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FREQUENCY OF REOVIRUS DETECTION IN BIOSOLIDS: COMPARISON OF THE EPA CFR 503 TECHNIQUE TO INTEGRATED CELL CULTURE - QUANTITATIVE PCR

BY

Elizabeth Gallagher
B.S., College of the Holy Cross, 2000

THESIS

Submitted to the University of New Hampshire in Partial Fulfillment of the Requirements for the Degree of

Masters of Science
In
Microbiology

December, 2009

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Date

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ABSTRACT

FREQUENCY OF REOVIRUS DETECTION IN BIOSOLIDS: COMPARISON OF THE EPA CFR 503 TECHNIQUE TO INTEGRATED CELL CULTURE - QUANTITATIVE PCR

by

Elizabeth Gallagher

University of New Hampshire, December, 2009

The public health threat from pathogens creates controversy for the land application of biosolids, a sewage treatment byproduct. Previous work has demonstrated that some enteric viruses are not detected with a plaque assay, the current method for virus detection in biosolids. The Integrated Cell Culture - Quantitative Polymerase Chain Reaction (ICC-qPCR) assay, which combined quantitative PCR with seven days incubation in cell culture, allows for detection of more viruses.

To compare method sensitivities, a biosolid sample was seeded with mammalian orthoreovirus. $3x10^5$ plaque forming units (PFU) per ml were detected by the plaque assay and 10^8 PFU equivalents per ml were detected by ICC-qPCR. To determine the ability of ICC-qPCR to detect mammalian orthoreovirus, twenty-four environmental samples were tested. No viruses were detected by the plaque assay based on the EPA method; however ICC-qPCR detected infectious mammalian orthoreovirus in thirteen samples. ICC-qPCR was more sensitive than the plaque assay.

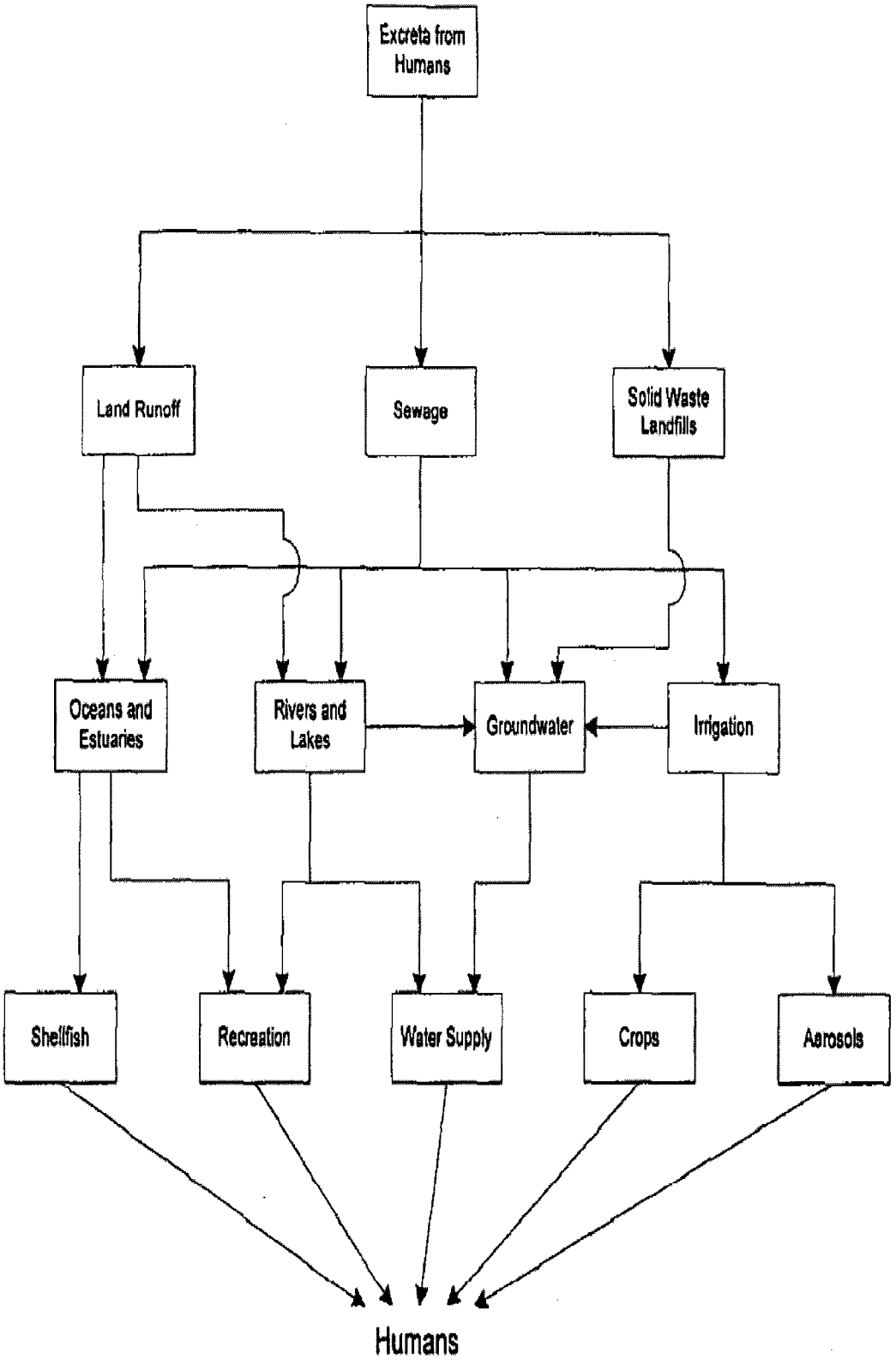
Introduction

I. Fecal Oral Route

In modern society, humans participate in a "large-scale recycling of feces back into the mouth" by the modern practices which process and distribute water and food (Fenner and White, 1986). In the 1940s, it was first realized that viruses could be transmitted through water by the fecal-oral route (Metcalf *et al.*, 1995). The realization that viruses could spread from feces into drinking water was an important step in preventing diseases. Figure 1 illustrates the fecal-oral pathway (Metcalf *et al.*, 1995). Pathways for feces to enter the fecal-oral route include leaking septic systems, urban and agricultural runoff, sewage outfall, vessel discharge, and insufficiently treated water (Fong and Lipp, 2005).

In 1945, cell culture was used to demonstrate that viruses are more resistant to disinfection than bacterial indicators of water pollution (Metcalf *et al.*, 1995). Virally contaminated water with no bacterial indicators has been the cause of documented gastroenteritis outbreaks (Fong and Lipp, 2005). Enteric viruses are common in sewage. These viruses are shed in high numbers and have a low infectious dose (Fong and Lipp, 2005).

Figure 1. The fecal-oral pathway is illustrated in this diagram.



II. Mammalian Orthoreovirus as a Waterborne Virus

A. Characteristics

Mammalian orthoreovirus is a member of the family Reoviridae and the genus *Orthoreovirus*. It is a medium sized virus (Fenner and White, 1986), with a particle to infectivity ratio of 15:1 (Wallis *et al.*, 1964). It is unclear if mammalian orthoreovirus is seasonal. However, several studies have isolated this virus throughout the year (AWWA, 1999; Matsuura *et al.*, 1988).

This virus has 10 to 12 double-stranded RNA linear genomic segments that can reassort. Viruses can undergo this genetic modification when passing through a host (Spinner and DiGiovanni, 2001). Each segment encodes a different gene.

Mammalian orthoreovirus has two round icosahedral capsids and does not have an envelope. The outer capsid is not able to penetrate cell membranes and infect the host. In the intestinal lumen of the mammals, the outer capsid is proteolytically uncoated and made infectious (Golden *et al.*, 2002). The virus then replicates in the cytoplasm of the cells that it infects (AWWA, 1999).

The double protein capsids make mammalian orthoreovirus resistant to disinfection, especially disinfection by methods affecting the outer part of the pathogen. These viruses are able to remain infectious for long periods of time under experimental conditions. Experiments have demonstrated infectivity in surface water for 200 days, in river water for 3 years, and in a cellophane tube suspended in a river for more than six months (Matsuura *et al.*, 1988). The virus

was also shown to survive aerosolization when crops were irrigated with water which contained virus (AWWA, 1999).

B. Pathology

There are three serotypes of mammalian orthoreovirus that infect many mammals, including humans (AWWA, 1999). The documented host range for mammalian orthoreovirus includes a large variety of mammals. This virus has been isolated from humans, chimpanzees, monkeys, mice, dogs, cats, horses, cattle, sheep, swine, bats, and chickens (Rosen, 1968; AWWA, 1999). Transmission from animals to humans is very likely (Nibert *et al.*, 1991).

Researchers believe that the virus infects human respiratory and intestinal tracts. Mammalian orthoreovirus has been linked to neonatal hepatitis, juvenile onset diabetes, fever, rash, pneumonia, eye infections, extrahepatic biliary atresia, meningitis, and myocarditis (Spinner and DiGiovanni, 2001; AWWA, 1999). Mammalian orthoreovirus has been isolated from patients with respiratory infections, gastroenteritis, and rashes (Ward and Ashley, 1977). Mammalian orthoreovirus infections can result in secondary bacterial infections which can become severe in immunocompromised individuals such as the young and the elderly (Spinner and DiGiovanni, 2001).

When three healthy adult male human volunteers were intranasally exposed to bovine reovirus, they became infected and shed virus in their feces. No significant symptoms were presented; however, two of the three volunteers had previous mammalian orthoreovirus type 3 antibodies (Kasel *et al.*, 1963).

These antibodies in the immune systems of the volunteers may have prevented major symptoms by effectively neutralizing the virus. In a similar study, eight human volunteers were infected with human reovirus, and the volunteers had a detectable homotypic hemagglutination inhibition (HI) antibody response, indicating an infection. Several volunteers had symptoms such as malaise, runny nose, cough, sneezing, pharyngitis, and headache (Rosen *et al.*, 1963).

In a study of adults with liver disease, elevated titers of antibodies to reovirus type 3 were detected in sera samples (Minuk *et al.,* 1987). These titers suggest a possible association of reovirus with certain types of liver disease. Another association of reovirus with human disease was a recent case of meningitis in a 6.5 week old baby. This child was infected with reovirus type 3 and the isolate produced lethal encephalitis in mice, strongly suggesting that the reovirus caused the disease (Tyler *et al.*, 2004).

Exposure to mammalian orthoreovirus is extremely widespread in humans worldwide. The majority of adults have serum antibodies to all three types of this virus (Fenner and White, 1986). The virus is shed by infected individuals in feces for several weeks and therefore is spread by the fecal-oral route (Fenner and White, 1986).

In mammals other than humans, reovirus is known to cause jaundice, meningitis, encephalitis, pneumonia, myocarditis, gastroenteritis, and biliary atresia. Reoviruses can also infect chickens, although the viruses are antigenically different. In chickens, the virus causes arthritis and suppresses the immune system (AWWA, 1999).

Laboratory experiments on mice provide insight into the pathogenesis of mammalian orthoreoviruses. Mice become ill upon injection. The viral type determines the route of viral spread within the mouse (AWWA, 1999). In mice injected with mammalian orthoreovirus type 2 and type 3, the virus spreads via the nervous system. In mice injected with type 1, the virus travels in the blood. The outer capsid protein is unique in each type and recognizes different cellular receptors (Flint *et al.*, 2000).

C. Prevalence

Mammalian orthoreovirus is abundant in environmental waters as evident from its frequent detection (AWWA, 1999). Scientists have detected mammalian orthoreovirus in wastewater, sludge, surface water, and groundwater.

Mammalian orthoreovirus was detected in 91% of wastewater influent samples over a nine year period in Wisconsin. It was the most common virus detected in influent and effluent water. Mammalian orthoreoviruses were detected at the highest concentrations upon comparison to any other virus in the wastewater influent water (Sedmak *et al.*, 2005). Additionally, mammalian orthoreovirus was the only virus type isolated from Lake Michigan (Sedmak *et al.*, 2005).

At an Australian wastewater treatment plant, virus levels were monitored for a year, and mammalian orthoreovirus was the most abundant virus detected. Mammalian orthoreovirus was detected in 85% of the effluent samples (Irving and Smith, 1981). Mammalian orthoreoviruses were more difficult to remove

from the wastewater than enteroviruses. When both enteroviruses and mammalian orthoreoviruses were detected in the influent water, mammalian orthoreoviruses were more likely to be detected in the effluent.

Eight of fifteen wastewater treatment plants in Puerto Rico had mammalian orthoreovirus in the effluent water, including two that did not have any mammalian orthoreovirus detected in the influent water. Of the treatment plants that detected mammalian orthoreovirus in the influent water, all but two of the treatment plants in the study detected this virus in the effluent water (Dahling *et al.*, 1989). Reoviruses were detected in 9 of the 15 plants in both the influent and the effluent waste streams. In several cases the amount of virus detected was greater in the effluent stream (Dahling *et al.*, 1989).

At a wastewater treatment plant in Ottawa, Ontario, reovirus was the most abundant isolate in both the pre-treatment and post-treatment samples (Sattar and Westwood, 1978). A multi-year study of the wastewater in Reading, Great Britain, isolated reovirus in every year of the study. Although isolated every year, reovirus was not the most common virus detected. It was the second most common virus detected. However, there was an underestimation of reovirus due to a technique favoring faster growing viruses, such as polioviruses (Sellwood *et al.*, 1981). Therefore reovirus may have been even more common than the study indicated.

Reoviruses were also isolated from the sludge at a wastewater treatment plant in Wisconsin. The highest levels were detected in waste activated sludge. Reoviruses were also detected at lower levels in return activated sludge, trickling

filter sloughing, and blended sludge. Additionally, reovirus concentrations in the digested sludge samples were greater than the concentrations of enteroviruses (Cliver, 1975). Recently in a French study on enteroviruses in sludge, the cell culture results indicated the presence of other viruses. Mammalian orthoreovirus type 1 was discovered to be the virus additionally present in the cell culture (Monpoeho *et al.*, 2004).

Research on viral levels in a Japanese urban river concluded that the most abundant virus was reovirus (Matsuura *et al.*, 1984). Forty-seven percent of the viruses isolated were reoviruses. The levels of reovirus surpassed even poliovirus directly after a live oral vaccine for poliovirus was given. This research also detected particle associated reovirus with more frequency than non-particle associated reovirus (Matsuura *et al.*, 1984). In an additional Japanese study, more reovirus was detected in the more populated areas (Matsuura *et al.*, 1988). The highest antibody titer was to type 2 mammalian orthoreovirus, which was also the most common virus type detected in the river (Matsuura *et al.*, 1988).

In a five year study at a different urban river in Japan, reoviruses were the most frequently isolated virus (Tani *et al.*, 1995). The viruses were isolated from this river throughout the five years.

Groundwater samples from 29 sites across the United States were evaluated for viruses on a monthly basis. Reovirus was the most common virus detected in these samples (Fout *et al.*, 2003).

III. Other Environmentally Important Viruses

Besides mammalian orthoreovirus, there are other viruses that are important in the environment. Adenovirus is a double stranded DNA virus and rotavirus is a double stranded RNA virus. Astrovirus, noroviruses, and the enteroviruses are all single stranded RNA viruses. Important enteroviruses include poliovirus, hepatitis A, coxsackievirus, and echovirus.

All of these viruses are transmitted via the fecal-oral route and can cause gastroenteritis. Some of these viruses also cause other clinically important diseases. Adenoviruses commonly cause respiratory infections. Infection with enterovirus can lead to flu-like illness. Poliovirus infection can cause paralysis. Hepatitis A infection can result in liver disease. Hand, foot, and mouth disease is most often caused by coxsackievirus. In addition, most of these viruses have been linked to viral meningitis.

IV. Biosolids

A. Definition/Considerations

"Biosolids (historically known as sewage sludge) are the solid organic matter produced from private or community wastewater treatment processes that can be beneficially used, especially as a soil amendment (EPA, 1999)."

