University of Alberta

Synergistic Effects of UV and Monochloramine Exposure on Enteric Adenovirus Inactivation Kinetics

By

Rachelle Lynn Ormond 

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Abstract

Adenoviruses have been detected in river water, seawater, and raw and treated wastewater. After rotaviruses, adenovirus 40 and 41 are the most common causes of gastroenteritis in children. The research objective was to compare viral assay methods and to evaluate the ultraviolet and monochloramine inactivation kinetics of adenovirus 41 (Ad41). The disinfectants were applied both separately and sequentially in order to evaluate the potential for synergistic effects. Viral titre was assessed using a cytopathic effect (CPE) based assay and a faster, more efficient integrated cell culture reverse transcription polymerase chain reaction (ICC RT-PCR) assay. Models were fitted to the UV, monochloramine, and sequential data. Statistical analyses of the results indicated that a synergistic effect was present when a 42 mJ/cm^2 UV fluence was followed by monochloramine exposure. The presence of a synergistic effect could reduce the UV fluence required thereby reducing water treatment costs.

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Abbreviations

PBS – Phosphate Buffered Saline

UV – Ultraviolet Irradiation

$TCID_{50}$ – fifty percent tissue culture infectious dose

rcf – relative centrifugal force

CPE – cytopathic effect

ICC RT-PCR – integrated cell culture reverse transcriptase polymerase chain reaction

DNA – deoxyribonucleic acid

RNA – ribonucleic acid

DFA – direct immunofluorescing antibody

DBP – disinfection by-products

1.0 Background and Literature review

1.1 Introduction

The purpose of this project was to validate viral assay methods based on real time, quantitative polymerase chain reaction (PCR) and to use these methods to evaluate the ultraviolet and monochloramine inactivation kinetics of the enteric virus Adenovirus 41 (Ad41). Ad41 causes gastroenteritis and has been found in environmental water samples and treated wastewater. The US EPA included adenoviruses in the Contaminant Candidate List 2 published in December 2006 (USEPA, 2006). Ad41 is more resistant to UV irradiation than other viruses. The disinfectants were applied both separately and sequentially in order to evaluate the potential for synergistic effects. The following sections provide background on the present study including the reason for selecting Ad41 as the virus of interest, the existing knowledge gaps in Ad41 inactivation literature, and the need for an improved viral assay method.

1.2 Virus Characteristics

Viruses are simplistic compared to most other microorganisms. They consist of an inner nucleic genome that contains either single or double stranded DNA or RNA and an outer protein layer. DNA and RNA are both comprised of four nucleotides: adenine, guanine, thymine, and cytosine in DNA; and adenine, guanine, uracil, and cytosine in RNA. Viruses require living cells in order to replicate. The virus attaches to a cell and penetrates it. DNA viruses use the viral enzyme reverse transcriptase during replication to produce mRNA, which is then transferred to proteins. The mRNA is then transformed to proteins. After the virus has replicated, it is released from the cell. An infected cell will display cytopathic effects (CPE) particular to the cell line and virus, such as rounding or clustering.

Cell lines originating from humans or animals can be used to support virus growth. A continuous cell line can replicate indefinitely when provided with the appropriate growth medium. Splitting the cells between flasks (passaging) increases the length of time the cells can be cultured. Passaging increases the health of the cells by decreasing

the cell density. Commonly used cell lines for the propagation of enteric viruses include primary liver carcinoma (PLC/PRF/5), colonic carcinoma (CaCo-2), and human embryo kidney (HEK).

1.2.1. Adenovirus Characteristics, Health Effects, and Occurrence

Adenovirus is a 60-90 nm, non-enveloped, icosahedral-shaped, double stranded DNA virus (Evans and Kaslow (1997)). There are 51 human serotypes of adenovirus in six species (Knipe and Howley (2007)). Adenovirus types 40 and 41 belong to species F, and are known to cause gastroenteritis. Symptoms include vomiting, fever, and diarrhea. Children and infants are particularly susceptible to infection. Adenovirus CPE are described as a swelling and rounding of the cells causing grapelike clusters and enlarged nuclei that eventually lead to cell lysis.

After rotaviruses, adenovirus 40 and 41 are the most common causes of gastroenteritis in children (Kidd, Rosenblatt and Besselaar (1986); Uhnoo et al. (1984)). Symptoms associated with gastroenteritis generally include diarrhea, abdominal cramps, and vomiting. Gastroenteritis is typically not a serious illness; however for infants, young children, the elderly, and immune compromised persons it can be serious. Adenovirus is shed in the feces of infected persons and can be transmitted through person to person contact, and contaminated food and water.

Adenovirus has been detected in river water, seawater, and raw and treated wastewater. The presence of adenovirus in the environment is likely due to incomplete removal and/or inactivation during wastewater treatment (Jiang and Chu (2004)). Lee et al. (2004) detected infectious adenovirus and other enteroviruses in samples from four rivers in South Korea. Chapron et al. (2000) collected 29 surface water samples of which eight were positive for infectious astrovirus, six for infectious enterovirus, and 11 for infectious Ad40 and Ad41. Carducci et al. (2006) detected adenovirus in raw and treated wastewater, river water, and seawater. Adenovirus has also been detected in treated drinking water and tap water in South Korea and South Africa (Jiang and Chu (2004)).

1.3 Viral Analysis Methods

There are two main methods of detecting infectious viruses in environmental samples: conventional cell culture and molecular methods. The most common cell

culture method is the endpoint dilution or 50 percent tissue culture infectious dose ($TCID_{50}$) test. In an endpoint dilution test, cells are infected with serial dilutions of the virus solution and observed for evidence of CPE. The viral titre is calculated using statistical methods based on the observed CPE. There are many disadvantages to cell culture methods; they are non-specific, time consuming, and material and labour intensive. CPE may be inconsistent and difficult to identify. The cell monolayer may become unhealthy before CPE is observed if the virus reproduces slowly and the cells may have to be passaged before CPE occurs if the viral titre is low. Cell culture methods are difficult to use for environmental samples because contaminants in the sample may cause cytotoxic changes that can be mistaken for CPE.

Molecular methods such as polymerase chain reaction (PCR) measure virus based on nucleic acid detection. PCR is used to amplify the targeted viral DNA and allows measurement of small quantities of virus. PCR is a relatively new technology that was developed in 1985 (Maier, Pepper and Gerba (2000)). The reverse transcriptase (RT) step is included when the target nucleic acid is RNA or mRNA. The RT enzyme is used to synthesize complementary DNA (cDNA) from the template RNA. The template cDNA is added to a PCR master mix containing forward and reverse primers, a fluorogenic probe (used in real time PCR only), and a DNA polymerase. In the first cycle of PCR, a DNA strand complementary to the cDNA strand is created resulting in a double stranded DNA molecule.

There are three steps to PCR: template denaturation, primer annealing, and primer extension. In step one, the double stranded DNA is denatured into two single strands (ssDNA). In the second step, oligonucleotide primers with a sequence that complements the ssDNA anneal to the ssDNA in the region targeted for amplification. PCR product extension is the final step; A DNA polymerase (such as Taq polymerase) is added to synthesize a complementary strand of ssDNA. At the end of the three steps there are two molecules of double stranded DNA. The DNA is amplified during each PCR cycle according to the initial number of DNA copies.

The DNA amplification includes three phases: exponential, linear, and plateau. The PCR amplification is initially exponential. Amplification slows as PCR reagents are degraded (linear) and finally stops (plateau). The most precise time to record the PCR

reaction is during the exponential phase where the reaction is well defined. In traditional PCR, agarose gel is used to measure the sample at the plateau phase. In real time PCR, the fluorogenic probe binds to the DNA and the increase in fluorescence is measured by the optical PCR system during the exponential phase.

Integrated cell culture PCR (ICC-PCR) combines both molecular and cell culture methods. Samples are inoculated onto cells and incubated for a predetermined time period. Infectious virus is biologically amplified by the cell culture step. After cell culture, the virus is harvested and analyzed by PCR. ICC-PCR provides an efficient, quantitative method for measuring infectious viruses (Reynolds, Gerba and Pepper (1996)). ICC-PCR also reduces time and costs. Ko, Cromeans and Sobsey (2003) developed a method to evaluate infectious Ad 2 and Ad 41 using mRNA ICC RT-PCR. The presence of mRNA indicates viral replication and therefore the presence of infectious virus.

A number of studies have indicated that ICC RT-PCR is superior to both cell culture and RT-PCR for detection of infectious virus in environmental samples. Chapron et al. (2000) used ICC-RT-PCR to measure astrovirus, Ad40, and Ad41 and found the method to be more sensitive than cell culture. The use of CaCo-2 cells to culture astrovirus prior to RT-PCR resulted in a positive detection for 15 samples that had previously been negative with RT-PCR (Mustafa, Palombo and Bishop (1998)).

1.4 Water Treatment Regulations

Population growth is putting pressure on the quality of water supplies. More wastewater is generated, leading to greater amounts of viruses released to the environment. Higher levels of water consumption have led to growing interest in wastewater reuse for indirect potable and non-potable uses, which requires a higher level of disinfection than discharge to receiving waters. Waterborne enteric viruses can cause serious or fatal illnesses. Developments in viral detection methods have enabled the identification of new enteric viruses that can be transmitted through water. Enteric viruses are of particular concern because they have high survival rates in the environment and can be found in wastewater, groundwater, and surface water (Bosch et al. (1997), Pintó et al. (1995)).

The removal and inactivation of enteric viruses during water treatment is regulated in many jurisdictions; however the inactivation kinetics are not well defined. The USEPA Contaminant Candidate List (CCL) identifies unregulated contaminants that may require regulation and prioritizes research efforts. Adenoviruses were included in the USEPA CCL 2 published in December 2006 (USEPA, 2006). Adenovirus treatment and occurrence were identified as information gaps.

The most common method of primary disinfection in North American water treatment plants is chlorination using free chlorine. The use of ultraviolet irradiation is steadily increasing. Chlorination using free chlorine can cause disinfection by-products (DBPs) to form when organic compounds are present in the water and is therefore not favourable for use in some source waters. The DBPs of most concern from a regulatory point of view are trihalomethanes (THMs) and haloacetic acids (HAAs). The USEPA's interim trihalomethanes rule and the Stage 1 and 2 Disinfectants and Disinfection By-Products Rules specify maximum allowable concentrations of residual disinfectants and DBPs in treated drinking water. Low doses of monochloramine or combined chlorine are typically added at the end of the water treatment process to provide a long lasting residual disinfectant in the distribution system. Monochloramine controls microbial contamination of treated water in the distribution system but is not regularly used as a primary disinfectant. Chloramines do not cause THMs or HAAs to form to the same extent as free chlorine.

When designing a water treatment plant, a balance must be achieved between microorganism inactivation goals and DBP formation. Chlorine is a stronger disinfectant than UV for adenovirus inactivation, but the use of UV eliminates DBP formation. UV exposure and monochloramine were selected for study with adenovirus because they are commonly used in drinking water treatment. The additional or synergistic viral inactivation achieved in the presence of chloramines could reduce the required UV fluence and provide sufficient adenovirus inactivation. A combination of UV and monochloramines could achieve disinfection goals while minimizing regulated DBP formation (*e.g.* THMs and HAAs).

1.4.1. United States Environmental Protection Agency (USEPA) Regulations & Guidelines

The USEPA regulations for drinking water treatment are discussed in this section. The principal rules and the amendments are as follows:

- Surface Water Treatment Rule
  - Interim Enhanced Surface Water Treatment Rule
  - Long Term I Enhanced Surface Water Treatment Rule
  - Long Term II Enhanced Surface Water Treatment Rule
- Stage 1 and Stage 2 Disinfectants and Disinfection By-Products Rule
- Groundwater Rule

The Groundwater Rule is a risk-based rule developed to minimize the potential of exposure to waterborne microbial pathogens in groundwater. The rule requires monitoring and protection of source water and 4 log removal of viruses using disinfection. The Surface Water Treatment Rule (SWTR) was introduced in 1989 with the purpose of preventing health effects from exposure to microbial pathogens. The SWTR specified maximum contaminant level goals (MCLGs) for viruses, bacteria and *Giardia lamblia* (a protozoan parasite) for surface water and groundwater under the direct influence of surface water. The SWTR also included specific treatment requirements (*eg.* filter effluent turbidity). The Interim Enhanced SWTR was introduced in 1998 to set further requirements for public water systems serving communities of 10,000 or more people. Modifications to the original SWTR included the addition of *Cryptosporidium parvum* (a protozoan parasite) removal requirements. The Long Term 1 Enhanced SWTR (LT1ESWTR) was introduced in 2002 and applies to public water systems serving communities of less than 10,000 people. The Long Term 2 Enhanced SWTR (LT2ESWTR) was introduced in 2006 and applies to public water systems of all sizes using surface water or groundwater under the direct influence of surface water as a source.

