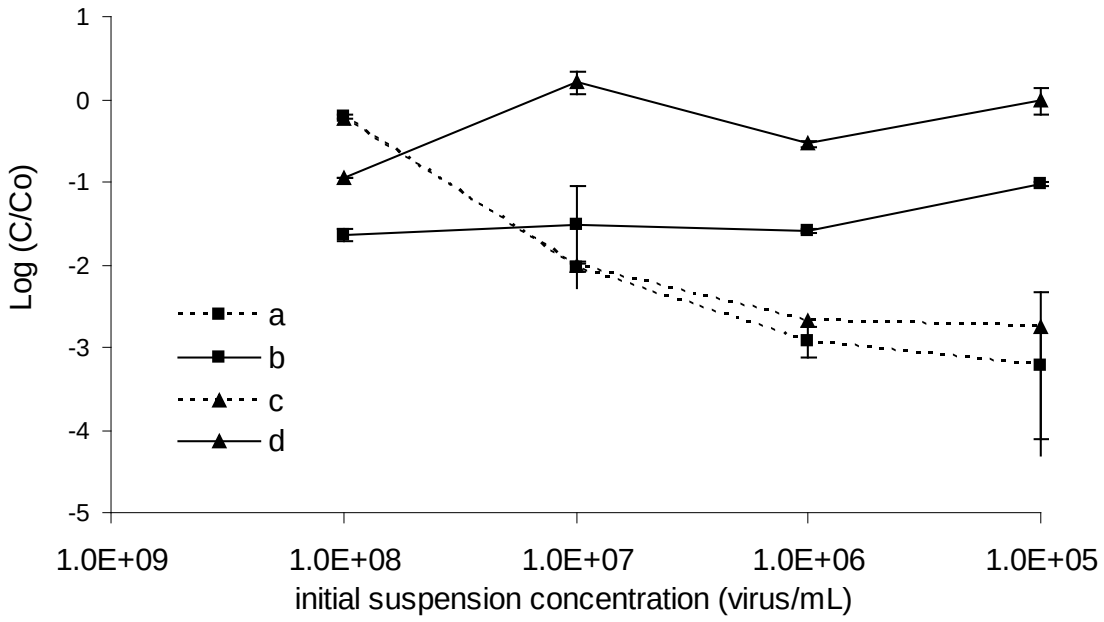


Figure 5.1. Recovery of HAdV sorbed to the polyethylene tubes. Line (a) and (c) is the fraction of HAdV remained in the PBS after tumbling in PE tube for 24 hours and experimental conditions for (a) and (c) were identical; AVL and BE was used to recover sorbed HAdV in set (a) and (c) of PE tubes, respectively (b) recovery of sorbed HAdV by AVL (d) recovery of sorbed HAdV by BE. Vertical bars indicate standard deviations of measurement values from duplicate experiments.