Settling and activated sludge are wastewater treatment processes which produce biosolids. A large number of wastewater treatment plants utilize both these methods. The activated sludge process enhances the removal of pathogens from the liquid portion of wastewater by concentrating the pathogens

in the solids, which can result in high viral concentrations in biosolids. Viruses remain infectious in the activated sludge process (Fenner and White, 1986). Even in treatment plants without an activated sludge process, biosolids can contain high levels of viruses. Disposal of biosolids with potentially high viral concentrations is a complicated issue due to political and health concerns.

Biosolids that are land applied may lead to viral contamination of crops or groundwater. Viruses tend to preferentially associate with solids (Metcalf *et al.*, 1995). Solid associated viruses are subject to desorption and migration following heavy rain and similar weather events complicating the environmental impact analysis of land application (Metcalf *et al.*, 1995). Viruses have been shown to remain infectious in non-aerated biosolids for more than six months in outdoor conditions. The viruses showed no significant reduction in concentration at 4°C during the course of the six month study (Pesaro *et al.*, 1995).

B. Regulations

The current regulations on the land application of biosolids are defined in the Code of Federal Register (CFR), Title 40 - Protection of Environment, Chapter I - Environmental Protection Agency, Part 503 - Standards for the Use or Disposal of Sewage Sludge. The regulations (EPA, 1992) define how sewage sludge is transformed into biosolids, and the rules for land application. The EPA distinguishes between two classes of biosolids, which differ in the amount of allowed pathogens.

Class A biosolids are required to have the levels of pathogens below

detectable levels. To achieve this goal, the treatment plant can treat the sewage sludge with any of the processes specially defined in the guideline and then test for bacteria, either fecal coliforms or *Salmonella*. These processes are designed to produce low levels of enteric viruses and viable helminth ova when the level of bacteria is low. Composting, heat drying, and thermophilic digestion are examples of approved processes. Processes not specifically defined in the guideline can also be used, however this requires additional testing. Either the process used to create the biosolids or the batch of biosolids must be tested for enteric viruses and viable helminth ova, in addition to testing for bacteria (EPA, 1992). Land application of class A biosolids is not restricted to particular site uses. Some examples of end uses for class A biosolids are food crop fertilizer or retail sales for home garden fertilizer.

Class B biosolids are allowed to have detectable fecal coliform levels. The fecal coliform density needs to stay below the limit set by the regulations. This limit produces biosolids that "do not pose a threat to public health and the environment" (EPA, 1992) as the environment will further reduce the pathogens. To help prevent a public health or environmental threat, the land application of class B biosolids is restricted to sites with specific uses. These uses include agricultural land that is not used for food and reclamation of destroyed mining areas. Class B biosolids cannot be used in areas that are open to the public (EPA, 1992). A wastewater treatment plant can produce Class B biosolids by either using a defined process that has been previously validated to produce low fecal coliform levels or by periodic fecal coliform testing. Some defined

processes for producing Class B sludge are digestion, drying, and lime stabilization.

V. The Total Culturable Virus Assay-Most Probable Number Method & The Plaque Forming Unit Assay

Cell culture has traditionally been the best method for the isolation of infectious virus from environmental samples (Fong and Lipp, 2005). Cultured cells can be used in two different types of assays. One assay is commonly referred to as the total culturable virus assay-most probable number (TCVA-MPN) method. The TCVA-MPN method is typically used for detecting viruses in surface water and groundwater. The second assay is known as the plaque forming unit assay (PFU). The plaque forming unit assay is used for detecting viruses in sewage sludge and biosolids.

In the TCVA-MPN, viruses replicate in a single layer of cells. Detection of viral replication is determined by the formation of cytopathogenic effects (CPE). Examples of CPE are cellular damage, cell rounding, and cell layer sloughing caused by cell death (Fong and Lipp, 2005). Cells grown on a culture dish, typically a 96 well plate, are infected at ten-fold dilutions. After incubation, the presence of CPE is scored as a positive result. The scores for each dilution are entered into an MPN calculation (Chapron *et al.*, 2000) and the viral concentration is determined. The calculation is found in the EPA's ICR microbial manual (Fout *et al.*, 1996).

Many enteric viruses do not produce CPE in cell culture, while other

viruses will form CPE only after repeated exposure to the same cell line (Fong and Lipp, 2005). In addition, a cell line infected with a mixed population of viruses may only support the replication of the fastest growing virus, resulting in lower detection of the slower growing viruses. In the Buffalo Green Monkey Kidney (BGMK) cell line, mammalian orthoreovirus replicates slower than other enteric viruses, forming CPE after 9-14 days of incubation (Spinner and DiGiovanni, 2001). When a quickly replicating enteric virus is present in an environmental sample along with mammalian orthoreovirus, the enteric virus replicates first and destroys any available cells in the process. Therefore the mammalian orthoreovirus is unable to replicate and goes undetected. However, when faster replicating viruses are not present, mammalian orthoreovirus has been detected successfully in BGMK cells.

All three strains of mammalian orthoreovirus show a common characteristic CPE in cell culture (Rosen, 1960). Mammalian orthoreovirus has demonstrated infection in cell lines from many different tissues, including respiratory, intestinal, heart, muscle, and brain (Golden *et al.*, 2002). For mammalian orthoreovirus to successfully infect a cell monolayer on a culture dish, proteolytic enzymes are necessary (Golden *et al.*, 2002). Trypsin is a commonly used enzyme for this purpose.

The other assay commonly used to identify viruses is the plaque forming unit assay. Because sludge contains both viruses and toxic substances, plaques can not be conclusively determined to be from viral origin. Certain toxins can cause a clearing of the cell monolayer resulting in a plaque not caused by a virus

(Schmidt *et al.*, 1978). Additional testing is required to positively confirm that the plaque is caused by a virus. The area around the plague is removed and used to inoculate a cell monolayer which is examined for one week for evidence of CPE (EPA, 2003).

The semi-solid state caused by the addition of agar in a plaque assay has been shown to reduce the sensitivity for the detection of certain enteric viruses. Some samples, containing mammalian orthoreoviruses, exhibit CPE in liquid culture, but fail to plaque in a semi-solid cell culture assay (Schmidt *et al.*, 1978).

Another problem associated with the plaque assay is sensitivity differences depending on the cell line and the virus. This is an issue with all cell based assays. In one example, rhesus monkey kidney cells (LLC-MK2) were shown to be more sensitive than buffalo green monkey kidney cells (BGMK), but both were less sensitive than Madin-Darby bovine kidney cells (MDBK) (Ridinger *et al.,* 1982). In another study BGMK cells were more sensitive to infection by reovirus than Hep-2, Caco-2 or RD cells (Sedmak *et al.*, 2005). Even if the cell line is susceptible to infection by the virus, the virus may not be able to plaque. For example, in a study by Agbalika *et al.,* reoviruses did not produce plaques using the BGMK cell line (1984).

Additionally, it is impossible to distinguish visually between plaques formed by different types of viruses. Sixty nine percent of the plaques detected in an environmental sewage sample using a BGMK plaque assay were not mammalian orthoreovirus (Ridinger *et al.*, 1982).

Plaque assays have many variables which complicate the comparison

between different types of plaque assays. The efficiency of the assay has been shown to be dependent on the number of cells seeded in the cultureware, the amount of viral inoculum added, and the incubation time (Payment and Trudel, 1985).

VI. Polymerase Chain Reaction & Quantitative PCR

A. Polymerase Chain Reaction (PCR)

PCR detects a viral target based on specific primer design (Fong and Lipp, 2005). Primers can be designed to detect a broad group of viruses, one virus strain, or just one viral serotype (Fong and Lipp, 2005). The primers and heat stable polymerase are combined in a PCR reaction. The primers bind to the target nucleic acid and the heat stable enzyme amplifies the target region. This amplification provides detectable quantities of the target nucleic acid. The efficiency of viral amplification from environmental samples by PCR is influenced by PCR inhibitors, the ability to recover the virus from the environmental matrix, and the amount of pure nucleic acid in the sample (Metcalf *et al.*, 1995).

PCR is less efficient for double stranded RNA viruses than single stranded RNA viruses because denaturation of the double strands is difficult (Metcalf *et al.*, 1995). Despite these difficulties, several studies have detected viruses with PCR that were not detected by cell culture (Fong and Lipp, 2005).

Reovirus has been detected using traditional PCR by several different research groups, each using a different gene segment as a primer. One research group has chosen the L1 gene segment, because this segment

encoded a portion of the RNA dependent – RNA polymerase and is highly conserved among all the strains (Leary *et al.*, 2002). Another group chose the Sigma 2 region because it is a region which is also highly conserved, encoding a major core protein (Muscillo *et al.,* 2001).

Successful detection of these viruses by PCR was an important technological advance due to the specificity in primer design. However, detection by PCR does not indicate infectivity, a major disadvantage of the PCR technique.

B. Quantitative PCR

Traditional PCR gives only a positive or negative result and does not quantify the amplified nucleic acid. Combining PCR with double stranded DNA fluorescent dyes or fluorescent reporter probes can quantify the concentration of virus in a sample. These fluorescent methods utilize the cycle threshold value to quantify the amount of infectious virus. The cycle threshold value is the cycle number when the fluorescent intensity of the reaction is greater than the background intensity. At the end of each temperature cycle during the PCR reaction the intensity of the fluorescence is measured. This method is also referred to as real time PCR because the level of fluoresce is measured after each cycle, and amplification can be visualized as it is occurring.

An example of a double stranded DNA dye is the SYBR Green assay. In this assay, a green fluorescent dye binds to double-stranded DNA and results in an increase in fluorescence during the double-stranded stage of nucleic acid

replication. Two examples of quantifying PCR with fluorescent reporter probes are the use of molecular beacons and the Taqman assay. Molecular beacons are strands of nucleic acid which form a hairpin loop shaped structure at the beginning of the assay. The fluorescing dye is located at one end of the loop and the quenching dye is located the other end of the loop. Therefore the fluorescing dye is close to the quenching dye at the beginning of the reaction and no fluorescence is emitted. During the assay, the labeled probe (beacon) hybridizes to the complementary target in the amplifying DNA and results in the fluorescent dye being located farther from the quenching dye and fluorescence produced. In the Taqman assay, a short probe contains both a fluorescent dye and a quencher dyer. Due to the short length of the probe, the quencher dye and the fluorescent dye are close together. The fluorescence is not produced until the DNA polymerase uses 5′ nuclease activity to cleave the probe when the strand elongation reaches the portion where the probe is located. The cleavage breaks apart the fluorescing dye from the quenching dye and fluorescence is produced.

Quantitative PCR has several advantages over traditional PCR. It does not require an agar gel to visualize results, which shortens the time frame to obtain results (Fong and Lipp, 2005). The transfer to an agar gel is a source of potential contamination in traditional PCR that is not present in quantitative PCR (Fong and Lipp, 2005). Another advantage is increased sensitivity to the target nucleic acid, which is important in low copy number experiments. However, increased sensitivity can lead to errors in results due to contamination from positive controls if good aseptic techniques are not used (Freeman *et al.*, 1999).

Quantitative PCR was the technique used in several studies to detect viruses in sludge. In France, researchers successfully used quantitative PCR to detect enteroviruses in sludge (Monpoeho *et al.*, 2000). Later, the same research group used quantitative PCR to compare amounts of enteroviruses before and after sludge treatment (Monpoeho *et al.*, 2004). This group has also developed a quantitative PCR assay that detects astrovirus in sewage from a wastewater treatment plant (Le Cann *et al.,* 2004).

Besides the group in France, other groups have detected viruses with quantitative PCR. Quantitative PCR was used to detect Adenovirus 40 in environmental samples from California that were seeded with the virus (Jiang *et al.*, 2005). In Germany, enteroviruses were detected by quantitative PCR in the activated sludge from a wastewater treatment plant (Pusch *et al.,* 2005).

VII. ICC-PCR

Like traditional PCR, quantitative PCR does not indicate infectivity. Combining cell culture with a PCR is known as Integrated Cell Culture-PCR (ICC-PCR) (Blackmer *et al.*, 2000). ICC-PCR overcomes the disadvantages of cell culture and PCR alone. While TCVA-MPN and plaque assays detect cellular death, which indicates viral infection, ICC-PCR has the ability to recognize genotypes and identify viruses present (Spinner and DiGiovanni, 2001). In addition, ICC-PCR is a sensitive method requiring minimal replication of the virus for detection to occur. This is an advantage over CPE, immunoassays, or cell culture detection, which requires a larger concentration of virus for detection

(Rosen, 1960).

The presence of inhibitors, the inability to assay large volumes, and the inability to measure infectivity are all limitations of PCR. Using the ICC-PCR method reduces these problems (Spinner and DiGiovanni, 2001). The use of cell culture helps to dilute out any PCR inhibitors that would otherwise have to be removed. Most removal methods, such as Qiagen® spins columns, are disadvantageous because they also reduce the concentration of virus. In addition to diluting out PCR inhibitors, cell culture provides an in-vitro amplification system. This amplification increases the numbers of viruses and enhances the sensitivity of the assay. When used with quantitative PCR (ICC-qPCR), amplification also provides a means of differentiating between infectious and non-infectious virus (Reynolds, 2004). The infectivity is measured by comparing the CT values detected by quantitative PCR of the sample before incubation to the CT values detected after the sample has been incubated. Obtaining a CT value before incubation is crucial to determining if infectious virus is present (Reynolds, 2004).

Additionally, ICC-PCR permits evaluation of a much larger percentage of the original sample as compared to traditional PCR. This is due to the larger volume of sample used in the cell culture portion of the assay. Several studies have compared the efficiencies of ICC-PCR to the plaque assay, the TCVA-MPN assay, or traditional PCR. One study compared ICC-PCR detection, traditional PCR detection, and cell culture/CPE detection of poliovirus and hepatitis A virus in environmental water samples. Cell culture and ICC-PCR were more sensitive

than traditional PCR, and detection by ICC-PCR was more rapid than cell culture detection (Reynolds *et al.*, 1997). Another study evaluating water samples seeded with poliovirus concluded ICC-PCR to be more rapid and more sensitive than viral detection with CPE. This study reproduced these results using primary sewage effluent from Hawaii (Reynolds *et al.*, 1996). In a study that examined river water and surface water from South Korea, ICC-PCR was more sensitive than the TCVA-MPN method. ICC-PCR detected enterovirus and adenovirus in 13 samples which TCVA-MPN did not detect virus (Lee *et al.*, 2005). In another Korean study at a separate University, ICC-PCR was compared to TCVA-MPN for adenovirus and enteroviruses. The ICC-PCR detected virus in more samples (Lee and Jeong, 2004).

When water samples from across the United States were tested for enteroviruses, adenoviruses, and astroviruses by comparing the TCVA-MPN method to ICC-PCR, ICC-PCR detected viruses in 48% more samples than TCVA-MPN (Chapron *et al.*, 2000). In another water study, water seeded with adenovirus adapted to the cell line, TCVA-MPN and ICC-PCR detection occurred in all the samples; however ICC-PCR detection occurred more rapidly (Ko *et al.*, 2003). In samples of sewage, marine water, and surface water, Hepatitis A virus and enteroviruses were detected by ICC-PCR more rapidly than by either direct PCR or TCVA-MPN (Reynolds *et al.*, 2001).

In a study where samples seeded with poliovirus were treated with UV and then tested for infectious virus using ICC-PCR and TCVA-MPN, only ICC-PCR detected poliovirus at the later time points (Blackmer *et al.*, 2000). In another

seeded study, researchers compared two different methods of ICC-PCR to a $TCID_{50}$ assay to detect hepatitis A virus. That study found detection of RNA intermediates by ICC-PCR was the most effective way to detect this virus. The advantage of the ICC-PCR was a clear positive after only 60 hours of incubation. After 60 hours, the $TCID_{50}$ was not positive (Jiang *et al.*, 2004).

Cell culture, direct PCR, and ICC-PCR were compared to detect enteroviruses and adenoviruses in sewage, sludge, river water, and shellfish in New Zealand. The ICC-PCR detected more viruses than the plaque assay method with the adenovirus but not with the enteroviruses. PCR detected more in both cases implying that some of the genomes were not infectious. However, only the media was tested in the ICC-PCR assay and not the potentially infected cells, which could have affected the results (Greening *et al.*, 2002).