The purpose of the LT2ESWTR is to minimize health effects due to microbial pathogens and to provide guidance on risk tradeoffs between disinfection by-products (DBPs) and illness due to microbial pathogens. Source water is categorized by the amount of *C. parvum* present in the water. *G. lamblia* and virus requirements were grandfathered into the LT2ESWTR from the SWTR. Unfiltered water systems must

achieve removal of *C. parvum* (2 or 3 log), *G. lamblia* (3 log), and viruses (4 log) using at least two disinfectants. Filtered water systems must meet the same *G. lamblia* and virus requirements but may be required to achieve up to 5.5 log removal of *C. parvum*.

The UV fluence required by the LT2ESWTR for log removal credit of microorganisms is contained in Table 1.1. Viruses have varying sensitivity to UV irradiation and are more resistant to UV than bacteria and protozoa. The UV fluence required to achieve four log removal of *C. parvum* and *G. lamblia* is 22 mJ/cm^2 while the specified fluence for viruses is 186 mJ/cm^2. The fluence for virus inactivation was established based on enteric adenovirus. Validation testing of UV reactors is also required as described by the "*Ultraviolet Disinfection Guidance Manual*" (USEPA, 2006). The manual is not a regulation but is intended to provide technical guidance for complying with the LT2SWTR. The Ct values (mg×min/L) for virus inactivation by chloramines at a pH of 6 – 9, presented in Table 1.2, were adapted from hepatitis A data (Sobsey, Fuji and Shields (1988)). A safety factor of 3 was included and the data were adjusted for temperature by doubling the Ct required for the same level of inactivation for every 10 °C drop in temperature.

Table 1.1 Fluence Required for Pathogen Inactivation (mJ/cm^2)

Pathogens	**Log Inactivation**							
	0.5	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	**5**	**4.0**
C. parvum	1.6	2.5	3.9	5.8	8.5	12	5	22
G. lamblia	1.5	2.1	3.0	5.2	7.7	11	5	22
Virus	39	58	79	100	121	143	163	186

Table 1.2 Chloramine CT Required for Viral Inactivation (mg×min/L)

Log Inactivation	**Temperature (°C)**				
	5	**10**	**15**	**20**	**25**
2 log	857	643	428	321	214
3 log	1423	1067	712	534	356
4 log	1988	1491	994	746	497

1.4.2. Canadian Regulations

Alberta Environment (AENV) has adopted the UV fluence and chloramine Ct requirements used by the USEPA for use in provincial drinking water treatment regulations. The "*Guidelines for Canadian Drinking Water Quality*" (GCDWQ) published by Health Canada are not legally binding, but provide a guide for provincial regulatory agencies. Due to the difficulty in detecting many microorganisms, the guidelines recommend the use of the fecal coliform *Escherichia coli* as an indicator of fecal contamination. The GCDWQ recommend that water treatment plants achieve a 3 log inactivation of protozoa and a 4 log inactivation of viruses. *E. coli* is not a good indicator of virus removal because it is much more susceptible to inactivation than viruses.

1.5 UV Inactivation Background

1.5.1. Mechanics of UV Light Inactivation

Light is transmitted in discrete packets of energy called photons, but also behaves as a wave with both a frequency and a wavelength. The Planck Law of Radiation describes the energy carried by light (Equation 1.1).

$$u = \frac{hc}{\lambda} \qquad \text{Eqn. 1.1}$$

where u is the energy (J) of one photon, h is the Planck constant (6.6261×10^{-34} J/s), c is the speed of light (2.9979×10^{8} m/s) in a vacuum, and λ is the wavelength (m).

The light emitted by UV lamps is incoherent, meaning that the emitted photons are not in phase with each other. The UV range of light can be divided into the following four groups according to wavelength: UVA (315 – 400 nm), UVB (280 – 315 nm), UVC (200 – 280 nm), and Vacuum Ultraviolet (100 – 200 nm). The UVC range is often referred to as the germicidal range because it is the most effective at inactivating microorganisms. The effective germicidal wavelength is 220 – 300 nm, with a peak at

approximately 260 nm. UVC light is absorbed by both proteins and nucleic acid. Microorganism inactivation and nucleotide absorbance peak at the same wavelengths indicating that nucleotide absorbance is the main mechanism of inactivation.

Adsorption of UVC light by nucleic acids causes a photochemical dimerization of the pyrimidine bases thymine and cytosine in DNA and uracil and cytosine in RNA (Bolton and Linden (2003)). Adjacent pyrimidine bases will bond chemically to form a pyrimidine dimer. The dimers disrupt the DNA or RNA structure so that the nucleic acid can not be replicated during cell mitosis. A virus that can not replicate is considered non-infectious, and therefore inactivated for the purposes of water treatment.

The efficiency of microorganism inactivation by UV irradiation is not affected by pH and temperature conditions. Turbidity and colour reduce the efficiency of UV irradiation by absorbing some of the UV photons. Some microorganisms have repair mechanisms and dark reactivation or photoreactivation can occur. Dark reactivation occurs when undamaged regions of double stranded DNA are replicated using information contained in an undamaged strand, or when the nucleotide containing the dimer is resynthesized. Inactivation by UV is irreversible in viruses with single stranded nucleic acids (Maier, Pepper and Gerba (2000)). Photoreactivation is facilitated by the photolase enzyme. The enzyme is activated by light in the 350 – 450 nm region and causes dimers to dissociate.

1.5.2. Application of UV Light

A collimated beam apparatus delivers a parallel beam of UV light to a target, such as a microorganism suspension in a Petri dish. A collimated beam apparatus consists of a housing containing a UV lamp, a pneumatic shutter, and a collimator. The inside of the collimator is black and absorbs any UV light that contacts it. A collimated beam of UV light travels down the collimator to the target when the shutter is opened. The applied UV light is generally referred to as the UV fluence.

The radiant energy and radiant power are used to calculate the fluence. Radiant energy is the total energy measured in Joules (J) that a source emits over time and radiant power is the rate at which radiant energy is emitted over time in all directions. The fluence rate (W/m^2) is the total radiant power incident from all directions onto the cross-

sectional area of a sphere. The fluence rate multiplied by the exposure time yields the fluence (J/m^2), often referred to as the UV dose, if the fluence rate remains constant. UV irradiance (W/m^2) is the total radiant power incident from one direction on an area's surface. In a collimated beam apparatus, the irradiance and the fluence rate are approximately the same. A calibrated radiometer can be used to measure the fluence rate of a collimated beam apparatus. The radiometer is placed such that the top of the radiometer is at the same level as the surface of the solution in the Petri dish. The reflection, Petri, water, and divergence factors are required to determine the average fluence rate delivered to the solution. The methods used to calculate the factors are described in detail by Bolton and Linden (2003) and are summarized in the following paragraph.

When light passes between two mediums, a portion of the beam is reflected. The fraction reflected (R) is 0.025 for air and water according to the Fresnel Law. The reflection factor corrects for the fraction of light entering the water and is calculated as (1-R) = 0.975. The Petri factor corrects for the variance in UV fluence rate delivered across the surface area of the water in the Petri dish. The Petri factor is the ratio of the average irradiance over the area of the Petri dish to the irradiance at the centre of the dish. An efficient collimated beam apparatus delivers a Petri factor of at least 0.9. The water factor (*WF*) corrects for the amount of UV absorbed by the medium at the wavelength of interest (Equation 1.2).

$$WF = \frac{\left(1-10^{-a\ell}\right)}{\left(a\ell \ln(10)\right)} \qquad \text{Eqn. 1.2}$$

where a = absorbance for a one cm path length (cm^{-1}), and l = vertical path length (cm) of the water in the Petri dish. The divergence factor (*DF*) corrects for the divergence of the UV beam as it travels through the solution in the Petri dish (Equation 1.3).

$$DF = \frac{L}{\left(L+1\right)} \qquad \text{Eqn. 1.3}$$

where L = the distance from the UV lamp to the surface of the suspension. The average fluence rate (E_{avg}) delivered by the collimated beam apparatus using a low pressure lamp is the product of the fluence rate at the centre of the Petri dish and each of the four factors (Equation 1.4).

$$E_{avg} = E_o \text{ x (Petri factor) x (1-R) x } (WF) \text{ x } (DF) \qquad \text{Eqn. 1.4}$$

where E_o is the radiometer reading at the centre of the Petri dish.

1.6 Monochloramine Inactivation Background

Chlorination and chloramination of drinking water were first used in 1908 and 1917, respectively (Letterman (1999)). Chlorine is an efficient disinfectant for use with bacteria and viruses, but protozoa are more resistant to chlorine inactivation. Chlorine is typically added to water as chlorine gas, solid calcium hypochlorite, or a sodium hypochlorite solution. If the amount of ammonia present in the water exceeds the chlorine added, chloramines compounds will be formed.

Chlorine present in solution without ammonia is referred to as free chlorine, and chlorine-ammonia compounds are referred to as combined chlorine. The total chlorine in solution is the sum of the free and combined chlorine. When an excess of ammonia is present, free chlorine will be less than one percent of the total chlorine. Monochloramine (NH_2Cl), dichloramine ($NHCl_2$), and trichloramine (NCl_3) are formed by a reaction between chlorine and ammonia, as demonstrated by Equations 1.5 – 1.7.

$$NH_3 + HOCl = NH_2Cl + H_2O + H^+ \qquad \text{Eqn. 1.5}$$

$$NH_2Cl + HOCl = NHCl_2 + H_2O \qquad \text{Eqn. 1.6}$$

$$NHCl_2 + HOCl = NCl_3 + H_2O \qquad \text{Eqn 1.7}$$

The dominant combined chlorine species depends on the pH and the chlorine to ammonia ratio. At a pH greater than 5.5, monochloramine will be the dominant species.

The applied monochloramine dose is measured as the concentration of disinfectant multiplied by the duration of exposure, and is referred to as the Ct product

with units of mass-time per volume (*i.e.* mg×min/L). The disinfectant concentration usually decays exponentially with time. The Ct is determined by calculating the area under the plot of concentration versus time for each measurement. The cumulative Ct ($C_{avg}t$) is determined by summing the Ct product previously measured in each time step. The shaded area of Figure 1.1 represents the $C_{avg}t$ after an exposure time of 100 minutes.

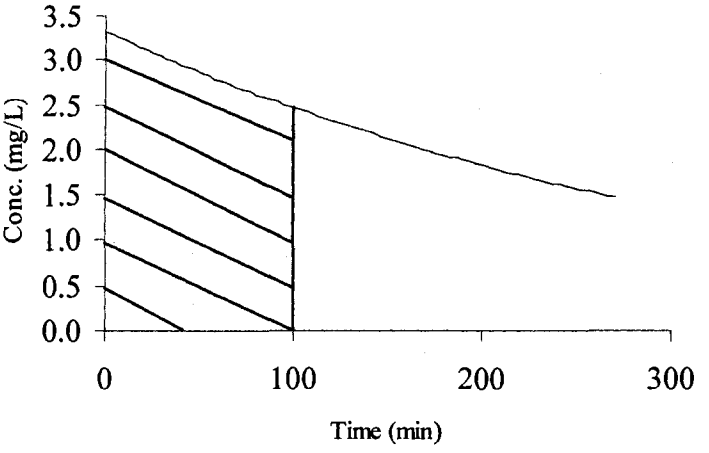


Figure 1.1 Typical Monochloramine Decay Curve

Two methods of chlorine inactivation of virus have been observed; Damage to the outer protein coat of poliovirus (Tenno *et al.* (1980)) and damage to the RNA of f2 bacteriophage (Olivieri *et al.* (1980)) and poliovirus (O'Brien and Newman (1979)). Chloramines initially attack the outer capsid of a virus before they can attack the nucleic acid. Polioviruses are inactivated by denaturation of the capsid protein, which prevents the virus from infecting cells (Fujioka (1983).

1.7 Modeling Disinfection Kinetics

In order to predict the dose required for inactivation of a microorganism, it is necessary to evaluate the disinfection kinetics. Disinfection kinetics relate the rate (dN/dt) at which a microorganism is inactivated to the disinfectant dose. The rate constant (k_r) can be defined in terms of disinfectant concentration and substituted into Equation 1.7.

$$\frac{dN}{dt} = -k_r N \qquad \text{Eqn. 1.7}$$

where N = the number of microorganisms. The integrated form of Equation 1.7 can then be used to describe the inactivation.

A number of assumptions are made in most kinetic models as follows: (a) no back mixing; (b) uniform mixing of organisms and disinfectant; (c) temperature and pH are fixed; (d) constant disinfectant concentration; and (e) adequate mixing such that liquid diffusion is not rate limiting. To completely evaluate the kinetics of some disinfectants, such as free chlorine, observations must be made at various temperature and pH conditions. The effects of decay must also be considered in some cases.

The relationship between log inactivation of microorganisms and disinfectant dose is often linear (Figure 1.2). In some cases, a lag time called shouldering may occur at the start of inactivation. Shouldering can be caused by clustering of microorganisms that initially slows the inactivation rate. A reduced inactivation rate, or tailing, may also occur at the end of the curve due to disinfectant resistance. It is possible for both shouldering and tailing to be observed on the same inactivation curve.