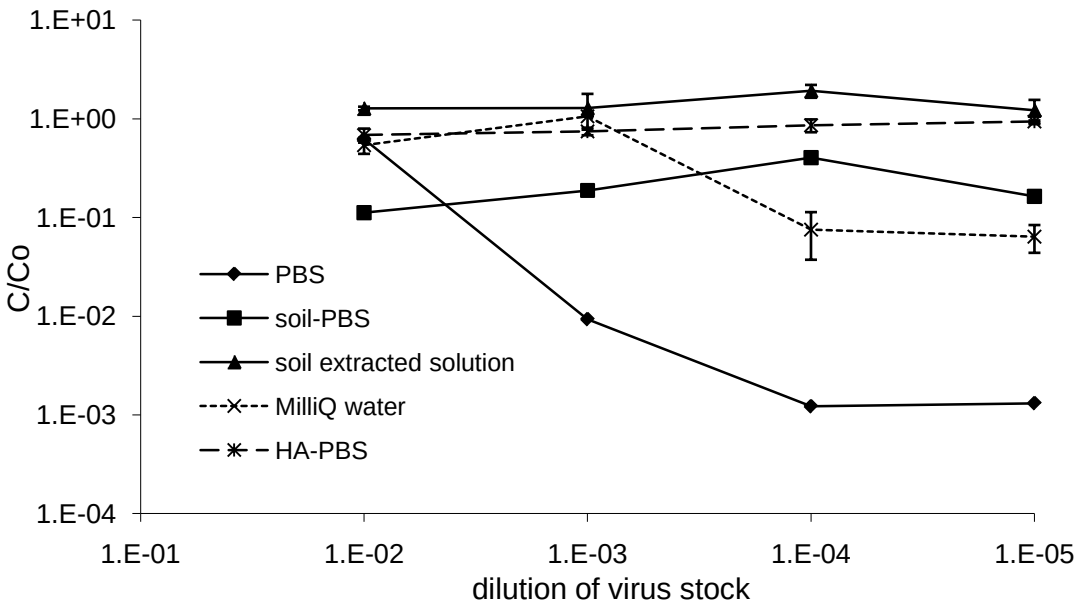
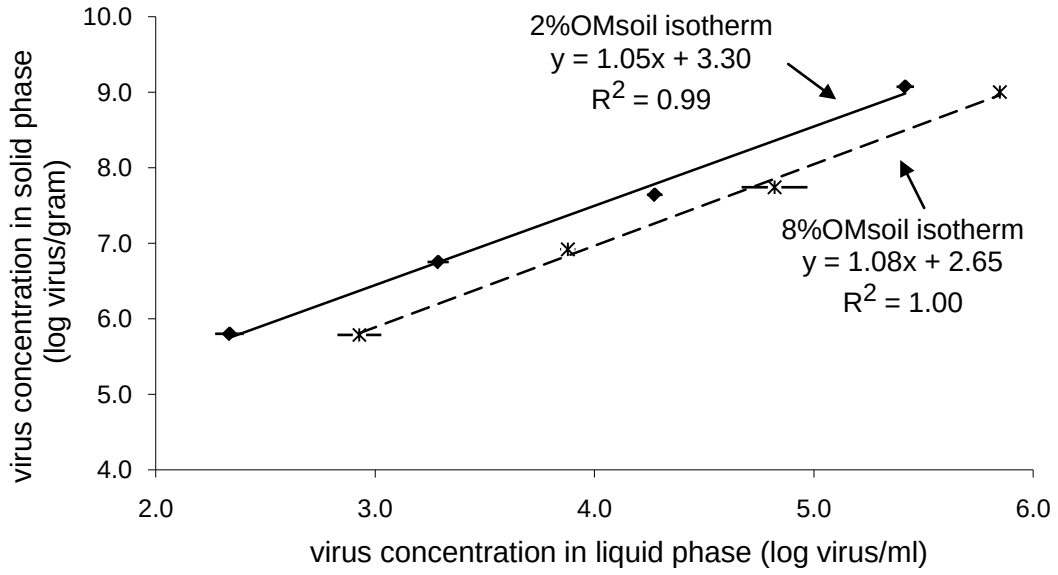


Figure 5.2. Fraction of HAdV remaining in different suspensions after 24 hours of tumbling in PE tube. Vertical bars indicate standard deviations of measurement values from duplicate experiments.

(a)



(b)