In another study, ICC-PCR had the same detection limit as traditional PCR for detecting adenovirus in shellfish tissues (Rigotto *et al.*, 2005). However the 72 hour incubation may not have been enough time to amplify the slowly multiplying viruses, especially if only a few viruses were present. This would reduce the advantage of the ICC-PCR over traditional PCR. Additionally, this study did not consider infectivity. The ability to determine infectivity is a benefit of ICC-qPCR.

The previously discussed studies have demonstrated the advantages of ICC-PCR over traditional methods. ICC-PCR is even more powerful when quantitative PCR is used in the PCR potion of the assay. One study compared quantitative PCR with traditional PCR to detect astrovirus, and then developed

an ICC-PCR assay using quantitative PCR. Using a dilution technique, they determined that quantitative PCR in the ICC-qPCR method was 2 fold more sensitive than traditional PCR (Grimm *et al.*, 2004).

A few studies have used ICC-PCR as the exclusive method for detecting the pathogen of interest. In Korea, tap water was examined for enteroviruses and adenoviruses using only this method (Lee and Kim, 2002). In South Africa, drinking water and surface water samples were tested for adenovirus using only ICC-PCR (van Heerden *et al.*, 2004). The same research group in South Africa also tested drinking water for the presence of enteroviruses using only ICC-PCR (Vivier *et al.*, 2004). Nested polymerase chain reaction (ICC-nPCR) was used as the sole method to detect human astrovirus, enteroviruses, rotavirus, and adenovirus type 40 and 41 in marine water samples from Massachusetts (Ballester *et al.*, 2005).

In conclusion, viruses enter into the environment by the fecal-oral route. At the endpoint of wastewater treatment process, biosolids are the point of exposure for these viruses. One of the important viruses that the public is potentially exposed to is mammalian orthoreovirus. Mammalian orthoreovirus is an important gastrointestinal virus due its persistence in the environment. Additionally studies comparing the occurrence of environmental viruses have demonstrated the prevalence of this virus. Detecting viruses in the environment has traditionally been accomplished with plaque assays and cell culture. PCR is a newer method for detecting viruses. PCR is sensitive and specific but does not demonstrate infectivity. Combining cell culture with PCR using ICC-PCR has

been shown to be effective for detecting viruses. Using quantitative PCR in the PCR portion of the ICC-PCR makes it an even more powerful tool. The research presented in this thesis compares the frequency of mammalian orthoreovirus detection using ICC-qPCR to the frequency of detection using a plaque assay.

Methods and Materials

Initial Propagation of Mammalian Orthoreovirus Type 1 Lang

Experiments were designed to detect viruses which had been spiked into sludge samples. Mammalian orthoreovirus Type 1 (Lang) was chosen for these experiments and obtained from the American Type Culture Collection (ATCC), Manassas, VA (catalog number: VR-230). Buffalo Green Monkey Kidney (BGMK) cells, a transformed mammalian kidney cell line, were used to multiply the virus. BGMK cells (ATCC, Rockville, MD) were grown in 75 cm^2 closed cell culture flasks to confluency using Minimal Essential Media (MEM) (Appendix 1) supplemented with 10% fetal bovine serum. Prior to infection, the cells were washed with Phosphate Buffered Saline (PBS). After washing, the cells were inoculated with mammalian orthoreovirus stock at a multiplicity of infection (MOI) of 2. The flasks were incubated at 37°C for 90 minutes, with periodic rocking to ensure cell hydration and viral absorption. Post absorption, MEM supplemented with 2% fetal bovine serum was added and flasks were returned to the incubator. Flasks were checked daily until approximately 75% of the cell monolayer was exhibiting cytopathic effects (CPE) which was determined by the sloughing off of the cell monolayer. When cell monolayer sloughing was observed, the flasks were placed in the freezer at -80°C until the liquid portion was frozen and removed from the freezer and placed at room temperature to thaw, known as freeze-thawing. This was repeated three times to liberate the virus from the

cells. Cellular debris was removed by centrifugation two times at 1000 x g (2100 RPM) with a Beckman JA14 rotor for 15 minutes and the supernatant containing the virus was aliquoted into storage tubes and stored at -80°C until use.

Adaptation of Reovirus Type 1 (Lang) to a Separate Cell Line

Mammalian orthoreovirus type 1 was adapted to the LLC-MK2 cell line. This cell line, a monkey kidney cell line, is different from the BGMK cell line used in the plaque assay. Five passages of mammalian orthoreovirus were done to adapt to the virus to the LLC-MK2 cell line. The cells were grown to confluency in 75 cm^2 closed cell culture flasks using MEM supplemented with 10% fetal bovine serum. The cells were inoculated with mammalian orthoreovirus at an MOI of 4. After each of the first four passages, the supernatant from the previous passage was used to infect the next set of flasks. After the fifth passage, the supernatant was divided into storage tubes and stored at -80°C.

Confirmation of CPE by PCR and Nested PCR

PCR and nested PCR were used to confirm that CPE was from mammalian orthoreovirus infection. The primers used are listed in Table 1. The master mix used was according to Katz (2005). A hot start cycle of 5 minutes at 95°C was used prior to the addition of the polymerase. Following the polymerase addition, 35 cycles of 95°C, 55°C, and 72°C were run for 1 minute each, followed by a final cycle of 72°C for 7 minutes. PCR amplicons were visualized on a 1.5 % gel electrophoresis stained with ethidium bromide. The gel was run at 100 mv.

The gel was examined for a fluorescent band at 416 base pairs using a gel documentation system.

Table 1. Primers used in traditional PCR

Upstream Primer for PCR	GCATCCATTGTAAATGACGAGTCTG
Downstream Primer for PCR	CTTGAGATTAGCTCTAGCATCTTCTG
Nested upstream primer	GCTAGGCCGATATCGGGAATTGCAG
Nested downstream primer	GTCTCACTATTCACCTTACCAGCAG

Sludge Collection for Seeded Experiments

For seeded experiments, 5 liters of biosolids were collected from the end of the secondary treatment train at a Concord, MA wastewater treatment plant. This plant serves approximately 5000 people and treats up to 1.2 million gallons per day during the summer months. The treatment process consists of a single-stage trickling filter with intermittent sand beds for winter season polishing. The biosolids collected were 3.4% solids. The biosolids were stored at 4°C until use.

Sludge Collection for Environmental Samples

Raw and treated sludge was collected from three different sites: Texas, Pennsylvania, and New Hampshire. The Texas and Pennsylvania samples were collected as part of CFR Part 503 testing and sent by overnight mail to our laboratory. The New Hampshire samples were obtained directly from the treatment plant and driven to the laboratory. The Pennsylvania plant uses

anaerobic digestion for treatment, the Texas plant uses lime stabilization, and the New Hampshire plant uses composting. One liter of sludge from each location was collected. All samples were stored at 4°C until use.

Percent Total Solids

The percent total solids of the sludge samples used in the seeded portion of the experiment were measured at the treatment plant. For all the other samples, this measurement was taken at the UNH lab. Using the EPA method (EPA, 2003), approximately 50 grams of sludge was measured into a ceramic weigh boat, previously dried in a dessicator. The sludge and weigh boat were placed in a drying oven at a temperature of 103-105°C for 24 hours. After 24 hours, the sludge and weigh boat were cooled in a desiccator and reweighed. The drying time was extended for one hour and the weighing was repeated until the loss in weight was no more than 4% of the previous weight. After determining the final weight, the percent solids were calculated (Appendix 2).

Elution of Viruses from Sludge Samples

Viruses were extracted from the sludge using the procedure in Figure 2. The procedure is based on EPA part 503 (EPA, 1992). 100 ml or 100 grams of sludge was combined with 100 ml of 10% sterile beef extract. The beef extract and sludge combination was blended in a Waring blender for 5 minutes. After blending, the sample was stirred for 30 minutes on a stir plate. After stirring, the sample was centrifuged at 10,000 x g for 30 minutes in a Beckman J2-21M

induction drive centrifuge. The supernatant was retained as the viruses were removed from the solids by the previous processes.

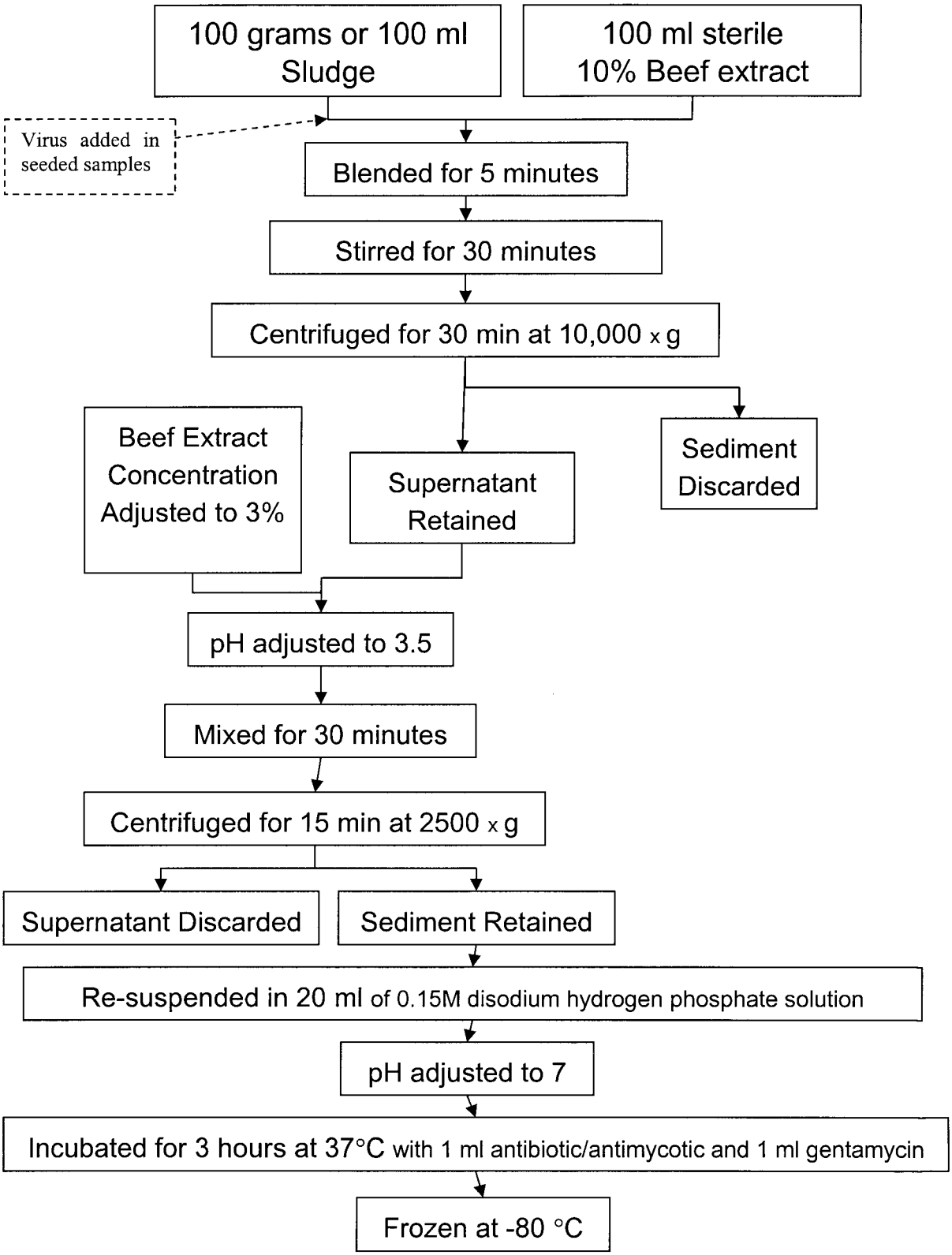
Sterile water was added to the supernatant to achieve a 3% concentration of beef extract. The pH was adjusted to 3.5 and the solution was mixed for 30 minutes. After mixing, the sample was centrifuged at 2,500 x g for 15 minutes. The precipitate was retained as the viruses are now concentrated in the solids.

The sediment was re-suspended in 20 ml of 0.15M disodium hydrogen phosphate solution. The pH was adjusted to a neutral pH of 7. The sample was incubated for 3 hours at 37°C with 1 ml of an antibiotic/antimycotic solution and 1ml of gentamicin. The incubation with antibiotics accomplishes bacterial decontamination. The concentrated eluent was frozen at -80°C until evaluation. The losses due to procedure have been outlined in Katz (2005) and are not significant for mammalian orthoreovirus.

Sample Seeding

In trials requiring seeded sludge samples, virus was added to the samples before the first step of the elution procedure. To achieve a final concentration of approximately 10^5 pfu/ml, 0.1 ml of mammalian orthoreovirus at 10^6 pfu/ml was added to 200 ml of sludge in the blender.

Figure 2. Procedure used to elute mammalian orthoreovirus from the sludge.



Sample Preparations

The samples were prepared for the seeded experiments using the procedure outlined in figure 3. From the 5 liters collected in Concord, MA for the seeded experiments, three different portions were removed. Each of these portions was seeded and eluted separately as described above.

Before use, sludge samples were thawed at 37°C until liquid and vortexed. 0.1 µl of chloroform per 1 ml of sludge was added to the thawed sludge. The sludge was centrifuged at 10,000 x g for 5 minutes. After centrifugation, the supernatant liquid was split into three aliquots. These aliquots were individually diluted three times to become the dilution series named: A, B, and C. The dilutions were done in phosphate buffered saline (PBS) adding 110 µl to 990 µl for 1 to 10 dilutions. The dilutions were 10^{-1} to 10^{-11} for the seeded samples

The seeded samples were designed with a four part code that started with the number that referred to the order in which the portion were removed from the 5L sample. The next character in the designation was "S" to indicated that the sludge was seeded with virus. The next digit stated which dilution series the sample belonged to: A-C. The final digit in the sample identification was the value of the dilution itself. For example, a seeded sample from the second portion, first dilution series, with a 10^{-5} dilution would have been 2SA5.

The environmental samples were prepared as shown in figure 4. Each sludge type was eluted as described in the previous section. The environmental samples then followed the same procedure as the seed samples. The samples were thawed, amended with chloroform, centrifuged, and split into aliquots.

These aliquots were individually diluted three times to become the dilution series. The dilutions were 10^{-1} to 10^{-3} for the environmental samples.

The sample designations for the environmental samples also started with a number. For these samples the first number indicated from which of the three locations the sample was taken. Number 1 referred to the Texas samples, 2 referred to the New Hampshire samples, and 3 referred to the Pennsylvania samples. The second digit in the environmental sample designation indicated their level of treatment, "U" for an untreated or raw sludge and "T" for a sludge that had been treated by a process approved by the EPA. The third and fourth digits were the same as with the seeded samples, referring to the dilution series and the dilution, respectively.

Figure 3. Procedure for seeded sample preparations.