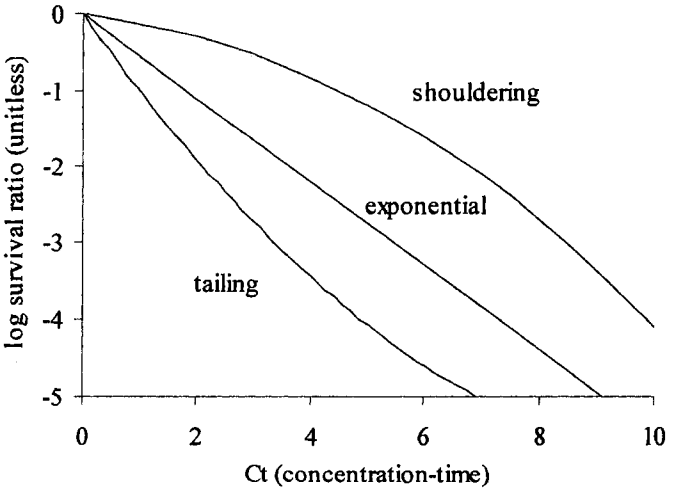


Figure 1.2 Microorganism Survival versus Disinfectant Ct Product

1.8 Previous Disinfection Studies

The typical UV fluence applied in water treatment plants is 40 mJ/cm^2 (Baxter (2007); this fluence is sufficient for most viruses but is too low to achieve four log inactivation of adenoviruses. The reason for the increased resistance to UV irradiation in adenoviruses compared to other viruses is unknown. Shin, Linden and Sobsey (2005) found that the size and genetic composition of enteric viruses and bacteriophages could not be used to predict sensitivity to UV irradiation. A number of studies have been completed to evaluate the effect of UV on various adenovirus serotypes. Studies on the UV fluence required to inactivate adenoviruses have produced inconsistent results.

The results of published studies for four log inactivation of adenovirus types 40, 41, 2, and 5 are summarized in Table 1.3. A comparison of the completed studies has indicated a difference between the UV fluence required to inactivate different serotypes, particularly between enteric adenoviruses (Ad40 and Ad41) and respiratory adenoviruses (Ad2 and Ad5). Baxter (2007) reported that Ad41 was more resistant than Ad2, and Gerba, Gramos and Nwachuku (2002) reported the opposite. Adenovirus has been consistently shown to require higher levels of UV to achieve the same inactivation as other viruses, including poliovirus and feline calcivirus (a norovirus surrogate) ((Meng and Gerba (1996); Thurston-Enriquez et al. (2003)).

The lack of standard adenovirus propagation and assay methods may explain the conflicting results in the literature. The harvesting, infection, purification, and extraction methods varied in each study. Ad41 inactivation by UV was lower using ICC RT-PCR (Ko, Cromeans and Sobsey (2005)) than $TCID_{50}$ (Meng and Gerba (1996)). Baxter *et al.* (2007), Thurston-Enriqez *et al.* (2003), and Meng and Gerba (1996) used PLC/PRF/5 cells and a $TCID_{50}$ assay. Baxter *et al.* (2007) also used Hek 293 cells, and confirmed the CPE observations with a fluorescent antibody assay. Ko, Cromeans and Sobsey (2005) (2005) used Hek 293 cells and ICC RT-PCR with 5 to 7 days of incubation prior to RT-PCR. Nwachuku *et al.* 2005 found that the sensitivity of various non-enteric adenoviruses to UV irradiation was not affected by the cell line, but could be affected by the number of freeze-thaw cycles. $TCID_{50}$ results are influenced by the number of replicates used per dilution, with the minimum being four and a maximum of ten.

Table 1.3 Summarized Results of UV Inactivation of Enteric Adenovirus from Previous Studies

Reference	Virus (strain, source)	Host Cells (source)	Purification Method	Viral Assay	UV Fluence (mJ/cm^2)	
					4 log inactivation	1 log inactivation
Baxter *et al.* 2007	Ad41 (prototype strain TAK)	PLC/PRF/5 and HEK 293 (ATCC)	five cycles freeze-thaw, clarified by centrifugation, cell pellet resuspended and treated with Vertrel XF followed by two cycles of cesium chloride	$TCID_{50}$ followed by fluorescent antibody detection	>120	45
Ko *et al.* 2005	Ad41 (VR-930, ATCC)	HEK 293 (ATCC)	chloroform extraction	cell culture mRNA RT-PCR	222	56
Jacangelo *et al.* 2002	Ad40 (Strain Dugan, ATCC)	PLC/PRF/5 (ATCC)	two cycles polyethylene glycol precipitation followed by chloroform extraction	CPE observation (24 days)	221	55
Thurston-Enriquez *et al.* 2003	Ad40* (Strain Dugan, ATCC)	PLC/PRF/5 (ATCC)	two cycles polyethylene glycol precipitation followed by chloroform extraction	CPE observation (24 days)	226	50
Meng and Gerba 1996	Ad41 (TAK, ATCC)	PLC/PRF/5 (ATCC)	five cycles freeze-thaw, Freon treated and centrifugation	$TCID_{50}$	112	24
	Ad40* (Dugan, ATCC)				124	30

Notes: - * Ad40 results are not directly comparable to Ad41 results, ATCC – American Type Culture Collection

Relatively few studies have been performed to evaluate monochloramine inactivation of adenovirus, partially because monochloramine is a weaker disinfectant than chlorine. Shin and Sobsey (1998) exposed poliovirus 1, MS-2 coliphage, and norovirus to monochloramine. A Ct value of 567 mg×min/L and 1,167 mg×min/L achieved 2 log inactivation of poliovirus 1 and MS-2 coliphage, respectively at pH 8 and 5 °C. In contrast, a chlorine Ct product of only 6.36 mg×min/L caused a 4 log inactivation of poliovirus 1 at pH 6 and 5 °C (Thurston-Enriquez *et al.* (2003)). Baxter *et al.* 2007 reported that a 4 log inactivation of Ad 5 and Ad 41 was achieved with a free chlorine Ct of 0.22 mg×min/L, while a monochloramine Ct of 350 mg×min/L was required to achieve a 2.5 log inactivation of Ad5 and Ad41 under the same conditions. Sirikanchana, Shisler and Mariñas (2008) found a Ct of approximately 1000 mg×min/L was required for 2 log inactivation of Ad2 by monochloramine.

Ballester and Malley (2004) found 264.5 mg×min/L was required to achieve a 1.2 log inactivation of Ad2 with preformed chloramines. Water that was seeded with adenovirus and NH_3 before addition of Cl_2 (sequential chloramines) provided an increased inactivation of 1 log inactivation with 40.5 mg×min/L, likely due to the initial existing free chlorine. When a 40 mJ/cm^2 UV fluence was followed by sequential (not preformed) chloramines exposure (27.2 mg×min/L), four log inactivation was achieved, rather than the additive 2 log inactivation that was expected. The use of sequential monochloramine followed by UV caused a smaller synergistic log inactivation of Ad2. A synergistic effect has been found on the inactivation of MS2 coliphage when low pressure UV was applied before monochloramine exposure (Shang, Cheung and Liu (2007)). The increase in log inactivation was higher at high UV fluences.

In contrast, other studies have not found a synergistic effect on Ad2 inactivation when UV exposure was followed by monochloramines (Baxter *et al.* (2007)). The typical UV fluence applied in a water treatment plant followed by monochloramines was found to be insufficient to achieve a four log inactivation by Baxter *et al.* (2007), but was recommended by Ballester and Malley (2004). Studies that evaluated sequential UV and free chlorine inactivation of MS2 coliphage (a common virus surrogate) and Ad5 found no synergistic effects (Coronell, Page and Marinas (2003); Shin et al. (2002)).

The low inactivation achieved by monochloramine makes it infeasible for use as a primary disinfectant because of the long contact times that would be required. Monochloramine is frequently used in water treatment to provide a residual in the distribution system. A knowledge gap in the existing adenovirus literature is the degree of additional inactivation monochloramine provides and the possibility of synergistic effects between UV and monochloramines on enteric adenovirus inactivation.

1.9 Problem Statement and Hypothesis

Two components will be examined in the inactivation of Ad41: the selected disinfectant and the viral assay. The use of chlorine as a primary disinfectant can cause the formation of DBPs in exceedance of acceptable limits. UV is a common alternative disinfectant, but it provides no residual and Ad41 is relatively resistant to UV. Traditional methods of measuring Ad41 in water are time consuming and expensive. An efficient method of Ad41 inactivation is required for safe drinking water. A reliable viral assay is necessary to evaluate the Ad41 removal.

It is hypothesized that application of UV followed by monochloramines will produce a synergistic effect and that the cumulative Ad41 inactivation will meet the regulated four log inactivation. The use of an ICC RT-PCR viral assay will reduce the time and costs associated with Ad41 measurement.

1.10 Thesis Objectives

The following objectives will be met:

1. Validate the use of the ICC RT-PCR method with real time, quantitative PCR for measuring virus inactivation in disinfection studies by comparing to the $TCID_{50}$ method.
2. Determine the inactivation kinetics of Ad41 using UV and monochloramine.
3. Evaluate the presence of synergy when UV and monochloramine are applied sequentially.

2.0 Experimental Methods and Materials

The following sections describe the methods and materials used. Sterilized materials were used whenever possible to avoid contamination. Materials were sterilized with an autoclave or disposable, pre-sterilized materials were used. Basic cell culture techniques, such as working in a biosafety cabinet were employed to prevent contamination.

2.1 Viral and Cell Culture Methods

2.1.1. Cell Culture

The cell line HEp-2 (ATCC CCL-23) was propagated as a host for a laboratory strain of Ad41 (ATCC VR930 Tak Strain). Stock HEp-2 cells were grown on 75 cm^2 BD Falcon tissue culture flasks containing a growth media comprised of minimal essential medium (MEM) with 10% fetal bovine serum (FBS). When cells reached approximately 80% confluence, growth media was removed and the monolayer was washed with phosphate buffered saline (PBS). Media comprised of MEM containing 2% FBS was used to maintain the cells. Table 2.1 specifies the other reagents added to the MEM. The media in the stock cells was exchanged after approximately five days and cells were passaged approximately every three weeks. Cells were incubated at 37 °C with 4 % CO_2.

Table 2.1 Cell Media Composition

Reagent	**Volume per 500 mL MEM**	**Purpose**
$NaHCO_3$	13.5	a buffering agent
HEPES	10	a buffering agent
Gentamicin	2	antibiotic to prevent the growth of gram negative bacteria
L-Glutamine	5	essential amino acid to provide cell energy

HEp-2 cells were propagated as needed. The media was removed from the flask and the cells were washed with 10 mL of PBS. The PBS was removed and 5 mL of 0.25% trypsin was added. The flask was rocked gently for one minute, followed by removal of 4 mL of trypsin and incubation at 37 °C. After ten minutes, 9 mL of growth media was added to the flask and the suspension was agitated with a pipette approximately 50 times to disperse the cells. Cells were split to new flasks at a ratio of one to ten, and growth media was added.

2.1.2. Adenovirus 41 Propagation

Ad41 stock was prepared by passaging the virus numerous times on HEp-2 cells. Cell monolayers were infected when approximately 80% confluence had been reached. The growth media was removed and the monolayer was washed with PBS. Ad41 stock was added to the cell monolayer and the flask was rocked to cover the monolayer with inoculum. The flask was incubated at 37 °C for 60 to 90 minutes and then maintenance media was added to the flask. Ad41 stock was produced in 75 cm^2 cell culture flasks. A 1 mL aliquot of Ad41 stock was added to the monolayer, followed by a ten day incubation. CPE was used to identify successful culture and propagation of Ad41. Viral particles were harvested by three cycles of freezing (-70 °C) and thawing (37 °C). Cells expansion and contraction during the freeze-thaw process allowed cell lysis and release of viruses into the media. The media and cells were aliquoted to conical tubes and centrifuged at 0.2 rcf for seven minutes. The supernatant was removed for further analysis and the cell pellet was discarded.

2.1.3. Virus Titration Using Fifty Percent Tissue Culture Infectious Dose (TCID50) with Direct Immunofluorescing Antibody Test (DFA)

The method described by Reed and Muench (1938) was used to calculate the $TCID_{50}$ with units of infectious units per milliliter (IU/mL). Sample calculations are included in Appendix A. $TCID_{50}$ analyses were performed using Becton Dickinson Labware Biocoat^R 24-well plates or NUNC Nunclon Surface 24-well plates. Tenfold serial dilutions of experimental samples or Ad41 stock were prepared and HEp-2 cells were infected with a 250 µL aliquot in replicates of four. The plates were examined daily

for the presence of CPE. After ten days, a direct immunofluorescing antibody (DFA) test was performed to confirm the presence or absence of Ad41 infected cells.

The IMAGEN™ Adenovirus K6100 procedure was followed for DFA testing. Cells were scraped from each well and pipetted with maintenance media into an eppendorf tube. The tube was centrifuged at 2.5 rpm for one minute and the majority of the supernatant was removed. The cell pellet was resuspended in the remaining one to two drops of supernatant, and pipetted onto a microscope slide. After the cell suspension had dried, the slides were fixed by soaking for 10 minutes in an acetone bath. The slides were air dried and 10 μL of IMAGEN™ Adenovirus reagent was pipetted onto the cells. The slides were incubated for 15 minutes at 37 °C in a moist chamber. After incubation, the slides were rinsed with PBS followed by a 5 minute PBS bath. The slides were then air dried and mounted. The slides were examined using an Olympus BX51 microscope with a fluorescence attachment. Ad41 infected cells appeared bright green and uninfected cells appeared red.