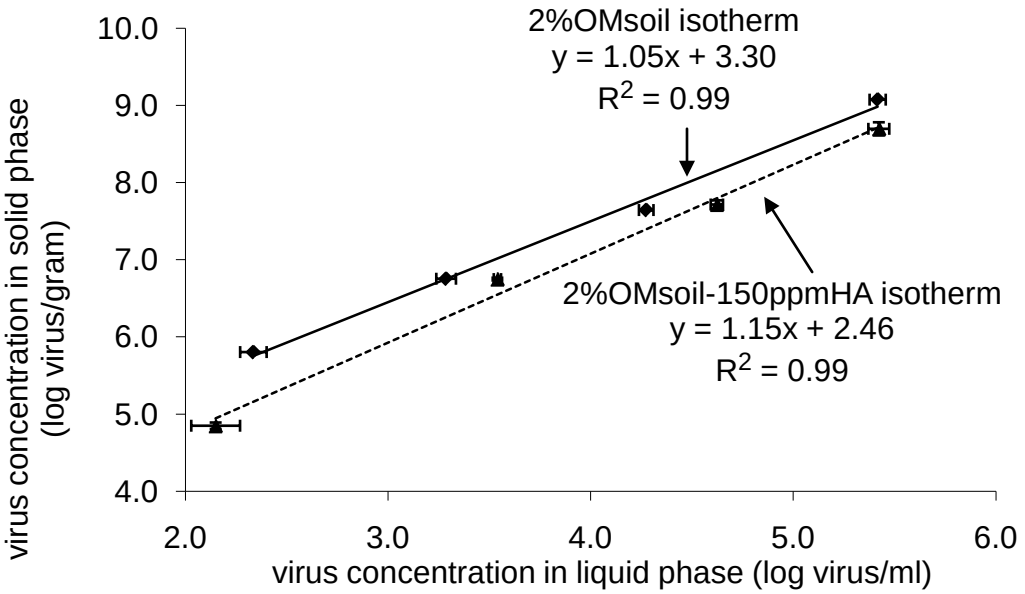


Figure 5.3. (a) Sorption isotherm of HAdV to 2% and 8% OM soils with suspension in PBS; (b) Sorption isotherm of HAdV to 2% OM soils with suspension in PBS and 150 ppm HA solution. Horizontal and vertical bars indicate standard deviations of measurement values from triplicate experiments.