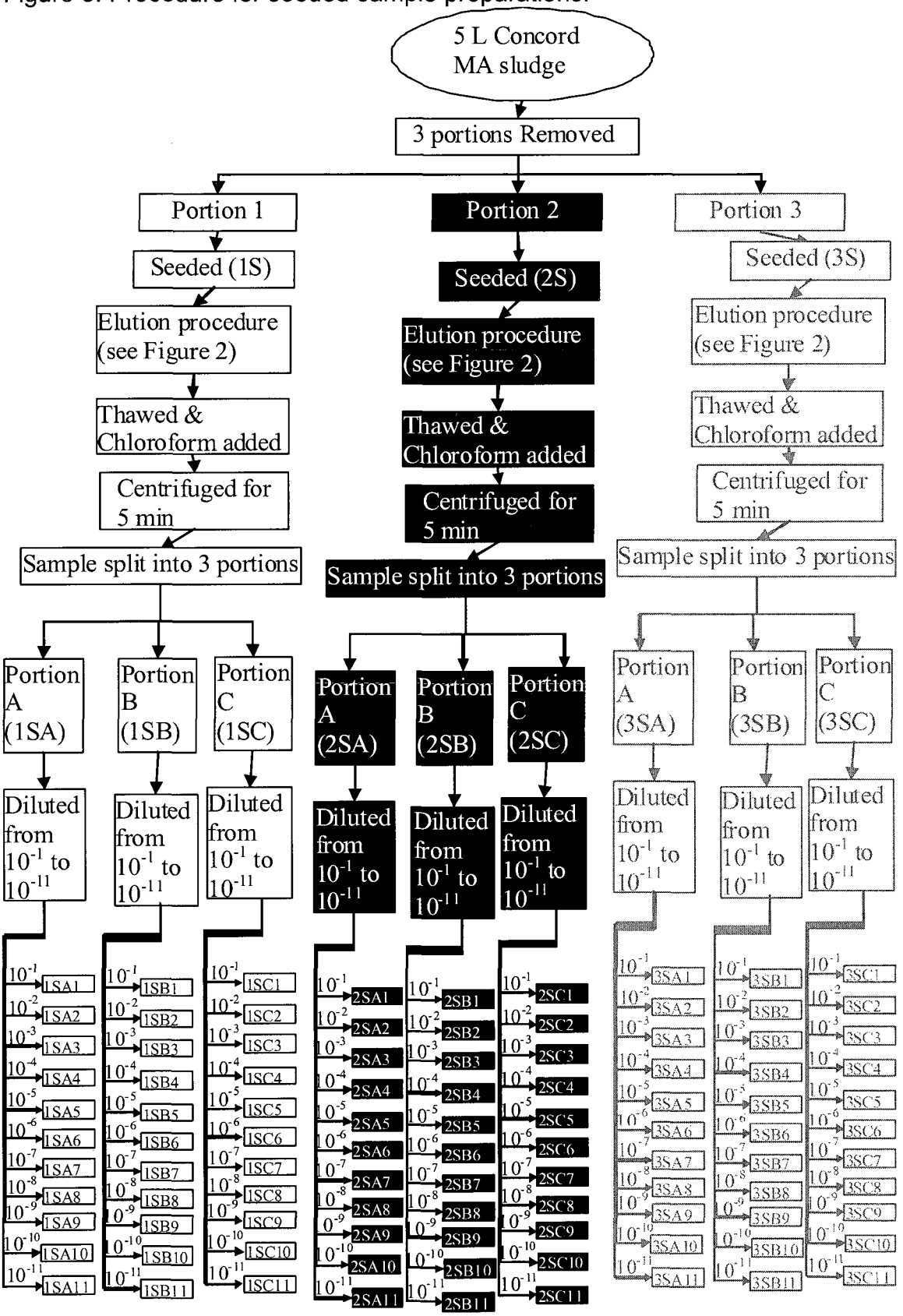
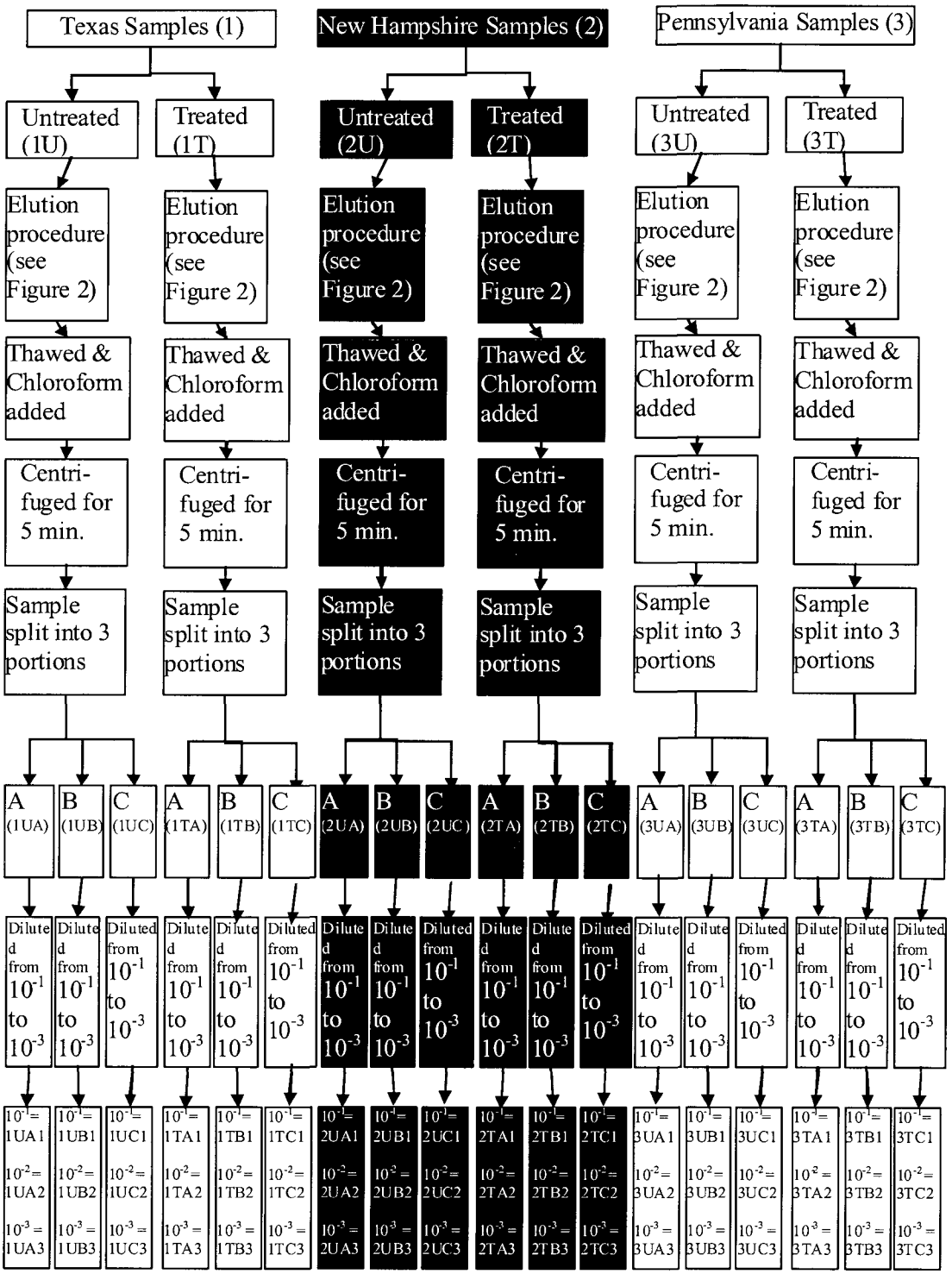


Figure 4. Procedure for environmental sample preparations.



Mammalian Orthoreovirus Plaque Assay

The concentration of virus was determined using the plaque forming unit method. For each sample three 10-fold dilutions were made. Plaques from each dilution were counted and averaged to determine plaque forming units/ml. The plaque assays were done according to Brabants (2003), but were modified for use in six well plates. In this procedure, Buffalo Green Monkey Kidney (BGMK) cells were grown to 95-100% confluency in 6 well plates. These plates were chosen because of the large surface area in the well. The large surface area creates more space between plaques, which is advantageous for counting individual plaques. The medium was removed and the cells were washed with MEM that had been warmed in a 37°C water-bath. After washing, a sample volume of 0.1 ml was used as an inoculum and added to each well. Once inoculated, the plates were rocked for 90 minutes to permit viral adsorption to occur. After adsorption, 4 ml of agar overlay containing 2% bacto-agar and 2X MEM were added to each flask. To enhance plaque formation, 100 µl of 1mg/µl trypsin was added to each well. The agar overlay was permitted to harden and plates were incubated at 37°C for 7 days. After 7 days, 1 ml of formalin was added to each well, and the plates were placed back in the incubator for 24 hours. After 24 hours, the agar overlay was removed with warm water and gentle tapping. A volume of 0.1 ml crystal violet was added to visualize the plaque forming units in the cell layer. The equation for plaque forming units is referenced in Appendix 3. The plaques forming units and the percent total solids

are used to calculate the PFU in 4 grams total solids, according to the calculation in Appendix 4.

Integrated Cell Culture / PCR

LLC-MK2 cells were grown to 75-90% confluency in 6 well plates using MEM with 10% FBS. The medium was removed, cells were washed one time with warm MEM, and 100 μl of the dilution was added to the cells. The samples were taken from the same tubes as were used in the plaque assay. Cells were rocked every 15 minutes for 90 minutes to allow for viral attachment. At the end of the rocking, 4 ml of MEM without trypsin was added. For the time equal to zero (T=0) plates, the medium was immediately removed and 1 ml of trypsin added. These plates were incubated for 20 minutes at 37°C to loosen the cells and placed in the freezer. The time equal to seven (T=7) days plates were placed in the incubator, upon which, after 24 hours, 100 µl of 1mg/ml trypsin was added to the plates and the plates were returned to the incubator for an additional six days. On the seventh day, the medium was removed, 100 µl of 1mg/ml trypsin was added to each well, the plates were incubated for 20 minutes, and the plates were placed in the freezer. After thawing the LLC-MK2 cells, RNA was extracted from the T=0 and T=7 plates.

RNA Extraction Method Selection

To select the extraction method with the least amount of loss, four kits from the Qiagen Company were compared. The kits were RNAeasy minelute

cleanup kit, QIAamp DNA blood mini kit, QIAamp viral RNA mini kit, and QIAamp minelute virus spin kit. Phenol-chloroform extraction and PCR without an extraction method were also compared. For the phenol-chloroform extraction, the method was based on the Cold Spring Harbor method (Sambrook *et al.*, 1999). Phenol and chloroform were added to the sample at 2.5 times the sample amount. The solution was centrifuged and the aqueous phase was kept. Phenol and chloroform were again added and the aqueous phase was kept until no protein was seen. Equal volumes of chloroform were added and the samples centrifuged until no protein was seen. Volumes of $1/10^{th}$ the amount of sodium acetate and 2.5 times the amount of 95% ethanol were added and the tube was frozen for 1 hour at -20°C, which allowed for the precipitation of the RNA. The resulting solution was diluted and read by a spectrophotometer to estimate the amount of nucleic acid.

RNA Extraction Procedure

The QIAamp viral RNA mini kit, manufactured by Qiagen (1999), was chosen for RNA extraction. The maximum amount of recommended sample, 140 µl, was placed in the column. RNA was absorbed to the QIAamp membrane provided in the spin column by using the supplied buffers. The residual contaminants were removed using the provided wash buffers. After washing, RNA was eluted off the membrane using a buffer. The virus elution was used in quantitative PCR immediately following extraction.

Quantitative PCR Primer Design

Primers and probe sets were designed with PrimerExpress, Applied Biosystems proprietary software. They were targeted to sections of the genome which diverge in the three types of mammalian orthoreovirus. Three sets of primers and probes were based on outer capsid protein (mu-1) in the m2 segment of the genome. NCBI blast was used to determine that the primers detected only the intended target organism.

Table 2. Primers used to detect the three types of mammalian orthoreovirus. Nucleotides are listed in 5'-3' direction.

Serotype	Forward Primer	Reverse Primer	Position of Amplified Region
1 (Lang)	gaggagggacacgcgtagtg	ccagatccagaacgaatctcatc	1114-1176
2 (Jones)	cggctacggtgtcaggatct	cgcgcgacgctattttg	1766-1824
3 (Dearing)	ctaccgctgtaccatcgttaagct	tggtacccctccgggatt	112-170

Table 3. Probes used to detect the three types of mammalian orthoreovirus which coordinate with the primers listed in Table 1. Nucleotides are listed in 5'-3' direction.

Serotype	Probe	Position
1 (Lang)	cttggatcagattgctc (tagged with FAM)	1137-1153
2 (Jones)	taatccgaaaggtattttgt (tagged with VIC)	1787-1806
3 (Dearing)	atcacctggaatgct (tagged with FAM)	137-151

Quantitative Real Time Reverse Transcription Polymerase

Primers were received from Applied Biosystems as dry and desalted at a concentration of 80,000 pmol. They were diluted to 50 µmol/L with molecular grade water. Probes were received from Applied Biosystems as 6000 pmol in 60

µl buffer and were diluted to 10 µmol with molecular grade water (Appendix 5). Primers and probes were dispensed into 0.2 ml micro-centrifuge tubes and frozen at 4°C. Master mix was prepared with 1x of TaqMan® One-Step RT-PCR Master Mix supplied by Applied Biosystems (part number: 4309169). This contained AmpliTaq Gold® DNA polymerase, Passive Reference I, and optimized buffer components. The master mix also contained 1x of MultiScribe™ Reverse Transcriptase and RNase Inhibitor from Applied Biosystems, 900 nM of the forward primer, 900 nM of the reverse primer, 250 nM of the probe, and sterile molecular grade water (Appendix 6). Five µl of the extracted DNA was combined with 25 µl of master mix. The sample was placed in the ABI Prism 7700 quantitative real time PCR thermocycler. The PCR run consisted of 48°C for 45 minutes, 95°C for 10 minutes, and 50 cycles of 94°C for 20 seconds, 55°C for 1 minute, and 72°C for 30 seconds. Fluorescence was detected at the end of each cycle to determine the cycle threshold value (CT), the cycle number at which the fluorescence generated within a reaction is greater than the background fluorescence. CT values for the T=7 samples were compared to the corresponding CT values for the T=0 samples. For the seeded samples, mammalian orthoreovirus type 1 primer set was used. For the environmental samples, each sample was separately combined with each primer set.

Calibration Experiment

Three dilution series of mammalian orthoreovirus type 1 were analyzed by plaque assay and on the quantitative thermocycler to determine the relationship

of plaque forming units to cycle threshold values without the in-vitro amplification step of cell culture. This analysis was repeated four times and plotted to determine the numerical relationship between the variables.

Quality Control

Strict quality assurance included documenting all reagents and recording their usage in a media notebook. Temperatures of refrigerators, freezers, and water baths were recorded twice daily and calibrated as necessary. The ABI Prism 7700 quantitative PCR thermocycler was calibrated monthly using a calibration plate provided by Applied Biosystems. Instrument wells emitting light were cleaned with alcohol and the background fluorescence was examined weekly.

In the plaque assay, each run had three negative control wells which contained no sample. In valid assays, negative control wells produced no plaques. Mammalian orthoreovirus was added to three wells for a positive control. The virus was at a concentration which would form plaques if the cells were susceptible and the virus was infectious. When samples were diluted with PBS, the PBS was tested in a plaque assay to confirm that it did not produce plaques.

In the cell culture portion of the ICC-qPCR, one positive and one negative well was run with every assay. The positive control was mammalian orthoreovirus type 1 and the negative control contained no virus. For the RNA extraction, the positive and negative controls from the cell culture portion were

included. An additional negative control referred to as the "spin control" was added. The spin control was a tube with all the buffers added which was placed in the centrifuge each time a spin was required. These three controls were included as part of the quantitative PCR assay. An additional negative control was added that contained only the master mix, and an additional positive control was added which contained the master mix and mammalian orthoreovirus. These five controls were included with every PCR assay.

Results

To determine if a consistent and detectable source of mammalian orthoreovirus was available for the study, the virus stock was tested with PCR primers of known effectiveness. The presence of sufficient virus in the mammalian orthoreovirus stock is confirmed by the resulting band at 416 base pairs shown in Figure 5. From the available methods for extracting RNA from samples, RNAeasy minelute cleanup kit, QIAamp DNA blood mini kit, QIAamp viral RNA mini kit, QIAamp minelute virus spin kit, and phenol-chloroform extraction, the most effective method of RNA extraction is the QIAamp viral RNA mini kit as shown in Table 4.

The relationship of plaque forming units (PFU) from the plaque assay to cycle threshold values (CT) from the quantitative PCR, without the in-vitro amplification step of cell culture, is plotted to determine the numerical relationship between the variables. A nearly linear relationship is established, that can be observed when both of the trials are displayed separately (Figure 6), and when the trials are averaged in the correlation curve (Figure 7). The lowest level of plaques that detected by the quantitative thermocycler during this portion of the study ranged from 1.25 to 5.90 PFU (Table 5). The calculation, in Appendix 8, converts the results of the quantitative PCR into plaque forming units per sample.