2.1.4. Nucleic Acid Extraction

Nucleic acid was extracted using a Cortex Biochem MagaZorb™ RNA Mini-Prep Kit. A 200 μL aliquot of harvested virus was transferred to a 2 mL, RNase-DNase free centrifuge tube. Proteinase K solution (20 μL) and lysis buffer (200 μL) were added to the sample and then the tube was incubated at 56 °C for 15 minutes to allow complete cell lysis and release of nucleic acid from the cells. After incubation, 500 μL of binding buffer and MagaZorb™ reagent (20 μL) were added to the solution to bind the lysed nucleic acid. The tube was incubated at room temperature for ten minutes with occasional end over end mixing. A magnetic rack was used to sediment the MagaZorb™ particles with the attached nucleic acid. The liquid was aspirated, and the MagaZorb™ particles were washed twice with 1 mL of wash buffer. The wash buffer was removed and RNase free water (100 μL) was added to the tube, which was mixed end over end for ten minutes at room temperature. The magnetic particles were then sedimented and the supernatant was transferred to RNase-DNase free tubes. Half of the extracted nucleic acid was stored at -70 °C and the other 50 μL was immediately treated with DNase to remove DNA.

2.1.5. Removing DNA from Total Nucleic Acid Extract

A DNase reaction was performed to remove any DNA extracted by MagaZorb™. DNA is not amplified by the RT-PCR step, but would be detected by the PCR step after; therefore a DNase reaction is necessary. The digestion was performed by incubating 50 µL of extracted RNA with 450 µL dH20, 50 µL dNase buffer, and 5 µL dNase for one hour at 37 °C.

2.1.6. Two Step Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

The reverse transcriptase (RT) step was performed using a Geneamp PCR System 2700. An RT master mix was made as described in Table 2.2. A 15 µL volume of the master mix and 5 µL of isolated Ad41 RNA were transferred to a 200 µL Eppendorf tube. The tube was placed into the PCR machine once the heating plate had reached 103 °C, and was incubated for 60 minutes at 42 °C followed by 15 minutes at 70 °C. The resulting cDNA was stored at -70 °C.

Table 2.2 Reaction Components for Reverse Transcription Master Mix

Component	**Volume per Reaction**	**Final Concentration**
5x first-strand buffer	4.0 µL	1X
DTT	1 µL	5 µM
dNTP	3 µL	200 nM
Random Primer (300 ng/µL)	2 µL	600 nM
RnaseOut	0.5 µL	20 units
SuperScript II	0.5 µL	100 units
dH_2O	4.0 µL	-
Total Volume	20 µL	-

The polymerase chain reaction (PCR) step was carried out using an ABI Prism 7500 with a real time Taqman probe. A PCR master mix was made by combining 12.5 µL of Taqman Universal PCR Master Mix per reaction with the appropriate primers and

probes and sufficient dH_2O to achieve a master mix volume of 20 µL per reaction. The primers (10 µM) and probe (5 µM) (1 µL each per reaction) used for detection of Ad41 were designed by Dr. Lilly Pang (unpublished) as follows: Adenovirus 40/41 Forward Primer (CCGACCCACGATGTAACCA), Adenovirus 40/41 Reverse Primer (CGGTCGACTGGCACGAAT), and Adenovirus 40/41 Probe (FAM-ACAGGTCACAGCGACTGACGCTGC-TAMRA). A passive dye, ROX, was included in the primer/probe as a control. A 5 µL volume of cDNA and a 20 µL volume of master mix were aliquoted to PCR optical tubes. The tubes were placed in an ABI Prism 7500 for the following thermal cycles: two minutes at 50 °C; ten minutes at 95 °C; and 45 cycles of 15 seconds at 95 °C followed by one minute at 60 °C.

The PCR results are expressed as a plot of change in fluorescence (Delta Rn) versus cycle number. The ABI Prism 7500 software was used to draw a line at 0.2 Delta Rn, known as the threshold. The cycle number at which the amplification line crossed the threshold (crossing point, CP) was recorded and quantified in units of RNA copies per PCR reaction using the standard curve. Increasing CP values indicate a decreasing amount of RNA. The RNA copies per PCR reaction can be converted to RNA copies per milliliter by multiplying by a factor of 4,440. A detailed calculation of the conversion factor is located in Appendix A.

2.1.7. Standard Curve for Quantification of Virus

An Ad2 standard with a viral titre of 10^7 DNA copies per 5 µL was serially diluted by a factor of ten in dH_2O. Duplicates of each dilution, (10^6 to 10^1 DNA copies per 5 µL) were analyzed with real-time PCR. Ad41 stock (passage 2) harvested from HEp-2 cells was also serially diluted by a factor of ten in dH_2O. Duplicates of each Ad41 dilution (10^{-6} to 10^{-1}) were analyzed by real time-RT-PCR. The Ad41 and Ad2 standard curves had similar slopes indicating that the PCR efficiency for both viruses was the same; therefore Ad2 can be used as a standard for absolute quantification of Ad41. Four Ad2 dilutions (10^6, 10^3, 10^4, and 10^1 DNA copies per 5 µL) were analyzed with every PCR run to quantify the Ad41 results.

The primers (20 µM) and probe (10 µM) (0.5 µL per reaction) used for detection of Ad2 were created by Lee *et al.* (May 2006) as follows: Adenovirus 2 Forward Primer

(CCAGGACGCCTCGGAGTA), Adenovirus 2 Reverse Primer (AAACTTGTTATTCAGGCTGAAGTACGT), and Adenovirus 2 Probe (FAM-AGTTTGCCCGCGCCACCG-TAMRA).

2.1.8. Integrated Cell Culture (ICC)-RT-PCR

The ICC RT-PCR assay combined cell culture and molecular methods. The method used was similar to Ko, Cromeans and Sobsey (2005) except that a real time quantitative PCR assay was used to measure infections on the cell cultures rather than an agarose gel method. A five day incubation was used to biologically amplify Ad41 present in disinfection samples. HEp-2 cells grown on 25 cm^2 flasks were infected with 1 mL aliquots of sample using the previously described methods. The infection process was modified for the monochloramine and sequential disinfection samples. The virus inoculum was removed and the cell monolayer was washed prior to addition of maintenance media. After five days the virus was harvested, and RNA extraction, digestion, and RT-PCR were performed as described above.

2.1.9. Transport and Storage

Virus stock was stored in a -70 °C freezer. Viruses were transported between the Provincial Health Laboratory and the University of Alberta campus in an insulated box. Transportation took approximately 20 minutes. Samples and stock were stored at -20 °C at the University of Alberta for up to 48 hours.

2.2 Disinfection Methods

2.2.1. UV Experimental Methods

Virus suspensions consisting of 1 mL Ad41 stock in 14 mL of PBS were exposed to approximately 25, 50, 75 or 100 mJ/cm^2 in triplicate. The suspension was placed in a quartz covered, 4.7 cm inner diameter, glass Petri dish containing a micro stir bar. Slow stirring was maintained throughout the duration of UV exposure. The exposures were conducted using a Calgon Carbon Corporation collimated beam apparatus containing a low pressure mercury lamp. A Gigahertz Optik Optometer P9710 radiometer calibrated to 254 nm, and covered by a quartz cover was used to measure the UV irradiance at the

centre of the Petri dish and at 0.5 cm intervals up to 0.5 cm beyond the radius of the Petri dish. The absorbance of the suspension was measured using a Shimadzu UV-2401 PC UV-VIS recording spectrophotometer. The exposure duration required to achieve the desired UV fluence was calculated using the Petri factor, water factor, divergence factor, reflection factor, absorbance, and the distance from the lamp to the surface of the suspension as described by Bolton and Linden (2003). The samples were collected in sterile culture flasks and frozen at -70 °C for analysis.

2.2.2. Monochloramine Experimental Methods

Oxidant demand free (ODF) water was prepared by ozonating Milli-Q water in 4-litre, glass jars. The jars were partially covered with aluminum foil and kept at room temperature for a minimum of 5 days before use. ODF glassware was prepared in a large plastic tub filled with distilled water. The water was ozonated and the glassware was allowed to soak overnight at room temperature. The glassware was then emptied of the ozonated water and place in a warm autoclave to dry. Glassware was covered with aluminum foil or ODF lids if not immediately used.

Chlorine residual was measured using a modified version of the DPD Colorimetric Method (4500-Cl G) described in "*Standard Methods for the Examination of Water and Wastewater*" (American Public Health Association, American Water Works Assocation, Water Environment Federation (1998). An Ultrospec 2000 UV spectrophotometer at 515 nm and 10 mm quartz cuvettes were used to measure the absorbance of samples. The absorbance of an 8.91 mg/L solution of potassium permanganate ($KMnO_4$) is equivalent to that of a 10 mg/L Cl_2 stock solution. Stock $KMnO_4$ solution was diluted to equivalent standards of 1, 2, 3 and 4 mg/L Cl_2 and a standard curve was prepared. A HACH Co. (Loveland, Co) Permachem Reagents DPD Total Chlorine Reagent pouch or Free Chlorine Reagent pouch was added to 10 mL of sample in a glass vial. The difference between the free and total chlorine measurement indicated the monochloramine residual.

A Cl_2 stock was prepared by diluting a 1 mL aliquot of 5% sodium hypochlorite ($NaOCl^-$) solution to 100 mL with ODF water. A 0.5 mL volume of Cl_2 stock was diluted to 100 mL with ODF water and the Cl_2 concentration was measured. A 1.67

mg/L as N stock solution of ammonium chloride (NH_4Cl) was prepared. The ammonium chloride was added to the reactor flask first, and Cl_2 stock was added at a ratio of 3 Cl_2:1 N. A 230 mL volume of ODF water and 20 mL of phosphate buffer was added to a glass flask to achieve a pH of 7. The monochloramine was allowed to form for 30 minutes.

Ad41 stock (25 mL) was added to the reactor flask after monochloramine formation. The flask was kept in a water bath for the duration of the experiment to maintain the temperature at 11 °C. Samples were collected in sterile plastic tubes containing a crystal of sodium thiosulphate. The samples were agitated to dissolve the sodium thiosulphate, and immediately frozen at -20 °C. The samples were transferred to a -70 °C freezer within 24 hours.

2.2.3. Sequential Experimental Methods

In the sequential experiments, Ad41 stock was exposed to a UV fluence of 42 mJ/cm^2 followed by monochloramine exposure. The methods described in sections 2.2.1 and 2.2.2 were used with some minor variations. The virus stock was not diluted in PBS during the UV exposures and 10, 15, or 20 mL of stock was exposed in a Petri dish. The UV exposed stock was pooled and stored at 4 °C overnight. The monochloramine exposure was conducted the next day, with 24 mL of virus stock in ODF water.

2.3 Quality Assurance/Quality Control

Control samples were included in order to evaluate variation. The controls that were included in the inactivation experiments and viral analyses are described in the following sections.

2.3.1. Inactivation Experiment Controls

Positive and negative controls were included in the inactivation experiments. Controls were treated in the same manner as samples. Positive controls consisted of virus stock that had been inactivated using an autoclave and negative controls consisted of viable virus stock. The controls were subjected to the same disinfection experimental procedures as the samples, except that the negative controls were not exposed to disinfectant. Controls were analyzed using ICC RT-PCR and $TCID_{50}$ methods. Method blanks consisting of ODF water in a sample collection tube containing sodium

thiosulphate were included in the monochloramine and sequential experiments to evaluate viral contamination.

2.3.2. Viral Analysis Controls

Controls were included during Ad41 analysis in order to evaluate contamination and equipment efficiency. Negative controls (sterilized dH2O) were included in viral extraction and digestion. Positive and negative controls were included in RT and PCR. The positive controls consisted of virus samples that had previously been confirmed as positive or were known to be positive (*e.g.* stock virus). Digested samples were analyzed by PCR without the RT step to confirm that all DNA was destroyed by digestion. A PCR standard curve was generated once during the UV experiments and for every PCR run thereafter.

3.0 Inactivation of Adenovirus 41 by UV Irradiation

3.1 UV Exposure Results and Discussion

The following sections discuss the log inactivation of Ad41 by UV irradiation. The inactivation results are presented and analyzed. The UV exposures were performed on November 21, December 5, and December 13, 2007 using Ad41 pooled stock (passages 3 and 4). The viral titre of the Ad41 stock before transport to the disinfection lab was determined to be 4.31 x 10^3 IU/mL using the $TCID_{50}$ combined with DFA method as previously described. The raw data for the disinfection experiments can be found in Appendix A, Table A.1.