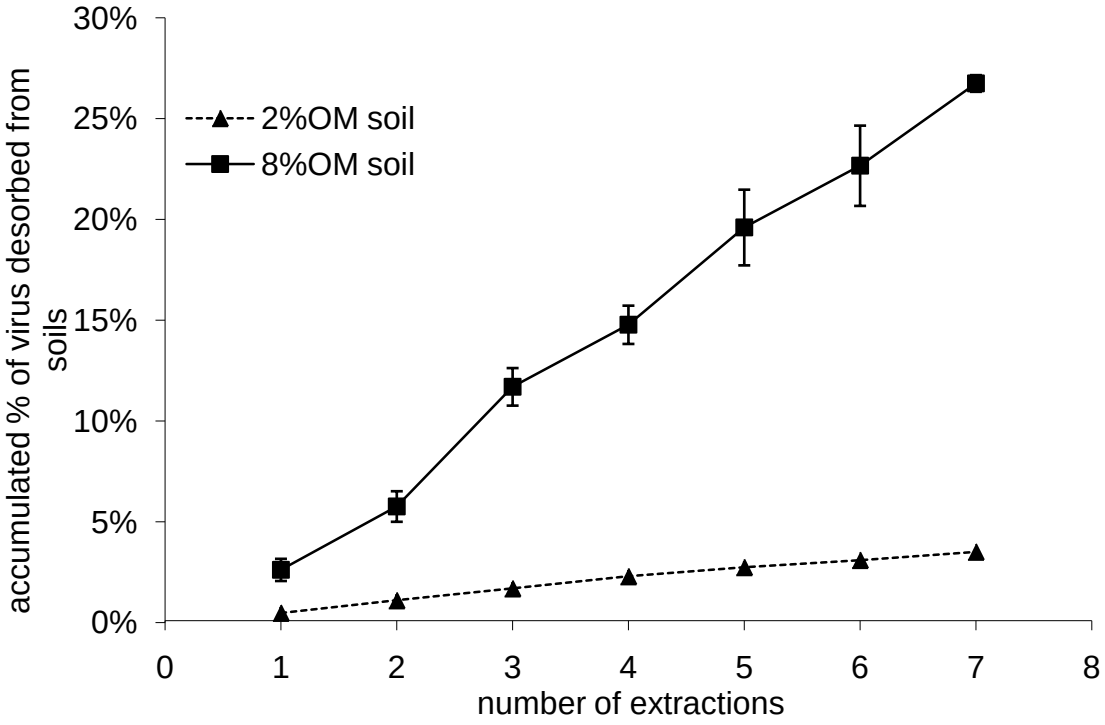


Figure 5.4. Accumulated percentage of HAdV desorbed from 2% and 8%OM soils after each sequential extraction. Vertical bars indicate standard deviations of measurement values from triplicate experiments.

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CHAPTER 6

ENGINEERING SIGNIFICANCE

Because of the simplicity of analytical procedure, traditional fecal indicators (fecal coliform, *E. coli*, enterococci, and bacteriophage) have been extensively used to indicate fecal pollution. For the same reason, these indicators were also used as the biological measurement parameter for many scientific/engineering studies, including fate and transport studies, to address microbiological pollution problems. With the recent advancement of molecular science and technology, many fecal associated pathogens, especially enteric viruses, can be quantified and identified without extensive labor, which lead to the possibility of addressing environmental pathogen related problems more accurately and specifically.

The aim of this study is to address the issue of microbiological pollution at land application sites by utilizing molecular techniques and engineering principles. The specific goals are to develop a better understanding on the quantitative levels of adenovirus in land applied solids (biosolids and bovine manure), and the fate and transport of adenovirus at land application sites. The significance of this study's results is presented in the following paragraphs.

The missing information on the quantitative levels of bovine adenovirus as well as polyomavirus in environmental samples was addressed by the qPCR assays developed in this study. Also, a comparison between the prevalence of bovine adenovirus/polyomavirus and other bacterial fecal indicators in fecal associated materials was performed to determine the suitability of using these two viruses as fecal

indicators. It was concluded that both of these viruses had lower concentration and prevalence than bacterial fecal indicator, but polyomavirus is more suitable than adenovirus for bovine fecal indication at land application sites due to its higher prevalence and lower genetic diversity. These results provide useful information for choosing bovine enteric virus as the fecal indicators and microbial source tracking tools.

Second, enterovirus has been used as the indicator for virus removal by sludge treatment. However, high quantitative and infectivity levels of adenoviruses in MAD biosolids presented in this study indicate that adenovirus could be a better indicator than enterovirus for the evaluation of sludge treatment efficiency. Results also showed that adenovirus is probably the most suitable enteric virus for human fecal indication at a land application site since it had the highest occurrence and concentration in biosolids.

Third, neither adenovirus nor somatic phage were detected in any of the lysimeter effluent samples, which indicates that the sandy loam soil subsurface system described in this study could almost completely remove all the indigenous viruses in biosolids. However, the rapid breakthrough of viral tracer showed that preferential flow in the natural soil systems could greatly enhance the transport of indigenous viruses in the subsurface especially in areas where tile drains are installed underneath the subsurface. Therefore, pre-tillage to disrupt the continuity of macro-pores is recommended to prior to land application. Also, since results showed the indigenous viruses could survive for almost 10 days after application, it would be beneficial to time manure/biosolid application rates to avoid application on wet ground, when tiles are flowing, or when there is a chance of significant rainfall within the next few days.

Finally, this study revealed the effect of organic matter on sorption of adenovirus. Organic matter is a major factor responsible for the uncertainty associated with predicting virus transport in soils and groundwater. The practice of biosolids land application on soils with lower OM content could reduce the risk of groundwater contamination by adenovirus based on the results from this study. Also, results showed adenovirus could sorb to polyethylene surfaces; therefore, sorption experiment between virus suspension and containers should be performed to determine the extent of virus sorption to container surface before proceeding to soil-virus sorption experiment.