Figure 8 is an overview showing that 10^{-8} was the most dilute sample of the seeded samples which contained mammalian orthoreovirus after 7 days in

ICC-qPCR compared to 10^{-5} in the plaque assay. Quantitative PCR from the initial time zero samples was compared to quantitative PCR in the samples that incubated for seven days to ensure that infectious virus was present and higher concentrations were found at the later time point, Figure 9. Figure 10 compares individual samples incubated for 7 days with ICC-qPCR to the PFU assay, demonstrating that the ICC-qPCR method detects the smallest dilution of virus in a particular sample. This figure shows that smaller amounts of the virus were detected with the quantitative thermocycler after cell culture than with the plaque assay. The results from the seeded experiments shown in figure 11 are plotted in a linear comparison showing the range of dilutions detected using ICC-qPCR.

Fifty-four percent of the environmental samples were positive by ICC-qPCR and none were positive by the plaque assay method as seen in Table 6. Table 7 presents the positive samples divided out by treatment and location, showing that each location had positive samples. Of the treated samples, only the digestion treatment was positive for mammalian orthoreovirus. Eleven percent of the treated samples were positive percent of the untreated samples were positive for mammalian orthoreovirus while eighty percent of the untreated samples were positive (Table 8). Considering only the location and not the treatment level at each location, 66% of the Texas samples, 56% of the New Hampshire samples, and 33% of Pennsylvania samples were positive for mammalian orthoreovirus (Table 9). The types of mammalian orthoreovirus detected varied among the different locations sampled. Mammalian orthoreovirus type 3 was detected at the Texas location and mammalian

orthoreovirus type 1 was detected at the New Hampshire and Pennsylvania locations (Table 10).

As mentioned previously, the correlation curve in Figure 7 can be used to estimate the PFU equivalents for the environmental samples. The curve shows the linear relationship between plaque forming units and cycle threshold values. Figure 12 plots all of the environmental samples that were positive for mammalian orthoreovirus on this curve with the estimates of log PFU for the environmental samples ranging from $1x10^{-2}$ to $1x10^{5}$. The error bars represent the standard error of the means from the correlation curve as previously plotted. Averaging the positive samples by treatment type and location and the plotting them on the correlation curve, the untreated Texas samples are plotted with the smallest PFU estimate and the untreated New Hampshire samples are plotted with the largest PFU estimate (Figure 13). In Table 11 lists the estimates of PFU/ml for the environmental samples ranging from $2.29x10^{2}$ to $7.43x10^{7}$ pfu/ml based on the comparison between PFU/ml and CT. The PFU is converted to PFU/ml using the equation in the Appendix 8. In Figure 14, the PFU/ml estimations are organized graphically by decreasing value and then separated visually by sampling group to illustrate that the majority of the high PFU/ml estimates are from the New Hampshire location and the majority of the low PFU/ml estimates are from the Texas location. Table 12 shows the percent total solids from the environmental samples was between 3.09% and 27.23%, as well as estimating the amount of virus in 4 grams total solids to be between $2.15x10^{2}$ and $1.71x10^{6}$ PFU/4 grams total solids.

Figure 5. A band at 416 base pairs on an electrophoresis gel confirms the presence of mammalian orthoreovirus after amplification through PCR.

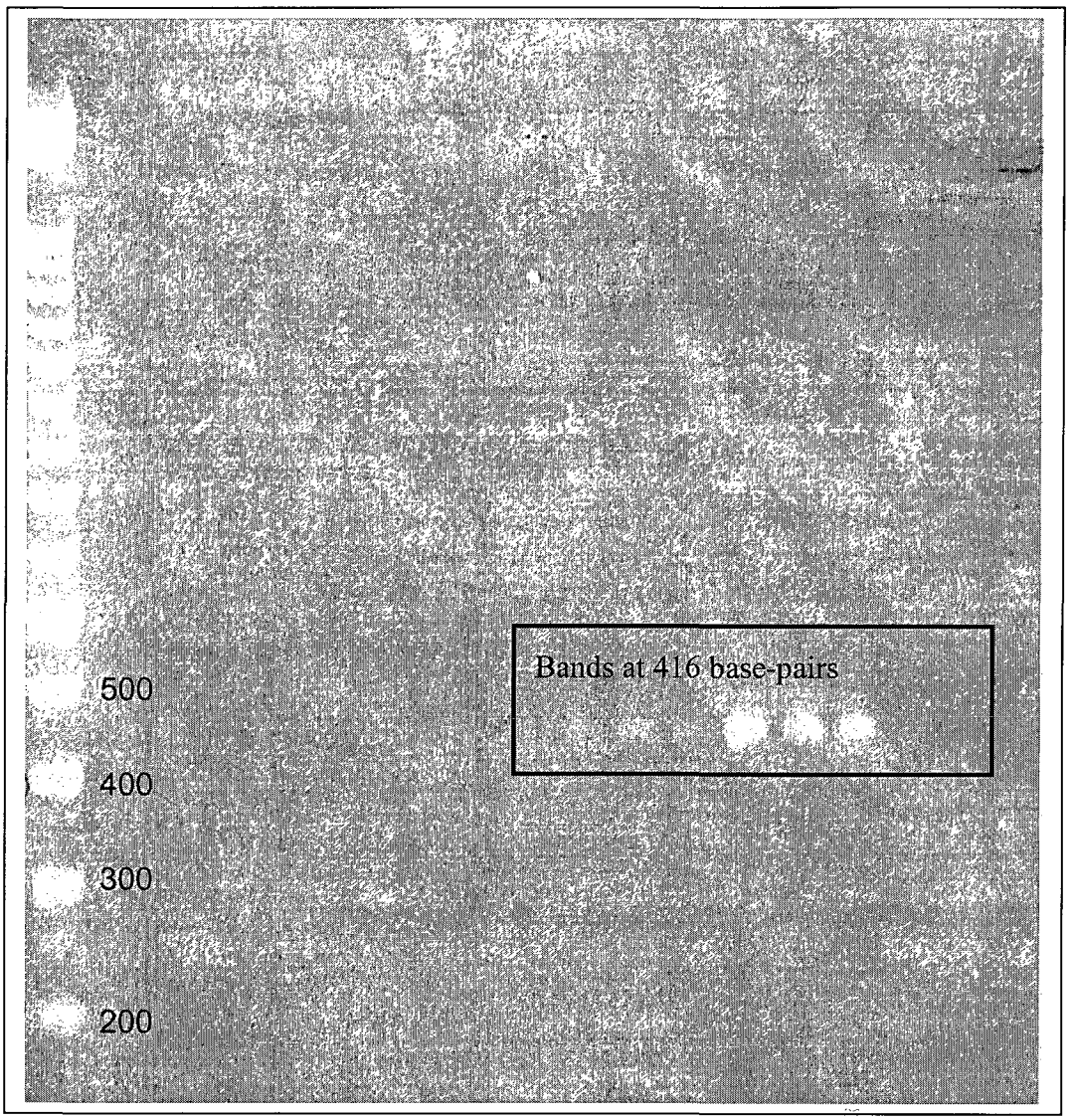


Table 4. The QIAamp viral RNA mini kit was the most efficient extraction method. By using this method before PCR, the PCR detected the most dilute sample of mammalian orthoreovirus. Therefore it was chosen as the method for extracting RNA from the cell culture product.

Extraction Method	**Most Dilute Concentration Detected**
No Extraction	10^{-3}
RNAeasy minelute cleanup kit	10^{-4}
QIAamp DNA blood mini kit	10^{-4}
QIAamp Viral RNA mini kit	10^{-5}
QIAamp minelute virus spin kit	No detection
Phenol Chloroform Extraction	10^{-3}

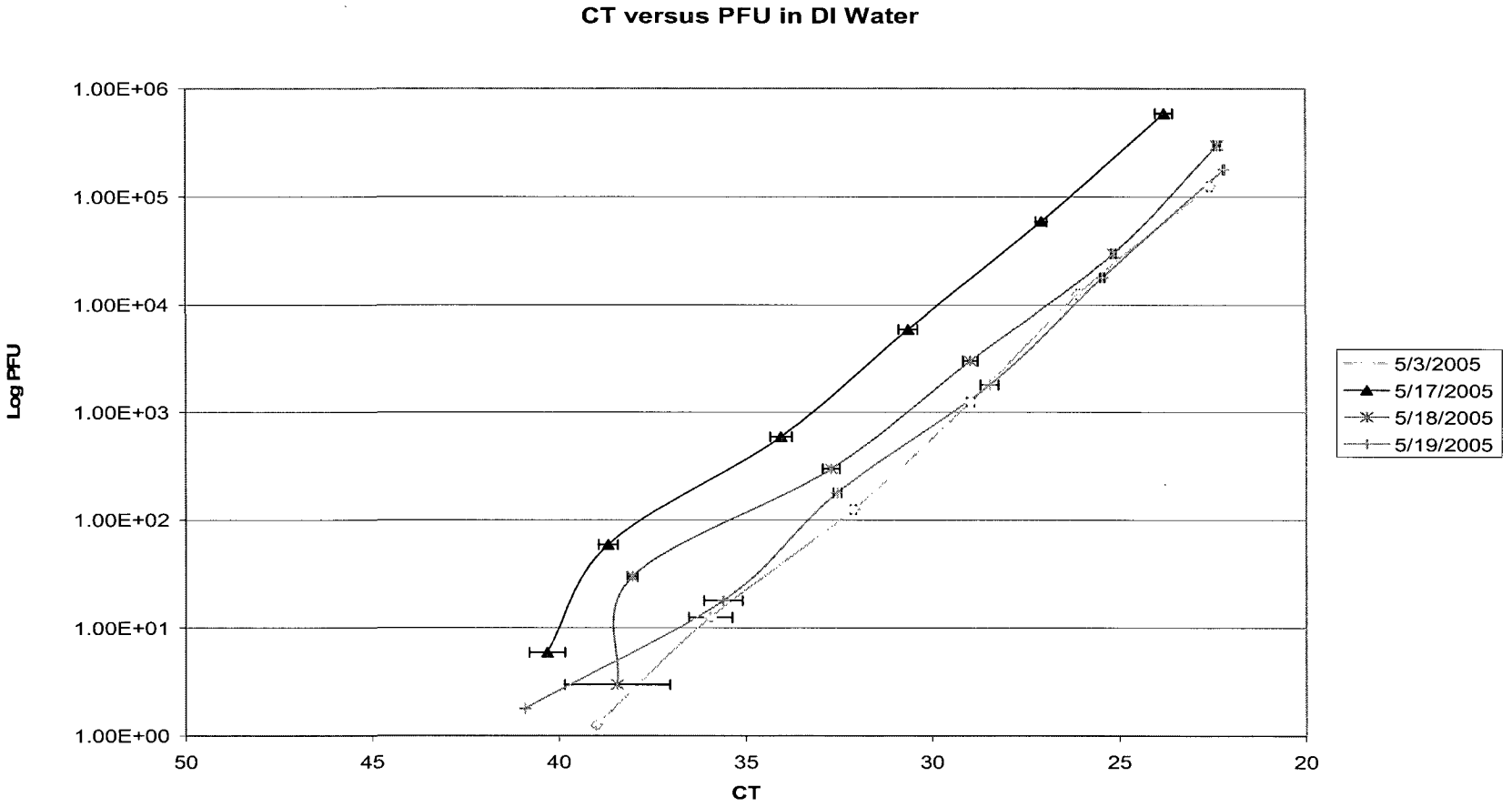


Figure 6. Each of the four separate trials comparing quantitative PCR to plaque assay demonstrated a nearly relationship between CT and PFU. The error bars show the standard error in CT values among the replicates in each trial.

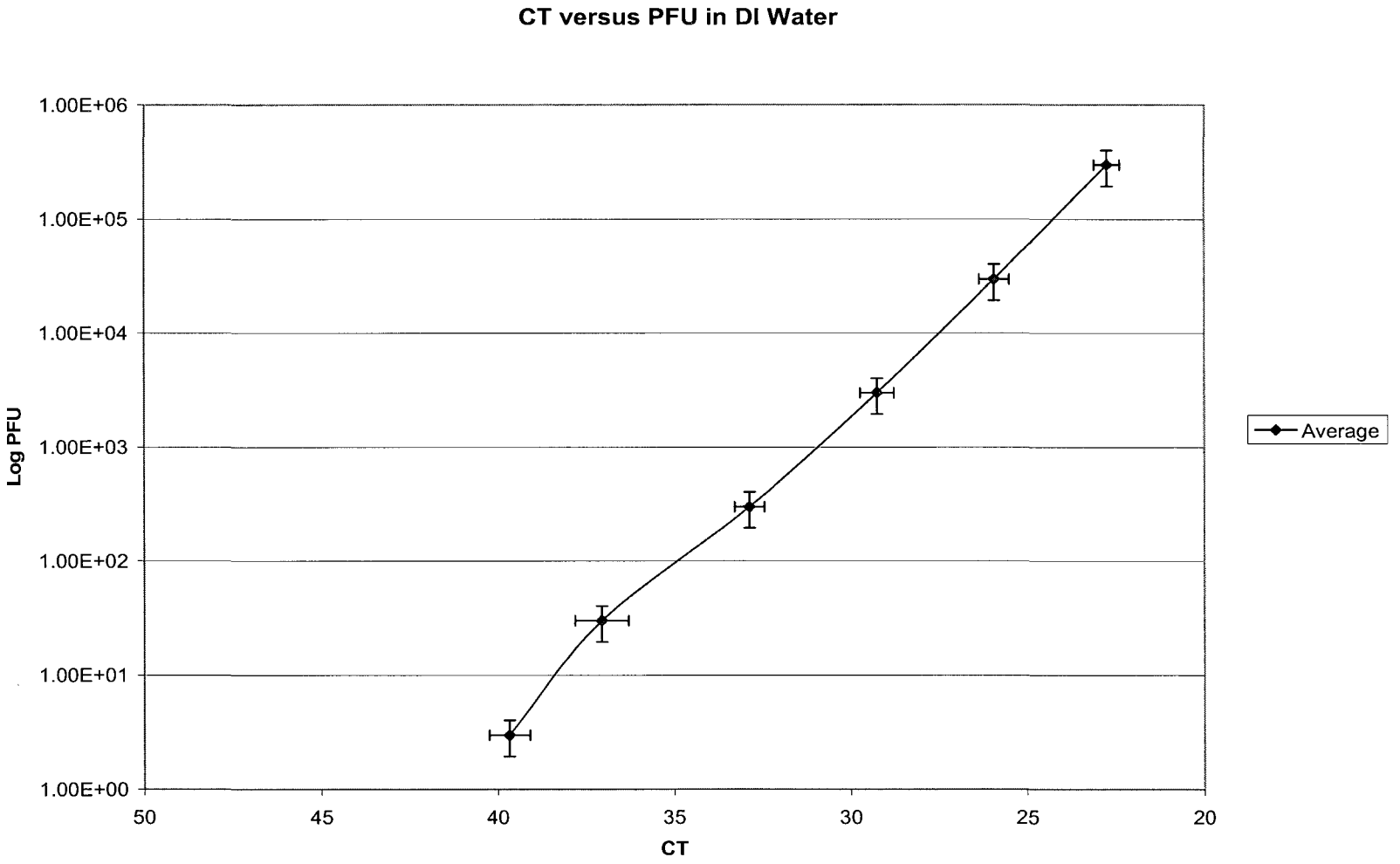


Figure 7. When the separate runs are averaged, a correlation curve can be determined and the relationship between PFU and CT can be shown on a graph. The error is plotted in both directions showing the standard error of the mean for both PFU values and CT values.

Table 5. The results of PCR sensitivity for the detection of mammalian orthoreovirus assayed in dilutions of water are shown below.