All samples were analyzed using real time, quantitative ICC RT-PCR and select samples were analyzed with direct RT-PCR and $TCID_{50}$. The ICC RT-PCR and $TCID_{50}$ results can not be compared directly due to the different units (copies per PCR reaction vs. IU/mL); however they can be compared when normalized as log inactivation. A PCR standard curve was created on November 14, 2007. An example of a PCR standard curve is contained in Appendix A (Figure A.3). The equation of the line fitted to the PCR standard curve (*CP* versus log *N*) was used to quantify the PCR *CP* results of the UV samples (Equation 3.1).

$$CP = -3.14Log(N) + 42.032 \qquad \text{Eqn. 3.1}$$

where *CP* = crossing point, and *N* = RNA copies per PCR reaction. The RNA copies per PCR reaction for each sample (*N*) was calculated and divided by the averaged RNA copies per PCR reaction of the corresponding negative controls (N_o). The log inactivation [Log (N/N_o)] was then determined.

The Chick-Watson model was fitted to the inactivation results. The rate constant (k_r) is substituted into Equation 1.7 as $k_r = kI$, where *I* is the UV fluence rate (Equation 3.2). Integrating Equation 3.2 from time zero to time '*t*' yields Equation 3.3.

$$\frac{dN}{dt} = -k(It)N \qquad \text{Eqn. 3.2}$$

$$\frac{N}{N_o} = e^{-klt} \qquad \text{Eqn. 3.3}$$

where N = viral titre at time 't' in RNA copies per PCR reaction for ICC RT-PCR results and IU/mL for $TCID_{50}$ results, N_o = initial viral titre, k = inactivation constant, and It = UV fluence. Equation 3.3 can be converted to a base ten logarithmic equation by dividing both sides by 2.30. The Chick-Watson model forces the intercept of the log inactivation versus fluence line through the origin.

3.1.1. Direct RT-PCR Analysis

The samples from the first UV trials were analyzed with direct RT-PCR (Table 3.1). No Ad41 was detected in the positive control samples. The Chick-Watson model was fitted to the RT-PCR data and the inactivation constant was found to be 0.006 cm^2/mJ (Figure 3.1). The highest log inactivation observed was 0.44. There was no statistically significant correlation between log inactivation using direct RT-PCR and the UV fluence at the 95% confidence level (R^2 = 0.17, p-value = 0.23). A significant fluence-response curve was not expected because RT-PCR detects both infectious and non-infectious nucleic acid (Richards (1999)), and UV exposure does not remove inactivated virus. At UV fluences greater than 300 mJ/cm^2, Bhattacharya et al. (2004) observed a correlation between UV fluence and hepatitis A inactivation using direct PCR. The correlation is likely due to the effect that large UV fluences have on viral nucleic acid.

Table 3.1 UV Inactivation of Ad41 Using Direct RT-PCR

UV Fluence (mJ/cm^2)	**Log (*N/No*)**	**Control Sample**	**Log (*N*)**
27	0.44	Negative	4.46
27	0.01	Negative	4.59
27	0.08	Negative	4.40
53	0.22	Positive	ND
53	0.21	Positive	ND
53	0.15	Positive	ND
72	0.23	-	-
72	0.37	-	-
72	ND	-	-
96	0.24	-	-
94	0.34	-	-
94	ND	-	-

Notes: ND – non-detect, '-' intentionally left blank

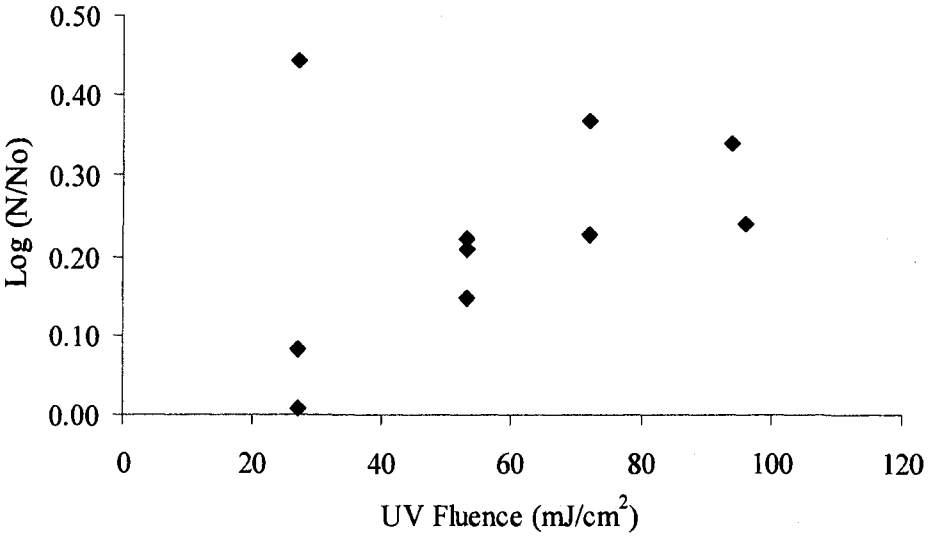


Figure 3.1 UV Inactivation of Ad41 Using Direct RT-PCR

3.1.2. Integrated Cell Culture RT-PCR Analysis

Negative controls (viable virus with no UV exposure) were conducted in all three sets of UV trials. The negative controls had a coefficient of variation (COV) of 7%. In the first and third trials, negative controls were assayed as non-detect. The cause of the non-detects was not known, and was attributed to laboratory error. Positive controls (heat inactivated virus) were only conducted in the first UV trials. RNA detected in the positive control would indicate that despite the biological amplification step, non-infectious viral RNA could still be detected. RNA was not detected in the positive control samples indicating that non-infectious virus was not detected using the ICC RT-PCR assay. The results of the control samples performed during UV exposures are included in Table 3.2.

Table 3.2 Results of Ultraviolet Irradiation Controls by ICC RT-PCR

Trial Set 1		**Trial Set 2**		**Trial Set 3**	
Control	**Log (*N*)**	**Control**	**Log (*N*)**	**Control**	**Log (*N*)**
positive	ND	positive	-	positive	-
positive	ND	positive	-	positive	-
positive	ND	positive	-	positive	-
negative	5.78	negative	4.94	negative	ND
negative	5.06^{R}	negative	5.05	negative	4.98
negative	5.74	negative	5.05	negative	ND

Notes: ND – not detected, - not performed, ^{R} –reanalyzed result

The results of UV log inactivation (Log (N/N_o)) as measured by ICC RT-PCR are provided in Table 3.3. Ad41 was not detected in a number of the samples from the first UV trials. The non-detect samples were reanalyzed using the complete ICC RT-PCR assay and the new result was used for further analysis. Samples from UV trial sets two and three with non-detectable Ad41 titres were not reanalyzed. It was decided that a consistent set of results was favourable. The non-detect samples could have been caused by a number of issues, such as an unsuccessful cell infection, or unhealthy cell monolayers.

Table 3.3 Ultraviolet Irradiation Log Ad41 Inactivation Using ICC RT-PCR

UV Trial Set	UV Fluence (mJ/cm^2)	Log (N/N_o)	UV Fluence (mJ/cm^2)	Log (N/N_o)	UV Fluence (mJ/cm^2)	Log (N/N_o)	UV Fluence (mJ/cm^2)	Log (N/N_o)
Trial Set 1	27A	0.47	53A	1.15	72A	2.01^R	96A	1.49
	27B	1.18^R	53B	1.02	72B	1.83^R	94B	2.22^R
	27C	0.89^R	53C	1.82^R	72C	1.22	94C	1.51
Trial Set 2	25A	0.53	50A	0.88	75A	1.05	100A	1.61
	25B	0.24	50B	0.92	75B	1.26	100B	1.26
	25C	0.11	50C	0.89	75C	ND	100C	2.52
Trial Set 3	25A	0.22	50A	0.37	75A	1.48	100A	1.17
	25B	0.11	50B	0.48	75B	1.40	100B	1.56
	25C	ND	50C	0.71	75C	1.25	100C	ND
mean	-	0.47	-	0.92	-	1.44	-	1.67
std dev	-	0.39	-	0.42	-	0.33	-	0.47
variance	-	0.15	-	0.18	-	0.11	-	0.22
COV (%)	-	83	-	46	-	23	-	28
maximum	-	1.18	-	1.82	-	2.01	-	2.52
minimum	-	0.11	-	0.37	-	1.05	-	1.17

Notes: ND – not detected, ^{R} – sample was reanalyzed, COV – coefficient of variation, std dev – standard deviation

Various statistical tests were applied to the UV data to evaluate the reproducibility of the disinfection and ICC RT-PCR methods. The means were compared using either a paired t-test (set two versus set three) or a two-sample t-test (set one versus set two and set three). A paired test was used when the UV fluence was the same in each trial set, and a two-sample test was used when the UV fluence was not the same. The mean log inactivation results of each set of UV trials were not statistically different at the 95% confidence level. The standard deviation of the log inactivation for each applied UV fluence was similar, with the coefficient of variation (COV) decreasing at the highest UV fluences. The COV of all the samples was 54%. The relatively small COV of the negative controls (7%) indicates that the ICC RT-PCR method was precise and the variability in the samples is largely due to the fluence response, which is explained by regression. The remaining observed variability in the log inactivation of Ad41 in the UV exposed samples can be attributed to random error or inherent method variability. The cell monolayer health, the number of cells present during infection, and the replication rate of the cells have major influences on the biological amplification step.

The Chick-Watson model was fitted to the ICC RT-PCR log inactivation data (R^2 = 0.58, p-value = 2 x 10^{-7}) and was found to be significant at the 95% confidence level. The ANOVA calculations can be found in Appendix B, Table B.1. The Chick-Watson model is a linear model with the intercept set to zero. The inactivation constant was calculated to be 0.0413 cm^2/mJ (Figure 3.2) using Equation 3.3, and ranged from 0.0365 to 0.0460 cm^2/mJ at the 95% confidence interval. The UV fluences required for 2, 3, and 4 log inactivation of Ad41 were approximately 110, 170, and 220 mJ/cm^2 based on the Chick-Watson model. The ICC RT-PCR results were also fitted to a linear model (R^2 = 0.59, p-value = 2.13 x 10^{-7}) with a slope (inactivation constant) and intercept of 0.0172 cm^2/mJ and 0.0531, respectively (Equation. 3.4, Figure 3.3).

$$Log\frac{N}{N_o} = k_L It + b \qquad \text{Eqn 3.4}$$

where k_L = the linear inactivation constant. Note that the linear inactivation rate constant, k_L, is not directly comparable to the Chick Watson rate constant, k, because of the difference in the base of logarithms (base 10 verus base e). To compare the rate constants, k_L must be multiplied by 2.30. A non-zero intercept indicates a loss of virus viability that was not normalized by the negative controls. The 95% confidence interval on the intercept included zero, which indicates that the non-zero intercept was not significant and the Chick-Watson model is a good fit to the data.

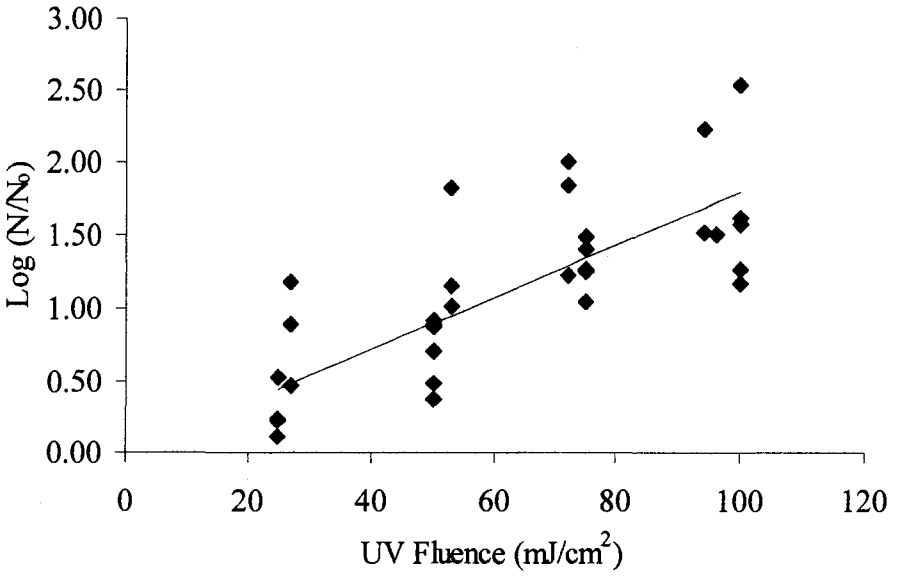


Figure 3.2 Ultraviolet Log Inactivation of Ad41 Using ICC RT-PCR Method Fitted with a Chick-Watson Regression Line

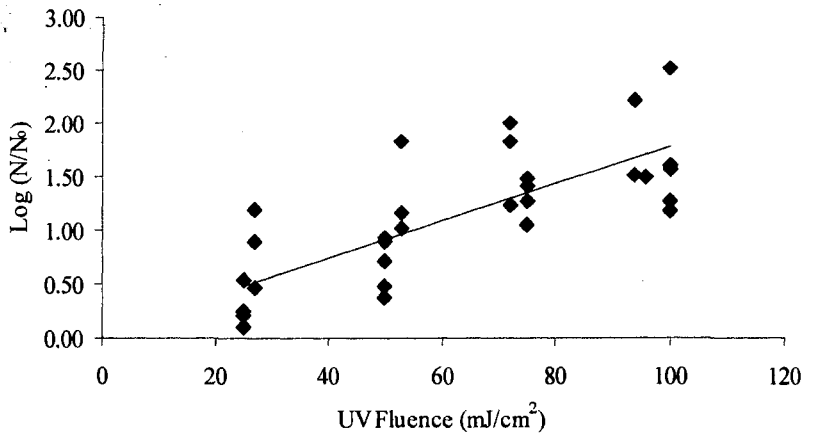


Figure 3.3 Ultraviolet Log Inactivation of Ad41 Using ICC RT-PCR Method Fitted with a Linear Regression Line (Equation 3.4)

The mean response intervals at the 95% confidence level were calculated on the Chick-Watson estimated data (Figure 3.4). There is a 95% certainty that the true mean response is within the mean response interval. The residual error was plotted against the applied UV fluence and Chick-Watson model estimated log inactivation (Figure 3.5). The residuals are evenly distributed, indicating that the model form is correct. The residuals were normally distributed with a mean of zero, and were assumed to be independent with a constant variance.

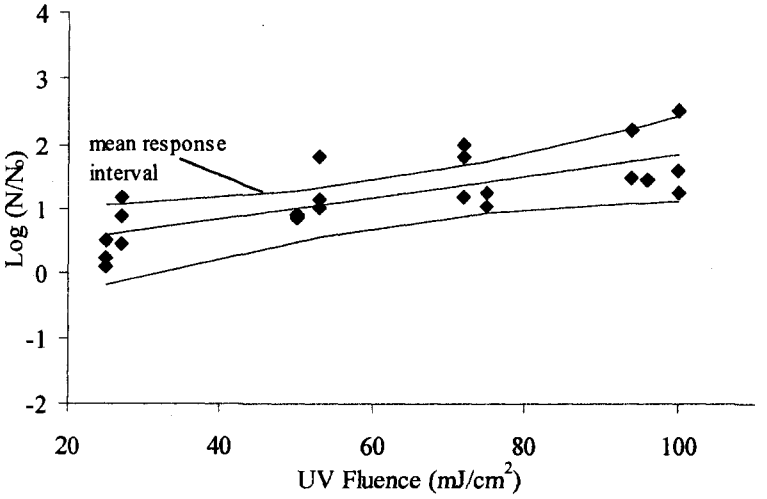


Figure 3.4 95% Confidence Intervals on Ad41 Log Inactivation Using ICC RT-PCR Method and Chick-Watson Model

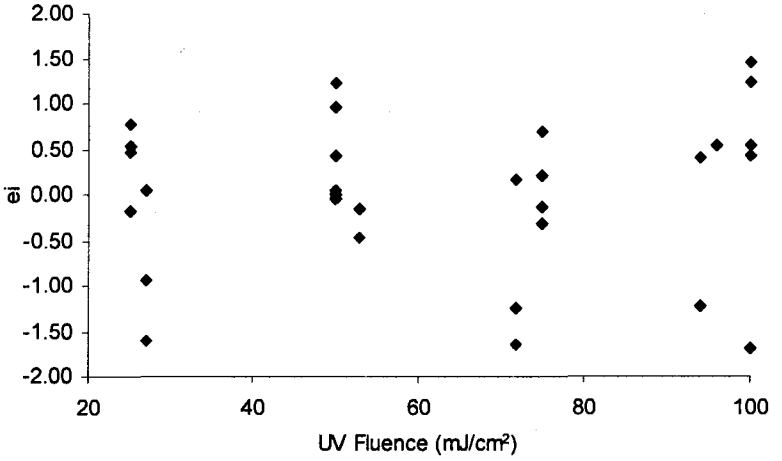


Figure 3.5A Residual Error (e_i) versus UV Fluence

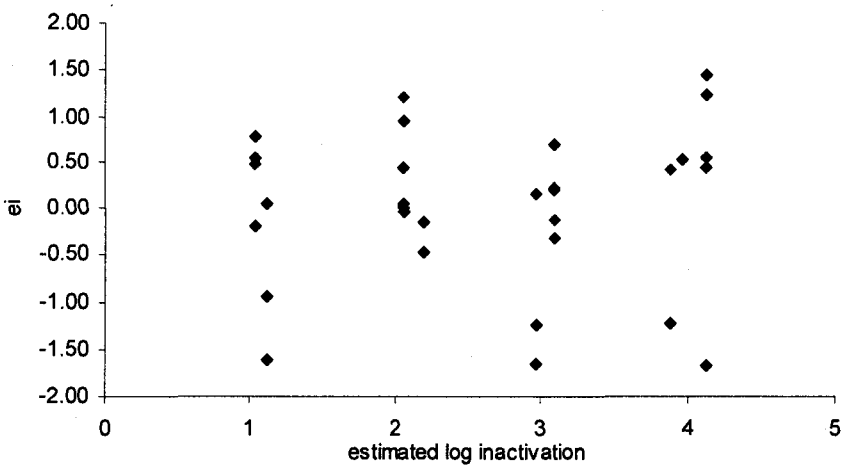


Figure 3.5B Residual Error (ei) versus Chick-Watson Model Estimated Log Inactivation

3.1.3. Fifty Percent Tissue Culture Infectious Fluence Analysis ($TCID_{50}$)

The CPE observation based $TCID_{50}$ method combined with a direct immunofluorescing antibody (DFA) test is labour and material intensive; therefore all of the samples were not be analyzed with this method. Four samples and one negative control were randomly selected from each set of UV trials for $TCID_{50}$ analysis. CPE was not observed in any of the disinfection or control samples. The absence of CPE in the

negative controls could have been caused by dilution of the virus stock during the disinfection experiments or CPE that was slow to form or difficult to identify. The HEp-2 cell line became overgrown after approximately four days, so that CPE that was slow to appear could not be observed. The use of a low viral titre stock in the disinfection experiments combined with dilution may also have prevented observation of CPE. DFA was required to confirm the presence or absence of Ad41 infectious particles on the cell culture. The log inactivation [Log (N/N_o)] as assayed by $TCID_{50}$ is displayed in Table 3.4 and Table 3.5.

Table 3.4 Ultraviolet Irradiation Negative Control Results Using $TCID_{50}$

UV Trial Set	Sample	Log(N)
1	Negative C	2.49
2	Negative C	2.16
3	Negative B	1.49

Table 3.5 Ultraviolet Irradiation Log Ad41 Inactivation Using $TCID_{50}$

UV Trial Set	UV Fluence (mJ/cm^2)	Log (N/N_o)	UV Fluence (mJ/cm^2)	Log (N/N_o)	UV Fluence (mJ/cm^2)	Log (N/N_o)	UV Fluence (mJ/cm^2)	Log (N/N_o)
1	27	1.17	53	1.12	72	2.38	95	2.67
2	25	0.17	50	1.12	75	1.12	100	1.17
3	25	0.58	50	1.83	75	3.33	100	2.05
Mean	-	0.64	-	1.36	-	2.28	-	1.96
Std Dev	-	0.5	-	0.41	-	1.11	-	0.75
COV (%)	-	79	-	30	-	49	-	38

Notes: COV – coefficient of variation, std dev – standard deviation, '-' intentionally left blank

Various statistical tests were applied to the UV data to evaluate the reproducibility and precision of the disinfection and the $TCID_{50}$ methods. The means of each set of trials were compared using either a paired t-test (set two versus set three) or a two-sample t-test

(set one versus sets two and three). The mean log inactivation results of each set of UV trials were not statistically different at the 95% confidence level. The COV was highest in the 25 mJ/cm^2 UV fluence exposures (79%). The same COV trend was observed in the ICC RT-PCR data. The COV of all the $TCID_{50}$ samples was 59% and the COV of the negative controls was 25%. The COV values for the ICC RT-PCR and $TCID_{50}$ exposed samples were similar indicating a similar level of precision in the methods.

The Chick-Watson model was fitted to the $TCID_{50}$ data (R^2 = 0.35, p-value = 0.034) (Figure 3.6). The $TCID_{50}$ inactivation constant was calculated as 0.0558 cm^2/mJ, and ranged from 0.0399 to 0.0716 cm^2/mJ at the 95% confidence interval. The 95% confidence interval included the ICC RT-PCR inactivation constant of 0.0413 cm^2/mJ. The UV fluences required for 2, 3, and 4 log inactivation of Ad41 were approximately 80, 120, and 160 mJ/cm^2 based on the Chick-Watson model. The $TCID_{50}$ results were also fitted to a linear model (R^2 = 0.37, p-value = 0.034) with a slope (inactivation constant) and intercept of 0.0198 cm^2/mJ and 0.3274, respectively (Figure 3.7). The inactivation rate constant, k_L, calculated with the ICC RT-PCR linear, non-zero intercept model was 0.0172 cm^2/mJ. As in the ICC RT-PCR data, a non-zero intercept was observed; however the intercept was not significantly different from zero at the 95% confidence level. The Chick-Watson model was used for further analysis of the data.

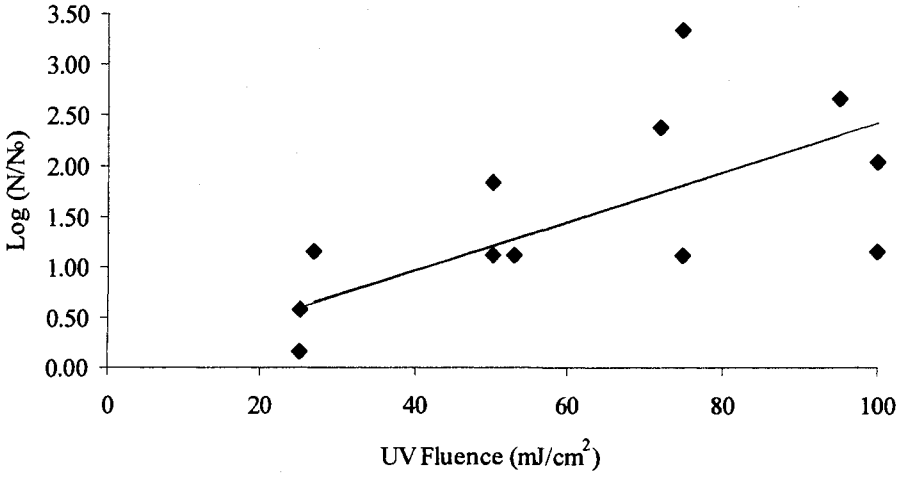


Figure 3.6 Ultraviolet Log Inactivation of Ad41 Using $TCID_{50}$ Method Fitted with a Chick-Watson Model

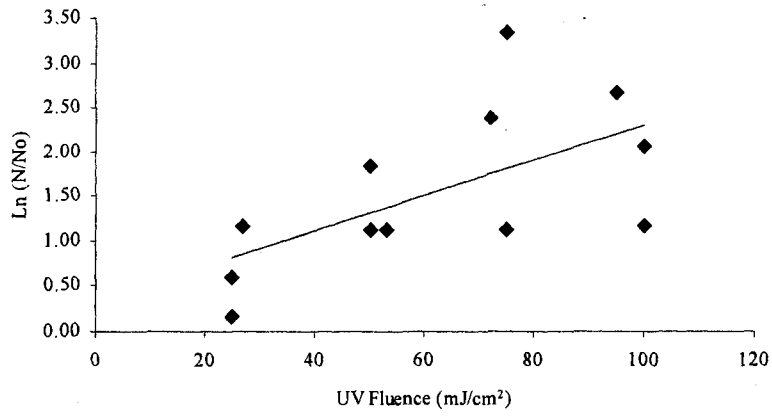


Figure 3.7 Ultraviolet Log Inactivation of Ad41 Using $TCID_{50}$ Method Fitted with a Linear Model with Non-Zero Intercept

The mean response interval at the 95% confidence level was calculated on the Chick-Watson estimated data (Figure 3.8). There is a 95% certainty that the true mean is within the mean response interval. The applied UV fluence and Chick-Watson model estimated log inactivation were plotted against the residual error (Figure 3.9). The residuals are evenly distributed, indicating that the Chick-Watson model form is a good fit to the data. The residuals were normally distributed with a mean of zero, and were assumed to be independent with a constant variance.