Date	PFU Detected in water
5/3/2005	1.25
5/17/2005	5.90
5/18/2005	2.98
5/19/2005	1.79

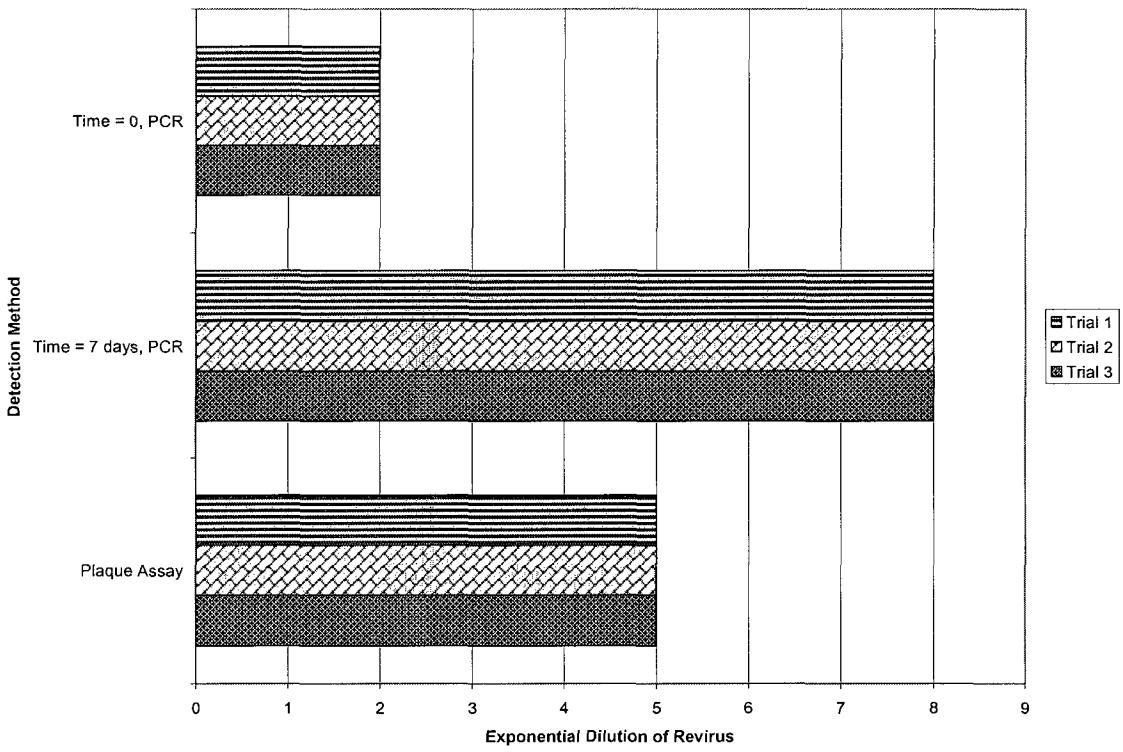


Figure 8. An overview of the seeded experiments shows the lowest dilutions that mammalian orthoreovirus was detected in by each method.

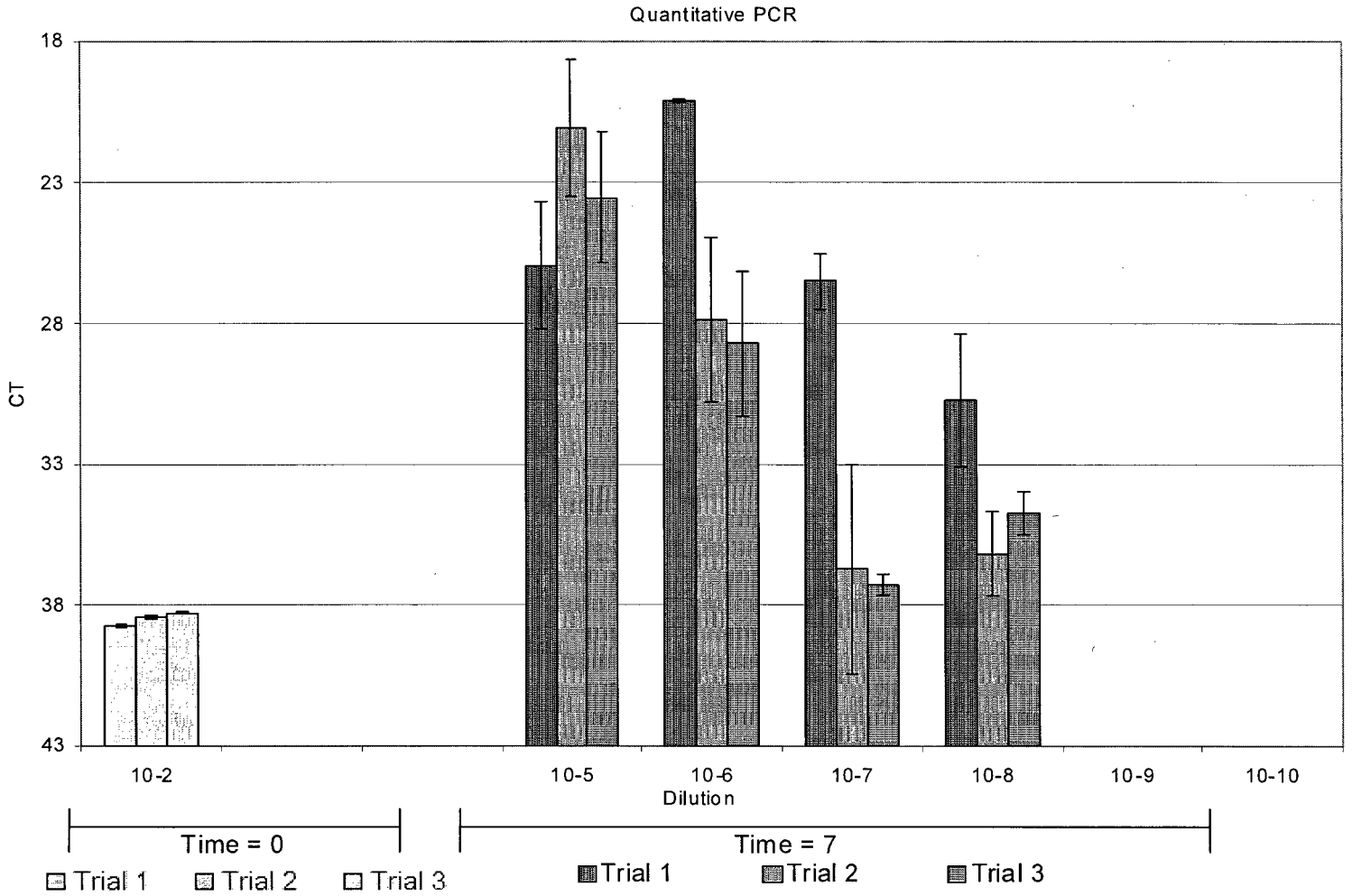


Figure 9. Quantitative PCR results from time equals seven days samples contained higher concentrations of mammalian orthoreovirus than the initial time zero samples, demonstrating that infectious virus was present.

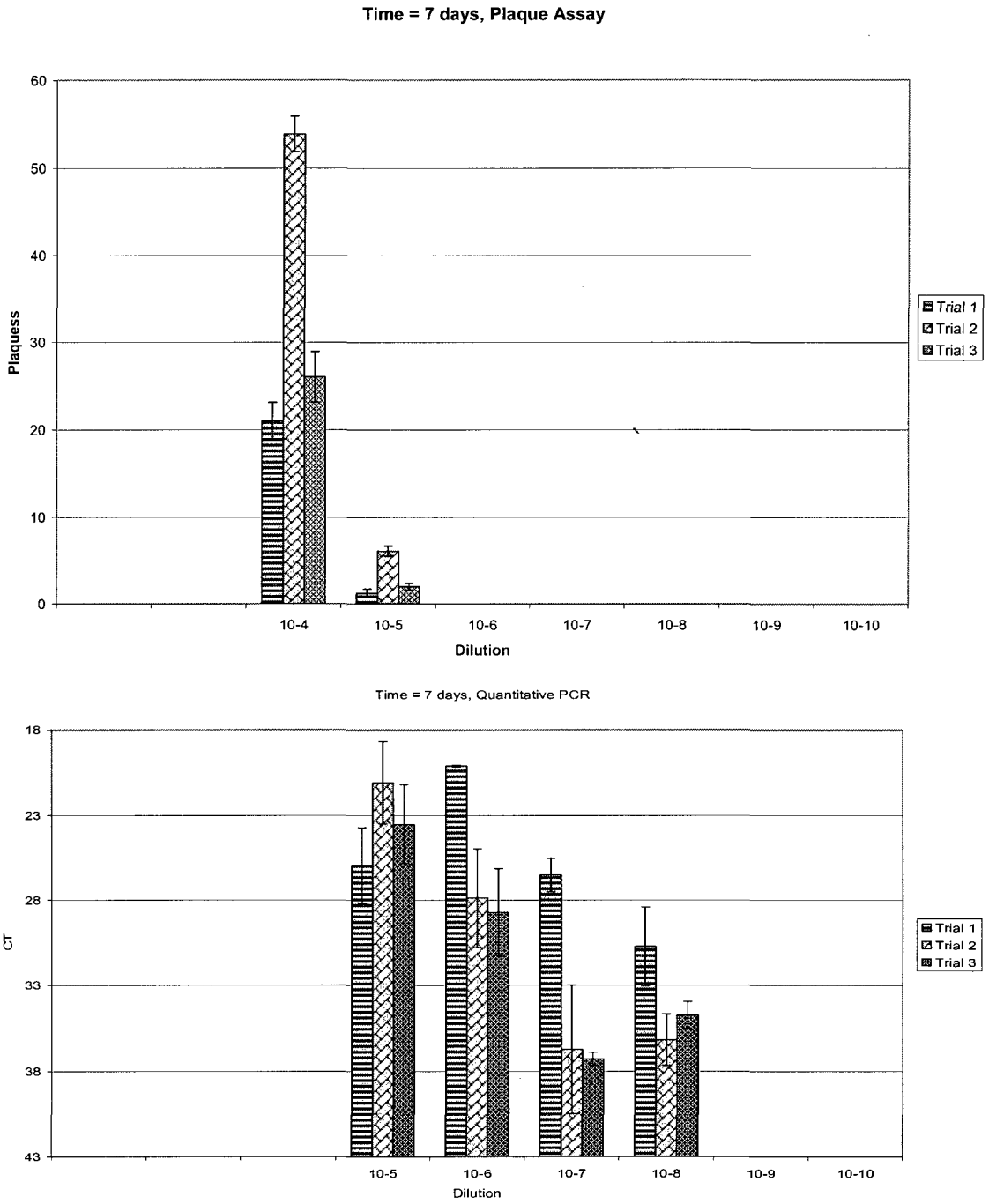


Figure 10. The top graph shows the plaque assay detected mammalian orthoreovirus to a dilution of 10^{-5} and the bottom graph shows the quantitative PCR detected the virus to 10^{-8}.

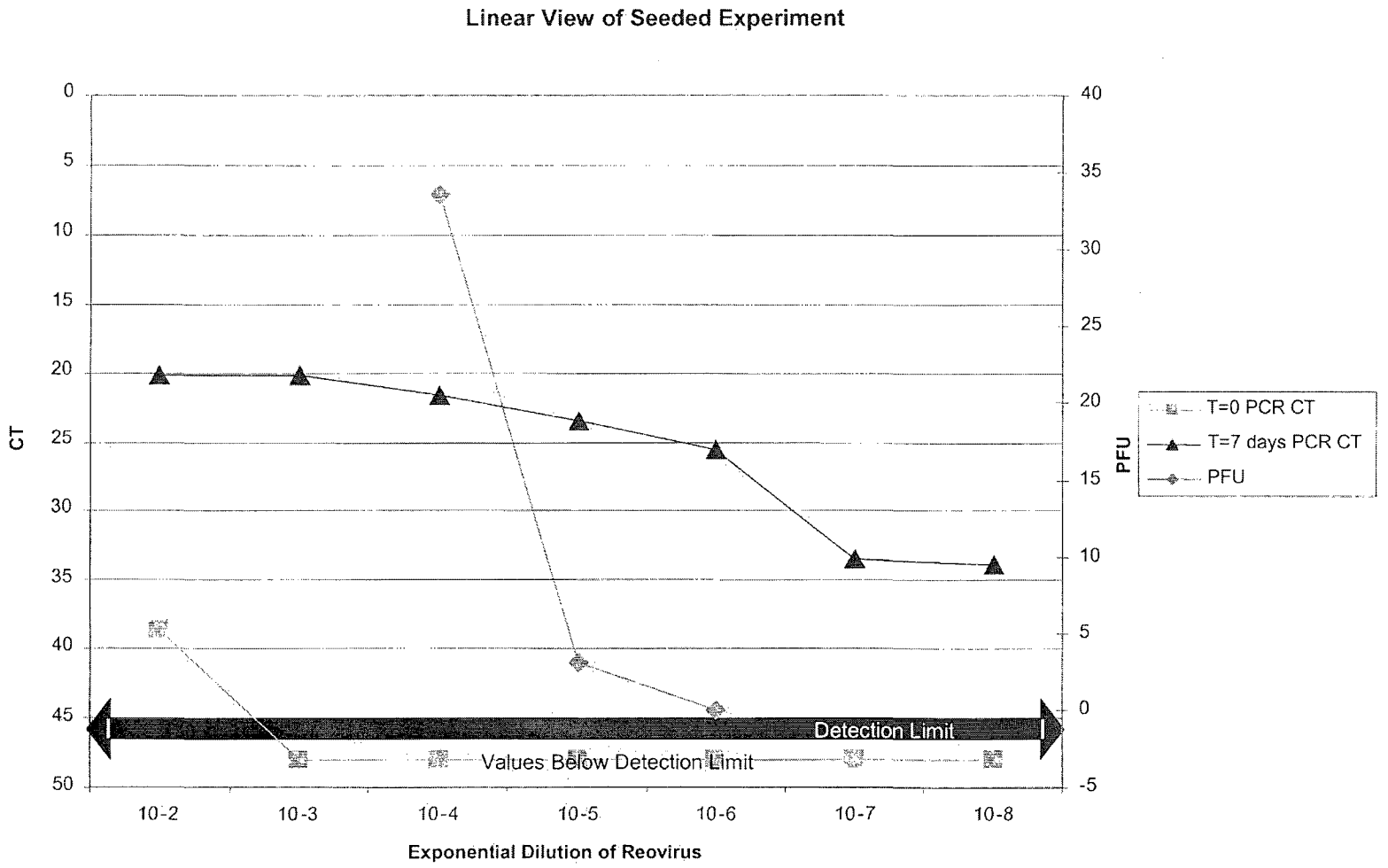


Figure 11. The linear graph of the seeded experiment show that CT can be plotted for all the dilutions detected at time = 7. For the plaque assay, only the dilutions with countable plaques can be plotted.

Table 6. Out of the 24 environmental biosolids samples that were tested for mammalian orthoreovirus, 13 tested positive by ICC-qPCR, and none tested positive by the plaque assay technique.

States Tested	Number of Samples Tested	Positive for Infectious Mammalian Orthoreovirus by:	
		ICC-qPCR	Plaque Assay
NH	9	5	0
TX	9	6	0
PA	6	2	0
Total	**24**	**13**	**0**
Percent Positive		**54%**	**0%**

Table 7. The environmental samples are divided into the treatment types which shows how many untreated are positive and how many treated are positive. The samples are broken out by location.

Location	**Treatment**	**Undiluted (Pos/Total)**	**10^{-1} (Pos/Total)**
Texas	Untreated	3/3	3/3
	Limed	0/3	
New Hampshire	Untreated	3/3	2/3
	Composted	0/3	
Pennsylvania	Untreated	1/3	
	Digested	1/3	

Table 8. When the samples are grouped by the treatment type (disregarding the locations), 80% of the untreated samples are positive and 11% of the untreated samples are positive.

Treatment	Positive by ICC-qPCR/Total Samples	Percent Positive by ICC-qPCR
Untreated	12/15	80%
Treated	1/9	11%

Table 9. When the samples are grouped by location and the treatment type is not taken into consideration, the Texas location has the highest percentage of positive samples.

Location	Treatment (includes untreated samples)	Positive by ICC-qPCR/Total	Percent Positive
Texas	Limed	6/9	66%
New Hampshire	Composted	5/9	56%
Pennsylvania	Digested	2/6	33%

Table 10. Mammalian Orthoreovirus detected based on sample location.