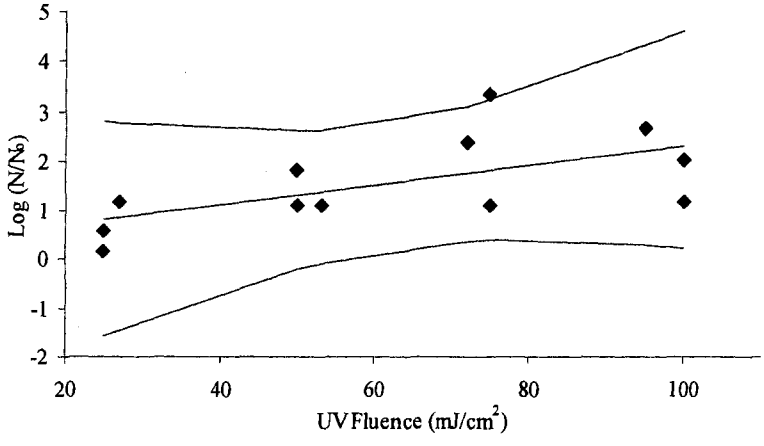


Figure 3.8 95% Confidence Intervals on Ad41 Log Inactivation Using $TCID_{50}$ Method and Chick-Watson Model

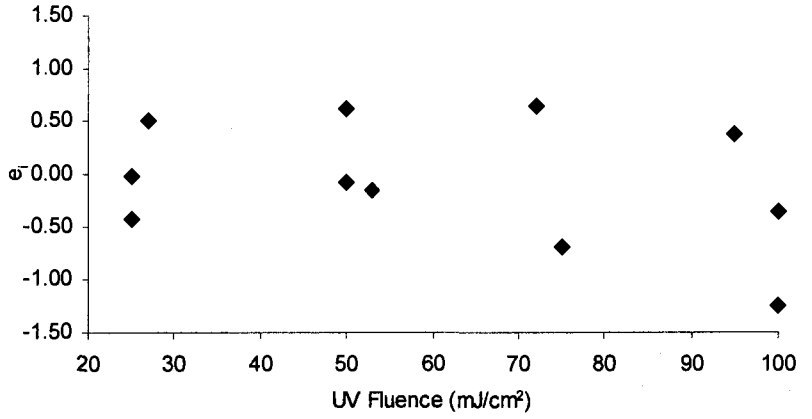


Figure 3.9(A) Residual Error (e_i) versus UV Fluence

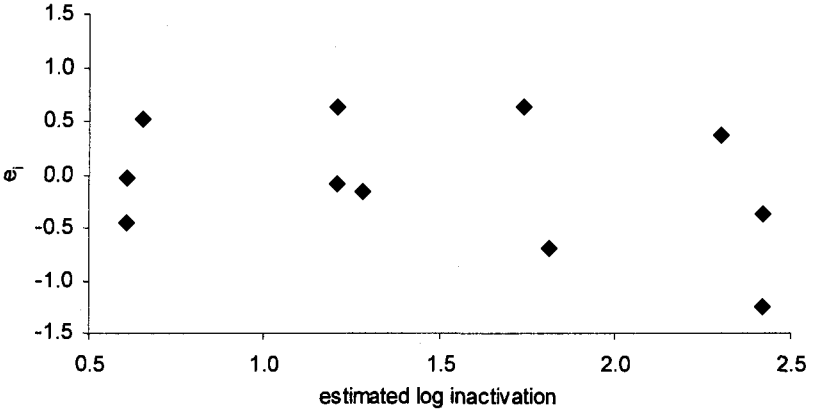


Figure 3.9(B) Residual Error (e_i) versus Chick-Watson Model Estimated Log Inactivation

3.2 Summary of UV Inactivation Experiments

Three UV inactivation experiments were performed using an Ad41 stock with a viral titre of 4.31×10^3 IU/mL. Inactivated samples were analyzed for Ad41 using $TCID_{50}$ and ICC RT-PCR methods. Select samples were also analyzed with direct RT-PCR. The main conclusions drawn from the experiments are as follows:

1. The applied UV fluence had no significant effect on Ad 41 log inactivation when samples were analyzed by direct RT-PCR.
2. RNA was not detected in the positive control samples assayed by ICC RT-PCR, indicating that the assay did not detect non-viable virus.

3. The Chick-Watson model was fitted to the UV inactivation data generated by ICC RT-PCR ($R^2 = 0.58$, p-value = 1×10^{-7}). The inactivation constant was calculated to be 0.0413 cm^2/mJ. The Chick-Watson model was also fitted to the UV inactivation data generated by $TCID_{50}$ ($R^2 = 0.35$, p-value = 0.014) and the inactivation constant was calculated to be 0.0558 cm^2/mJ.

4. The ICC RT-PCR 95% confidence interval is contained within the $TCID_{50}$ 95% confidence interval (Figure 3.10). The ICC RT-PCR mean response was therefore not significantly different from the $TCID_{50}$ mean response. The slopes of the ICC RT-PCR and $TCID_{50}$ regression lines were compared using a t-test for comparison of two slopes (Zar, 1984). The slope of the $TCID_{50}$ regression line was greater at the 95% confidence level (p-value = 0.02). The t-test has more statistical rigor than the confidence interval comparison because it includes the degrees of freedom associated with both tests; therefore the mean responses were not the same.

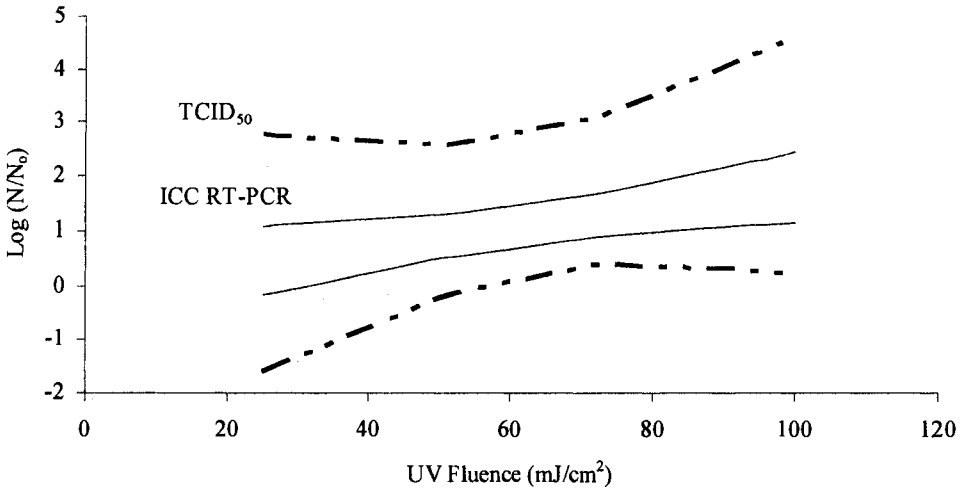


Figure 3.10 Comparison of 95% Confidence Intervals on Ad41 Log Inactivation Using $TCID_{50}$ and ICC RT-PCR Assays

5. The UV fluences required for 2, 3, and 4 log inactivation of Ad41 were 110, 170, and 220 mJ/cm^2 based on the ICC RT-PCR Chick-Watson model.

6. The UV fluences required for 2, 3, and 4 log inactivation of Ad41 were 80, 120, and 160 mJ/cm^2 based on the $TCID_{50}$ Chick-Watson model.

7. The non-zero intercept observed in the linear model fitted to $TCID_{50}$ and ICC RT-PCR results was not significant at the 95% confidence level.

4.0 Inactivation of Adenovirus 41 by Monochloramine Exposure

4.1 Monochloramine Inactivation Results and Discussion

The following sections discuss the inactivation of Ad41 using monochloramine as the only disinfectant. The inactivation results are presented and analyzed. The monochloramine trials were performed on January 24, February 11, and February 28, 2008 using Ad41 stock. The raw results of the monochloramines experiments are located in Appendix A, Table A.1. A new stock was used for each set of trials consisting of passage 6, pooled passages 3 and 4, and pooled passages 4 and 5 for trial set one, two, and three, respectively. The viral titre of each Ad41 stock before transport to the disinfection lab was determined to be on the order of 10^5 to 10^6 RNA copies per PCR reaction (10^8 to 10^9 RNA copies per mL, see Appendix A for conversion details) using RT-PCR. The viral titre of the Ad41 stock was on the order of 10^4 IU/mL when assayed with $TCID_{50}$. All monochloramine exposed samples were analyzed using real time, quantitative ICC RT-PCR and select samples were analyzed with $TCID_{50}$. The ICC RT-PCR and $TCID_{50}$ results can not be compared directly due to the different units (RNA copies per PCR reaction vs. IU/mL); however they can be compared when normalized as log inactivation.

4.1.1. Integrated Cell Culture RT-PCR Analysis

A PCR standard curve was created for each PCR run. The equation of the line fitted to the PCR standard curve (Appendix A, Figure A.3) was used to quantify the PCR *CP* results of the monochloramine exposed samples (Equation 4.1).

$$CP = -mLog(C) + b \qquad \text{Eqn. 4.1}$$

where m = slope, C = viral titre, b = intercept, and CP = crossing point. The RNA copies per PCR reaction for each sample (N) was calculated and divided by the averaged RNA copies per PCR reaction of the corresponding negative controls (N_o). The log inactivation [Log (N/N_o)] was then determined.

Positive and negative controls were conducted in all three monochloramine trial sets (Table 4.1). RNA was detected in all four of the positive control samples from set one, one of four control samples from set two, and none of the two control samples from set three. The log viral titre of the positive controls ranged from 1.42 to 2.61 RNA copies per PCR reaction. The log viral titre of the samples collected from the reactor flasks ranged from 2.62 to 5.61 RNA copies per PCR reaction. In the ICC RT-PCR assay, viable virus is biologically amplified during the cell culture step so that the non-viable virus is only a small portion of the sample RNA. RNA detected in the positive control indicated that non-infectious virus could still be detected with the ICC RT-PCR assay. Bhattacharya et al. (2004) determined that viral RNA may still be synthesized by RT depending on the locations of strand breakage, cDNA synthesis, and the PCR amplification primers. Contamination of the positive controls in the laboratory could also have caused the RNA detection.

Table 4.1 Monochloramine Control Results Using ICC RT-PCR

Control	Trial Set 1		Trial Set 2		Trial Set 3	
	$C_{avg}t$ (mg×min/L)	Log (N)	$C_{avg}t$ (mg×min/L)	Log (N)	$C_{avg}t$ (mg×min/L)	Log (N)
Positive	0	2.61	0	ND	0	ND
	49	1.76	89	ND	72	ND
	192	1.98	320	1.42	-	-
	309	2.70	351	ND	-	-
Mean	-	2.26	-	-	-	-
Std Dev	-	0.46	-	-	-	-
COV (%)	-	20	-	-	-	-
Control	**Time (min)**	**Log (N)**	**Time (min)**	**Log (N)**	**Time (min)**	**Log (N)**
Negative	0	4.72	0	5.87	0	5.61
	17	4.73	30	5.39	30	5.76
	58	4.68	60	4.48	60	5.52
	141	4.58	90	4.84	90	5.57
	270	4.48	120	4.49	-	-
Mean	-	4.64	-	5.01	-	5.61
Std Dev	-	0.11	-	0.61	-	0.10
COV (%)	-	2	-	12	-	2

Notes: the negative controls were not exposed to monochloramine, ND – not detected, std dev – standard deviation, COV – coefficient of variation, '-' intentionally left blank

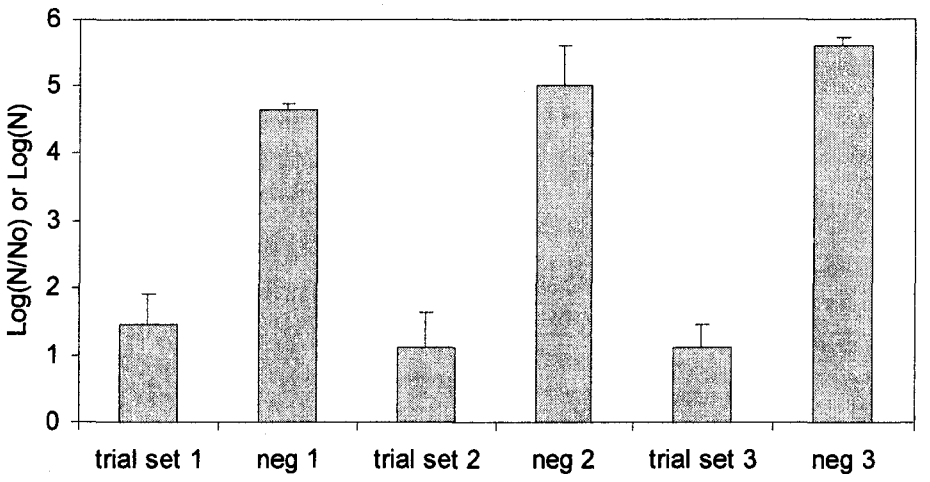
The monochloramine log inactivation (Log (N/N_o)) as measured by ICC RT-PCR is displayed in Table 4.2. In trial sets one and three, three reactor flasks were used and three to four samples were collected from each flask. In trial set two, four reactor flasks were used and three to five samples were collected from each flask. Various statistical tests were applied to the monochloramine data to evaluate the reproducibility and precision of the disinfection and ICC RT-PCR methods. A two-sample test was used to compare the mean Ad41 inactivation from each of the three monochloramine trial sets. The mean log inactivation results of each monochloramine trial set were not statistically different at the 95% confidence level, indicating that the disinfection and viral assay methods were reproducible.