State	Mammalian Orthoreovirus Type(s) Detected
Texas	Type 3 (Dearing)
New Hampshire	Type 1 (Lang)
Pennsylvania	Type 1 (Lang)

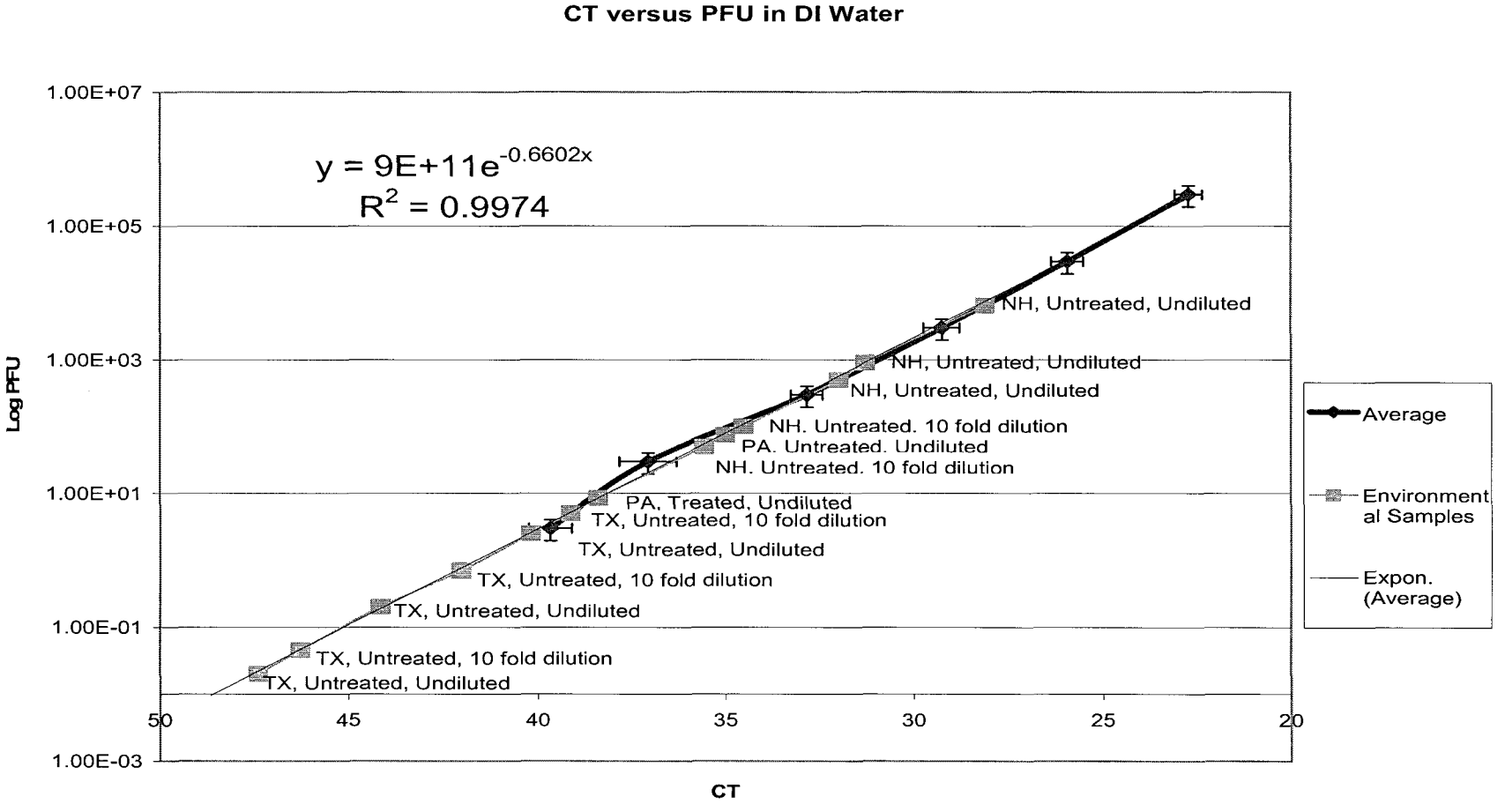


Figure 12. A line can be drawn through the mean calibration curve and extended to estimate PFU values when only CT is known. The experimental samples were given "PFU" values by using the curve even though they did not form plaques.

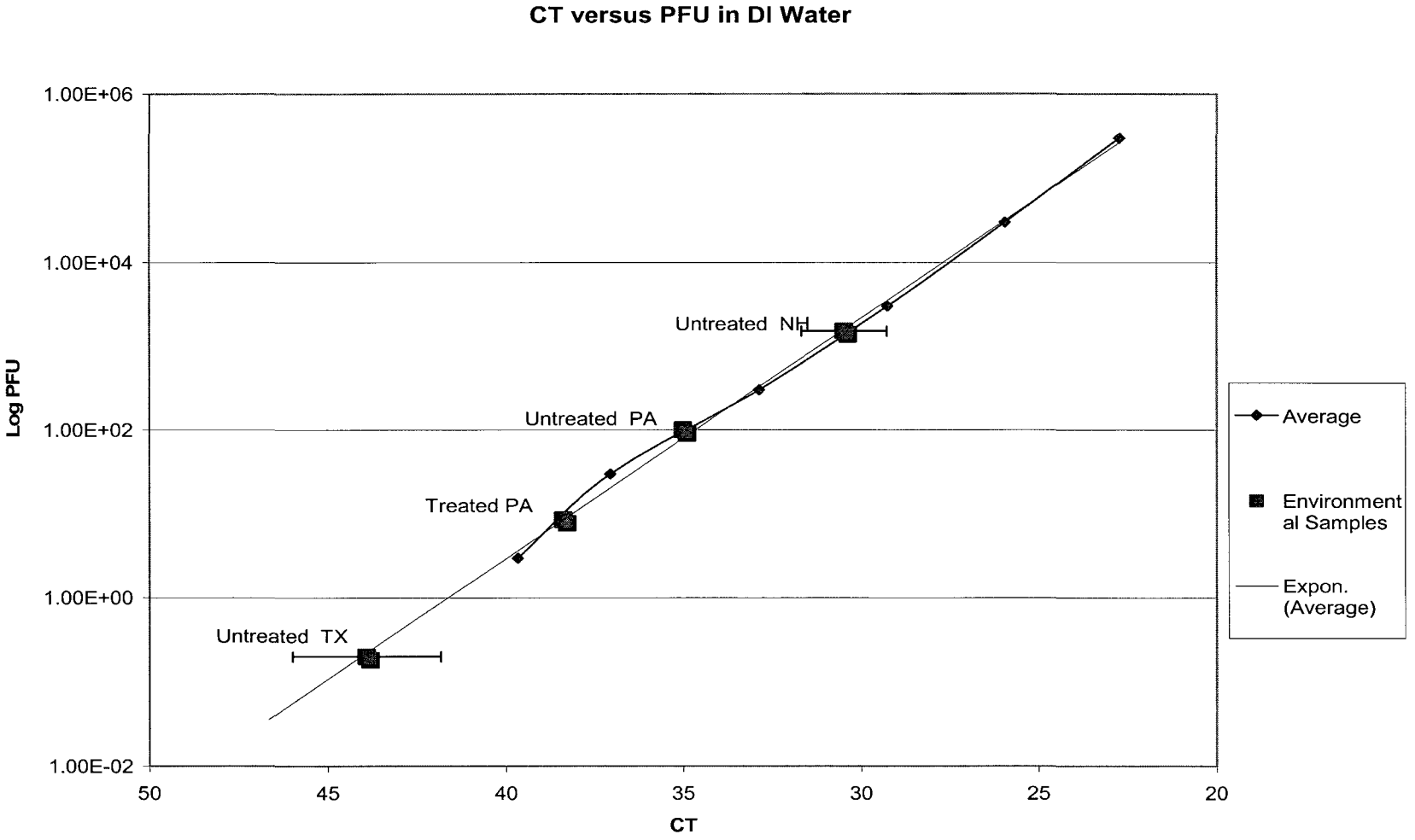


Figure 13. The environmental samples can be grouped and then placed on the calibration curve.

Table 11. The cycle threshold value for the each positive sample can be correlated with a PFU value based on the correlation experiment. The PFU/ml can then be calculated based on the equation in Appendix 8. This PFU/ml is an estimate of the resulting PFU concentration after incubation on cells.

Sample Type	Sample	CT by ICC-qPCR	PFU by Plaque Assay	PFU/ml after 7 days on cells (estimated with standard curve)
Untreated New Hampshire	2U-A0	28.11	0	7.43E+07
	2U-C0	31.31	0	1.03E+07
	2U -B0	32.02	0	5.71E+06
	2U -C1	34.53	0	1.14E+06
	2U -A1	35.57	0	5.71E+05
Untreated Pennsylvania	3U-A0	35.01	0	8.57E+05
Treated Pennsylvania	3T-A0	38.39	0	9.71E+04
Untreated Texas	1U-C1	39.14	0	5.71E+04
	1U -C0	40.19	0	2.86E+04
	1U -A1	42.02	0	8.00E+03
	1U -B0	44.15	0	2.29E+03
	1U -B1	46.28	0	5.14E+02
	1U -A0	47.40	0	2.29E+02

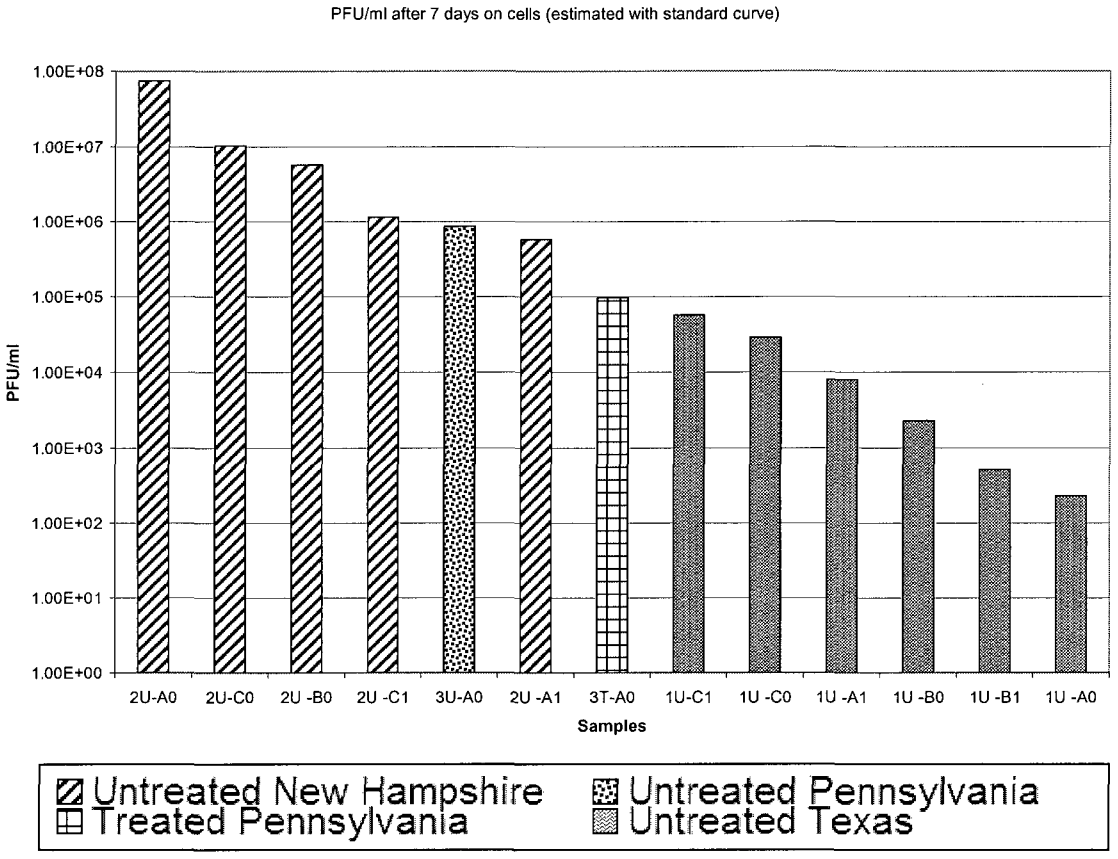


Figure 14. The estimated PFU/ml of the environmental samples can be graphed in order of concentration and distinguished by location and treatment. This PFU/ml is an estimate of the resulting PFU concentration after incubation on cells.

Table 12. The PFU per 4 grams of solid can be estimated from the environmental samples (averaged by location and treatment). This estimation is calculated from the PFU/ml which is calculated from the PFU obtained from the calibration curve. This PFU is an estimate of the resulting PFU concentration after incubation on cells.

	Estimated PFU/5ul Sample, Amount run in PCR	PFU/ml	% Solids	PFU/4 Gram Total Solids
1-U (TX)	**0.20**	2.29×10^{2}	3.90%	3.30×10^{2}
2-U (NH)	**1500**	1.71×10^{6}	3.09%	1.71×10^{6}
3-U (PA)	**0.10**	1.14×10^{2}	3.79%	2.15×10^{2}
3-T (PA)	**8.50**	9.71×10^{4}	27.23%	9.72×10^{4}

Discussion

In this study, mammalian orthoreovirus was chosen as the target virus because it was shown to be prevalent in previous environmental studies (AWWA, 1999; Cliver, 1975; Dahling *et al.*, 1989; Fout *et al.*, 2003; Irving and Smith, 1981; Matsuura *et al.*, 1984; Matsuura *et al.*, 1988; Monpoeho *et al.*, 2004; Sattar and Westwood, 1978; Sedmak *et al.*, 2005; Sellwood *et al.*, 1988; Tani *et al.*, 1995). This study was the first to demonstrate that ICC-qPCR can detect mammalian orthoreovirus in sewage sludge. Further, the ICC-qPCR method was able to detect more mammalian orthoreovirus than the plaque assay, which is the current standard method. The ability of ICC-qPCR to detect mammalian orthoreovirus is supported by the historical success of the ICC-PCR method (Blackmer *et al.*, 2000; Chapron *et al.*, 2000; Jiang *et al.*, 2004; Ko *et al.*, 2003; Lee and Jeong, 2004; Lee *et al.*, 2005; Reynolds *et al.*, 1996; Reynolds *et al.*, 1997; Reynolds *et al.*, 2001).

The effectiveness of ICC-qPCR is based on the combination of the cell culture and quantitative PCR. The sample is inoculated into a cell culture and before and after inoculation quantitative PCR readings are done (Reynolds, 2004). To determine if a cell line is infected with the target virus, quantitative PCR readings on the initial inoculation (Time = 0 days) are compared to quantitative PCR readings on the inoculated cells after seven days (Time = 7 days). Study samples are compared using the cycle threshold (Ct) values, which

represent the cycle number at which the fluorescence generated by the PCR reaction is greater than the background fluorescence. In the PCR reaction, target nucleic acid sequences are replicated exponentially in each cycle. Larger initial concentrations of virus will reach the threshold sooner, resulting in smaller Ct values.

If the Ct value after cell culture replication was smaller than the initial Ct value, it was deduced that infectious virus was present in the original inoculum. Using this principle, both seeded and environmental samples were evaluated for infectious virus. Samples that were positive for infectious virus using ICC-qPCR were negative for infectious virus using the plaque assay. Previous studies agree that ICC-PCR has better detection of poliovirus, hepatitis A virus, enterovirus, adenovirus, and astrovirus than cell culture, CPE, and TCVA-MPN (Chapron *et al.*, 2000; Lee and Jeong, 2004; Lee *et al.*, 2005; Reynolds *et al.*, 1996; Reynolds *et al.*, 1997). Greening *et al.* (2002) compared detection of enteroviruses and adenoviruses and similarly found that ICC-PCR was more useful than the plaque assay. The negative plaque assay results reveal the limitations of the plaque assay for evaluating sewage sludge. Infectious virus could be missed and possibly cause illness in an exposed individual.

In the part of the study where sludge was seeded with virus, in all of the seeded 10^{-3} to 10^{-8} dilutions virus was not detectable at T=0 days by ICC-qPCR. By T=7 days, virus was detectable in every dilution. The detection of virus at T=7 suggests that each sample contained at least one infectious virus.

In a perfunctory interpretation of the results, each dilution would be seen

as positive. However, by assuming that the viral particles were distributed equally in the viral stock solution and the dilution scheme, each 10 fold dilution would contain proportionately less virus than the previous dilution set. Interpreting the results as proportional dilutions allows for the seeded sample dilutions to be treated as individual samples with decreasing amounts of virus present, as represented in Figures 8 and 9.

Using a 10 fold dilution scheme, the most dilute sample with a positive result represents the detection limit. The overview of the seeded experiments, Figure 6, illustrates the detection limits of the plaque assay, PCR, and ICC-qPCR. The bar labeled "PCR after the 7 days in cell culture" represents the ICC-qPCR detection limit, illustrating that ICC-qPCR has the lowest detection limit and is the most sensitive method. By comparing the methods, treating each dilution as a separate sample, the ICC-qPCR has a more sensitive detection limit than the plaque assay. The samples were taken from the exact same tube for both assays. This sampling method minimized dilution inaccuracies, allowing for a more accurate comparison.

However, there is a larger sample amount tested in a plaque assay than in a PCR tube, and this comparison is biased to the plaque assay. If only a few infectious viral particles are present, a smaller sample may not contain the viral particle needed to produce a positive result. Comparing the smaller sample size used in ICC-qPCR to the larger sample size used in the plaque assay is a conservative approach to comparing the effectiveness of ICC-qPCR.

An important advantage to ICC-qPCR is that almost every dilution

generates a numerical value. In contrast, for a plaque assay, the only statistically accurate dilutions are the dilutions that have plaques between 20 and 200 (EPA, 1992). In a plaque assay, plates with small numbers of infectious viruses are difficult to interpret and plates with large numbers are impossible to count. This concept is demonstrated with the Figure 8 which represents the results from the seeded samples. With the ICC-qPCR readings, a clear trend is observed throughout the samples. The Ct values decrease as the samples become more dilute. The wide range of dilutions that generate a numerical value as a result of ICC-qPCR is an advantage because previous experience with the sample type is not needed to choose the appropriate range of dilutions (EPA, 1992).

A difficulty with plaque assays is that cell damage from a non-viral component of the sample could be misinterpreted as cell lysis (Schmidt *et al.*, 1978). Because ICC-qPCR directly detects the viral nucleic acid, it does not have the same problems. If there were any toxic effects from the sample, the toxicity would have occurred in cell culture. The importance of toxicity in cell culture is reduced for several reasons. First, the overall result would not be affected if some cells are lost due to toxicity. Additionally, the cell culture media can both buffer and dilute a toxic agent (Spinner and DiGiovanni, 2001). Finally, the toxic agents in the sample can be removed through harsher methods without concern for damaging the fragile external structure of the virus because removing the viral capsid proteins does not affect detection by PCR (Fong and Lipp, 2005).

The strength of the ICC-qPCR method is specificity and sensitivity. There are several cost and time factors to consider when comparing the method. The

expense of cell culture needs to be taken into consideration. The cell culture materials needed for a plaque assay tend to be more expensive than the cell culture portion of ICC-qPCR. However, the quantitative PCR equipment requires a costly initial investment (Qiagen, 1999). Another expense to the PCR portion of the method is the purchase of the primers and probes. A further consideration is processing time. The initial time to complete the methods is similar for negative samples (EPA, 1992; Qiagen, 1999). However a positive result with the ICC-qPCR is a definitive answer, while positive results from plaque assays require more testing and can add to the testing time (EPA, 1992). This specificity and the sensitivity from the low detection limit are the major advantages of ICC-qPCR.

Seeded experiments alone can not validate methods because samples in nature often do not act similarly to artificially created samples (LeBlanc, 2004). In this study, the environmental samples served to further confirm the usefulness of the ICC-qPCR assay. The initial environmental samples are not displayed in the tables and figures because all of the initial environmental samples were negative using plaque assay and ICC-PCR. Initial negative samples indicated that mammalian orthoreovirus detected later was from replicated virus. The virus replicated from the samples originated from a small amount of undetected virus present in the initial sample. This demonstrates that the ICC-qPCR method is more sensitive than the plaque assay method, but also that the cell culture portion of the method is crucial to its success. The criticality of the cell culture portion of the method is supported by previous studies (Reynolds *et al.*, 1997;

Reynolds *et al.*, 2001).

At least one of the untreated samples from every environmental sampling location had samples that were positive for mammalian orthoreovirus after seven days using the ICC-qPCR method. This uniform distribution through all the untreated sludge agrees with previous research that mammalian orthoreovirus may be common in the environment (AWWA, 1999; Cliver, 1975; Dahling *et al.*, 1989; Fout *et al.*, 2003; Irving and Smith, 1981; Matsuura *et al.*, 1984; Matsuura *et al.*, 1988; Monpoeho *et al.*, 2004; Sattar and Westwood, 1978; Sedmak *et al.*, 2005; Sellwood *et al.*, 1988; Tani *et al.*, 1995).

In addition to untreated sludge, treated sludge was tested. The three locations used in the course of the study were chosen because each of them had different treatment types. The locations that treated the sludge by composting or liming did not yield any positive results from the treatment samples. The location that treated the sludge by aerobic digestion did yield a positive sample. Aerobic digestion has traditionally been considered a less effective treatment method for removing pathogens (Spillman *et al.*, 1987). Detecting mammalian orthoreovirus most often in samples with the historically least effective method further supports the ICC-qPCR method.

Detecting mammalian orthoreovirus in the untreated sludge does not necessarily indicate health risk, as there is further treatment before release to the environment (EPA, 1999). However, the significance of a positive result in treated sludge has to be given careful consideration because it indicates a potential for exposure.

The first consideration in determining the health risk is sample size. Only 100 grams out of several tons of sewage sludge at the treatment plant were tested. It is possible that the only virus present in the entire sludge pile at the treatment plant was present in the portion tested, or that only a small section of the sludge was contaminated. Conversely, it is possible that the virus was distributed more evenly throughout the sludge. Given the diverse nature of sludge piles, it is unlikely that the sludge was evenly mixed (MacGregor, 1981). Therefore a positive result could indicate a small area of contamination, and the actual public health risk could be very small. Consideration needs to be given to the total amount of sludge, the uniformity of that material, the amount tested, and the ultimate fate of the sludge before determining if there is an actual public health risk (Sidhu, 2009).

Further experiments were done on samples that yielded a positive ICC-qPCR result. The positive samples were diluted 10-fold and re-tested using ICC-qPCR. The results indicated that 83% of the samples positive in the undiluted samples were positive at the more dilute concentrations. Positive results in samples that were diluted ten fold indicate at least a ten fold increase in detectable virus during the incubation period. Because of the greater increase in detectable virus in these samples, further experimentation should be done on these samples to pinpoint a minimum incubation time for highly contaminated locations. Reynolds *et al.* (2001) found that detection with ICC-PCR was dependant upon initial virus concentration. It may be possible to shorten the incubation time when a large quantity of viruses are initially present and still obtain a positive result, thus

increasing assay efficiency.

The environmental samples varied in the mammalian orthoreovirus type detected. The type of mammalian orthoreovirus detected in New Hampshire and Pennsylvania was type 1, and in Texas, type 3. This is not meant to be a representative survey of regional variation in viral type, but nonetheless could indicate possible regional differences. Genetic diversity in avian reovirus has been shown to closely correlate with geographic sites (Lui *et al.*, 2003). A study researching the genetic differences between the mammalian orthoreovirus strains found no geographic pattern, but the study focused on phylogenetic relationships rather than type distributions (Leary *et al.*, 2002). The differential detection of the virus types may also be a reflection of different original starting concentrations or differential replication rates among the mammalian orthoreovirus types (Spinner and DiGiovanni, 2001). This result also demonstrates the ability to use the three sets of primers and probes to differentiate between mammalian orthoreovirus types, which could be an advantage in epidemiological research. Leary *et. al* (2002) used a similar set of primers to distinguish between the strains of mammalian orthoreovirus using PCR. This study did not require primers as it was not done using quantitative PCR. Alternatively, one set of primers and probes could be created that encompassed all three types. A suggestion for this primer and probe set would be the RNA dependent RNA polymerase region which has been used successfully to create primers for mammalian orthoreovirus in traditional PCR (Leary *et al.*, 2002).

For the ICC-qPCR method, experiments were completed to correlate Ct with plaque forming units (PFU) as measured by the plaque assay. Figure 3 shows the individual lines that were formed when the results of several trials were graphed on a scatter plot. The average of the individual lines creates the relationship between Ct and PFU shown in Figure 4. The small error bars on this figure indicate a consistent relationship between Ct and PFU. Other researchers have found similar relationships between Ct and PFU for a variety of virus types including respiratory syncytial virus (Falsey *et al.*, 2003), dengue virus (Ito *et al.*, 2004), and West Nile virus (Hunt *et al.,* 2002). In Figure 9, the relationship is extended to accommodate the low concentrations of virus that were detected in the environmental samples. Plotting the environmental samples with their Ct value allows for an estimation of the PFU value, which can be used to calculate an estimated PFU/ml value.

The calculated PFU/ml based on this estimation method for the positive environmental samples is shown in Table 11. The calculated result is the amount of virus present in the sample *after* seven days incubating in cell culture, not the initial amount of virus. However, a relationship exists between the amount of virus in the sample before incubation and the amount of virus in the sample after incubation (Reynolds *et al.*, 1997; Reynolds *et al.*, 2001). The relationship between final concentrations to initial starting concentrations was not determined in this study and would require further exploration. In this study, samples were evaluated on a semi-quantitative basis using a series of dilutions. Ultimately it may be possible to correlate the amount of virus at some incubation

time to an original starting concentration to make ICC-qPCR truly quantitative.

The positive samples may also be averaged by location and treatment level and plotted on the correlation line as shown in Figure 11. This figure reveals that the New Hampshire untreated samples have the highest level of estimated PFU, the untreated Texas samples that were positive have the lowest level, and the Pennsylvania samples are in the middle level. The high estimated level of mammalian orthoreovirus in the raw New Hampshire sample may be due to the very small lag between the sampling at the treatment plant and testing. Haramoto *et. al* (2008) have found that viral recovery decreased dependant upon storage method, temperature, and time. The shorter holding time for the New Hampshire samples may have preserved the virus better than the other treatment plant samples which had a longer holding time.

Figure 10 is a graphical representation of the PFU/ml of the samples in order of concentration. When the estimated concentrations of the samples are compared within treatment type and location, untreated samples from Texas and New Hampshire have a 2 to 3 log difference between the estimation of the highest value and the estimation of the lowest value. This difference could be due to a variety of factors. For example, some samples may have contained viruses with a higher replication rate than in other samples. Samples containing a mixed population of viruses may only support the replication of the fastest growing virus, resulting in lower detection of the slower growing viruses (Spinner and DiGiovanni, 2001). Additionally, the cell culture may have been more susceptible to the some of the viruses. Some viruses are able to form CPE only

after repeated exposure to the same cell line (Fong and Lipp, 2005). The figure provides a broad estimate of the amount of virus present, and emphasizes that mammalian orthoreovirus was present in the ICC-qPCR detection method and not present in the plaque assay method.

The US EPA requires the viral concentration in sludge to be reported as PFU per 4 grams total solids (EPA, 1992). Since the percent total solids of the positive samples is known, this value can be estimated for these samples. The amounts of virus shown in the results (Table 12) are surprisingly high, but the same caveats apply to this estimation that applies to the calculation of pfu/ml.

Reovirus has long been considered a good virus to research in environmental studies because of its prevalence in water and sewage sludge (AWWA, 1999) and its resistance to disinfection compared to other environmental viruses (Wallis *et al.*, 1964). Prior to this study, detection by plaque assay has underestimated the amount of mammalian orthoreovirus present. This study has shown that integrated cell culture with quantitative reverse transcriptase PCR can be used to determine mammalian orthoreovirus presence and estimate its concentration. More infectious mammalian orthoreovirus can be detected and quantified with this new method. The ICC-qPCR method could help public health officials to be more aware of potentially contaminated sludge and prevent viral disease.

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Appendix 1

Minimal Essential Media Recipe:	
Ingredient	Amount
Eagles MEM	4.7 g
L-15	7.4 g
HEPES	4.245 g
L-glutamine	0.292 g
Sodium Bicarbonate	0.75 g
Non-essential Amino Acids	10 ml
Add ingredients to 1 L of water and adjust pH to 7.2-7.4	

Appendix 2

Percent Total Solids:

$$\frac{\textit{Final Weight of Dish} - \textit{Inital Weight of Dish}}{\textit{Weight of Dish and Solids} - \textit{Initial Weight of Dish}}$$

Appendix 3

Plaque Forming Units per ml:

$$\frac{PFU}{ml} = \frac{number\ of\ plaques}{amount\ plated} \times \frac{1}{dilution\ factor}$$

Appendix 4

PFU per 4 Grams Total Solids:

$$PFU/4\ grams\ TS = 0.8 \times \frac{\#\ plaques}{Volume\ of\ inoculum} \times Negative\ reciprocol \times Volume\ of\ remaining\ Portion \times Fraction\ of\ Confirmed\ Plaques$$

Appendix 5

Primer Dilution:

$$80{,}000\,pm\left(\frac{1\,\mu m}{pm}\right) = 0.8\,\mu M$$

$$\frac{0.8\,\mu M}{x} = \frac{50\,\mu M}{1L} = 1.6ml\,H_2 0$$

$$\frac{80{,}000\,pm}{1.6x10^{-3}\,L}\left(\frac{10^{-6}\,\mu M}{pm}\right) = \frac{50\,\mu M}{L}$$

added 1.5 ml molecular grade H_20 in clean room hood

Appendix 6

Probe Dilution:

$$\frac{6000\,pm}{60\,\mu L} = \frac{100\,pm}{\mu L}\left(\frac{10^{-6}\,\mu M}{pm}\right) = \frac{10^{-4}\,\mu M}{\mu L}\left(\frac{10^{6}\,\mu L}{L}\right) = \frac{100\,\mu M}{L}$$

1:100 dilution required: 50 µL probe + 450 µL molecular grade H_20 = 500 µl of 10µM/L

Appendix 7

Master Mix Quantities:			
Component	Initial Concentration	Amount in 1 reaction	Final Concentration
Universal RT Master Mix	2x	12.500 µL	1x
40x Multiscribe and RNAse inhibitor	40x	0.625 µL	1x
Forward Primer	50 µM	0.450 µL	900 nM
Reverse Primer	50 µM	0.450 µL	900 nM
Probe	10 µM	0.625 µL	250 nM
Sterile Molecular Grade Water		5.350 µL	
Sample		5.000 µL	
Total Reaction Volume		25.000 µL	

Appendix 8

To complete the correlation curve, calculations were to standardize to the sample amounts in the plaque assay and in the quantitative PCR assay. The plaque assay has a sample size of 100 µl and the quantitative PCR has a sample size 5 µl. The sample used in the quantitative PCR was previously extracted. The calculation assumes 100% recovery for the extraction method, which conservatively estimates the amount of nucleic acid detected by the quantitative PCR.

PFU from PFU/ml to use in the Calibration Experiment:	
Reason For Manipulation	**Calculation**
Calculate PFU/µl from PFU/ml	Divide by 1000
140 µl of the PFU/µl was added to the spin column	Multiply by 140
Assume 100% recovery from spin column (80µl recovered)	Use same number
5µl of the 80µl recovered is used in the PCR	Divide by 16 (5 into 80)
PFU/ml from PFU to use in the Calibration Experiment:	
Reason For Manipulation	**Calculation**
5µl of the 80µl recovered in the spin column was used in the PCR (assuming 100% recovery)	Multiply by 16
140 µl were originally added to the spin column	Divide by 140
There are 1000 µl in 1 ml	Multiply by 1000
The amount was in the plate was 1000 µl which presumably contained all the virus	Multiply by 100