Table 4.2 Monochloramine Ad41 Log Inactivation Using ICC RT-PCR

Trial Set 1		Trial Set 2		Trial Set 3	
$C_{avg}t$ (mg×min/L)	Log (N/N_o)	$C_{avg}t$ (mg×min /L)	Log (N/N_o)	$C_{avg}t$ (mg×min/L)	Log (N/N_o)
44	1.18	71	0.71	91	0.75
156	1.20	257	0.57	170	0.76
346	1.46	518	0.68	315	0.69
604	1.28	81	0.28	636	1.58
49	1.29	305	1.37	99	0.88
169	1.75	573	1.56	187	1.05
374	2.26	86	1.31	348	1.29
657	1.82	303	1.10	708	1.77
63	0.72	451	1.10	95	0.96
141	0.90	980	1.43	177	1.33
307	2.03	1205	1.49	372	1.12
542	1.50	78	0.57	711	1.27
-	-	666	2.19	-	-
-	-	853	1.16	-	-
Mean	1.45	-	1.11	-	1.12
Std Dev	0.45	-	0.51	-	0.34
COV	0.31	-	0.46	-	0.30

Notes: ND – not detected, COV – coefficient of variation, '-' intentionally left blank, std dev – standard deviation

The standard deviation and COV for each monochloramine set of trials was similar. The COV of the samples from all three inactivation trial sets was 38%. The relatively small COV of the negative controls (11%) indicates the method has a high precision and the variability in the samples is due to the response to varying Ct values. The average Ad41 log inactivation and the average negative control results for each set of monochloramine trials are shown together to illustrate the amount of noise associated with the experimental methods (Figure 4.1). The error bars represent one standard deviation. The standard deviation of the negative control in trial set two is larger than that of the monochloramine inactivation dataset. In trial sets one and three, the error associated with the negative controls is smaller than the error associated with the inactivation data, indicating the methods were precise.



Notes: The y-value of the trials is Log (N/N_o) and the y-value of the negative controls is Log (N_o)

Figure 4.1 Average Monochloramine Ad41 Log Inactivation Using ICC RT-PCR Method and Average Negative Control

It was necessary to include decay in the models fitted to the monochloramine inactivation data due to the high monochloramine demand that was observed. The high demand was likely due to the presence of cell debris in the unpurified virus stock. Monochloramine decays exponentially as described by Equation 4.5.

$$C = C_o e^{-k't} \qquad \text{Eqn. 4.5}$$

where k' is the decay constant (min^{-1}), C_o is the disinfectant concentration observed at time zero, and t is the exposure time. The decay constant for each of the ten reactor flasks was determined by fitting Equation 4.5 to the monochloramine concentration data and minimizing the sum of squares due to error (SS_E) with the Microsoft Excel Version 3.0 Solver function (Appendix C, Table C.1). The decay curve observed in flask one of monochloramine trial set three and the best fit line is presented on Figure 4.2.

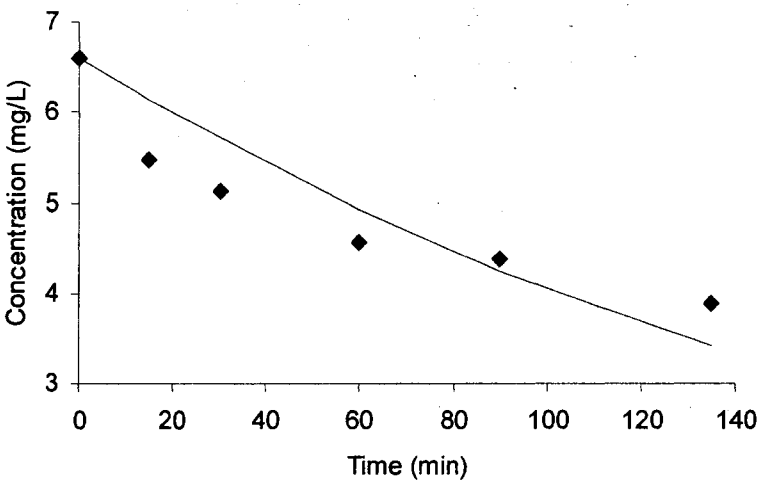


Figure 4.2 Observed Monochloramine Concentration in Flask One, Trial Set Three and Best Fit Line

The monochloramine data were fitted to the Chick-Watson and Hom models. The Chick-Watson model was adjusted to include the effect of chlorine decay as presented by Thurston-Enriquez et al. (2003) (Equation 4.6).

$$Ln\frac{N}{N_o} = \frac{-k}{k'n}\left(C_o^n - C_t^n\right) \qquad \text{Eqn 4.6}$$

where n = empirical constant. The integrated form of the Hom model (Equation 4.7) has been used to describe the inactivation of poliovirus with chloramines (Haas et al. (1995)) and *Cryptosporidium* with ozone (Finch et al. (1993)).

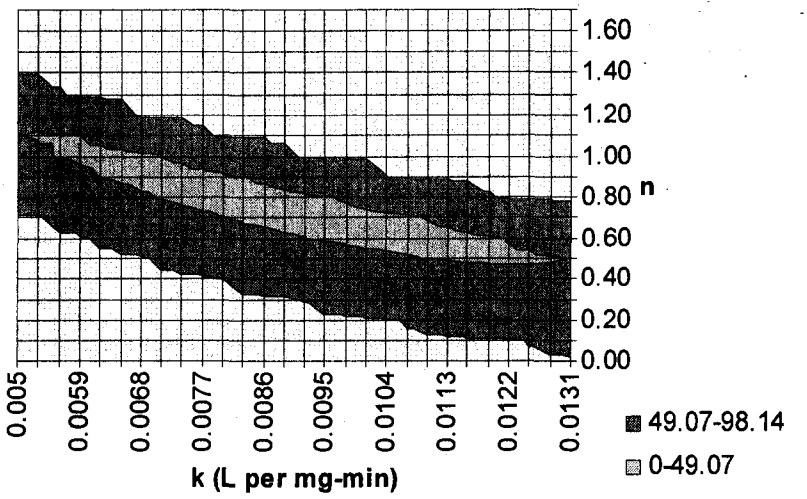
$$Ln\frac{N}{N_o} = -kC^n t^m \qquad \text{Eqn 4.7}$$

where m = empirical constant. The Hom model adjusted to include the effect of chlorine decay, was fitted to the monochloramine data (Equation 4.8, Haas et al. (1995)).

$$Ln\frac{N}{N_o} = -\left(\frac{m}{nk'}\right)^m kC_o^n\left[1 - e^{\left(\frac{-nk't}{m}\right)}\right]^m \qquad \text{Eqn 4.8}$$

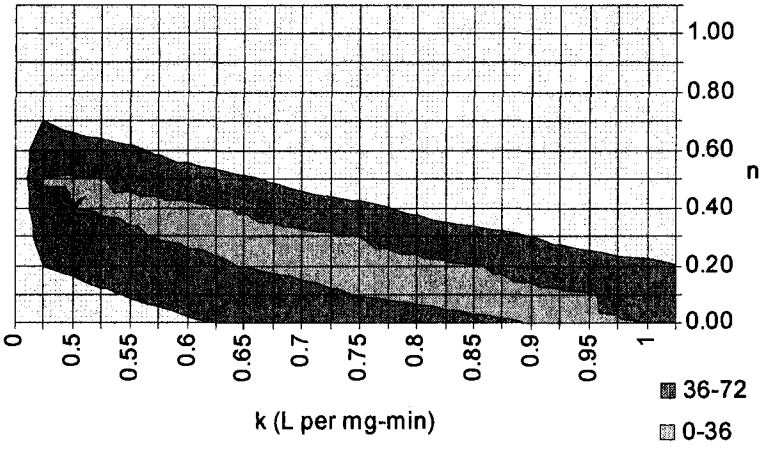
When a model that is non-linear in the parameters is used, analysis of variance (ANOVA) can not be used to determine the significance of the model. Instead, joint confidence regions (JCRs) and residuals plots must be used to evaluate the precision and form of the model. A surface contour is drawn by changing the value of the model parameters in small increments to observe the effect on the SS_E. The JCR is a 2-D reflection of the 3-D surface contour. The first interval of the JCR is the critical SS_E, which indicates the 95% confidence region of two model parameters. The confidence region is considered to be approximate due to the non-linearity of the model. A small, bean-shaped JCR indicates precise parameter estimates and low parameter correlation (Berthouex (2002)).

The Chick-Watson and Hom models were fitted to the inactivation data by minimizing the SS_E with the Microsoft Excel Version 3.0 Solver function. The Chick-Watson constants *n* and *k* were determined to be 0.775 (unitless) and 0.0086 L/mg×min, respectively. The Hom constants *n*, *m*, and *k* were determined to be 0.228 (unitless), 0.243 (unitless), and 0.724 L/mg×min, respectively. JCRs were plotted for both models. The Hom JCR was drawn by changing the value of n and k, and keeping the value of m fixed at 0.243. Both JCRs were long and thin indicating high correlation between the model parameters (Figures 4.3 and 4.4). The area of the Chick-Watson JCR is smaller than the area of the Hom JCR, indicating more precise parameter estimates with the Chick-Watson model.



Notes: the innermost interval indicates the 95% confidence region

Figure 4.3 Chick-Watson Joint Confidence Region for Monochloramine Data



Notes: the innermost interval indicates the 95% confidence region

Figure 4.4 Hom Joint Confidence Region for Monochloramine Data

The Chick-Watson and Hom model residuals (e_i) were plotted against the estimated log inactivation and cumulative Ct product (Figures 4.5 and 4.6). A descending band is apparent in the plot of Chick-Watson residuals versus Ct product indicating an error in the model form. No pattern was observed in the Hom residuals plots, indicating the model form was accurate. The sum of the residuals for the Chick-

Watson and Hom models was 8.84 and 0.05, respectively. Ideally, the residuals should be evenly distributed about zero such that the sum of the residuals equals zero.

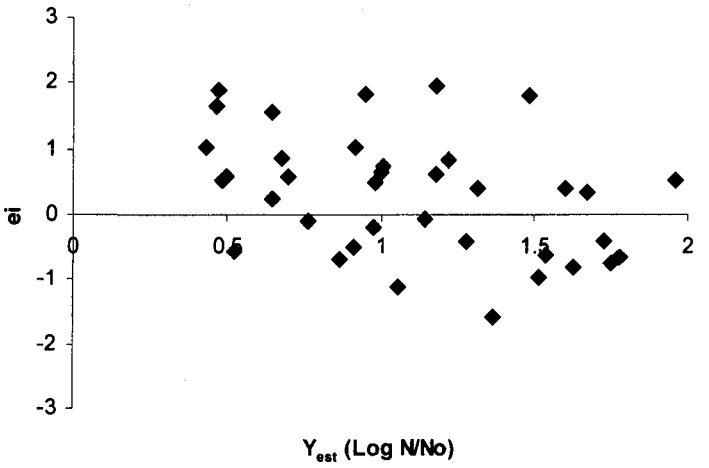


Figure 4.5(A) Chick-Watson Residual Error (e_i) versus Estimated Log Inactivation

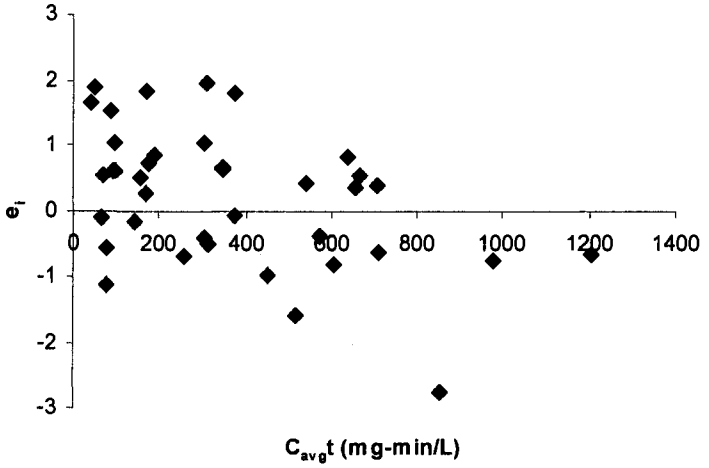


Figure 4.5(B) Chick-Watson Residual Error (e_i) versus Monochloramine $C_{avg}t$ Product

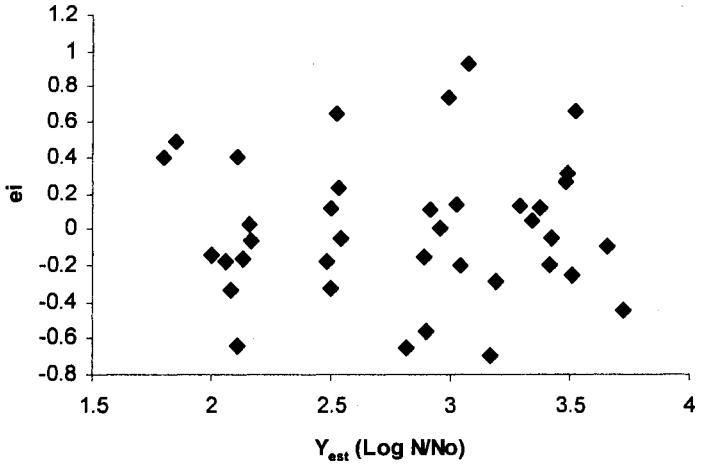


Figure 4.6(A) Hom Residual Error (e_i) versus Estimated Log Inactivation

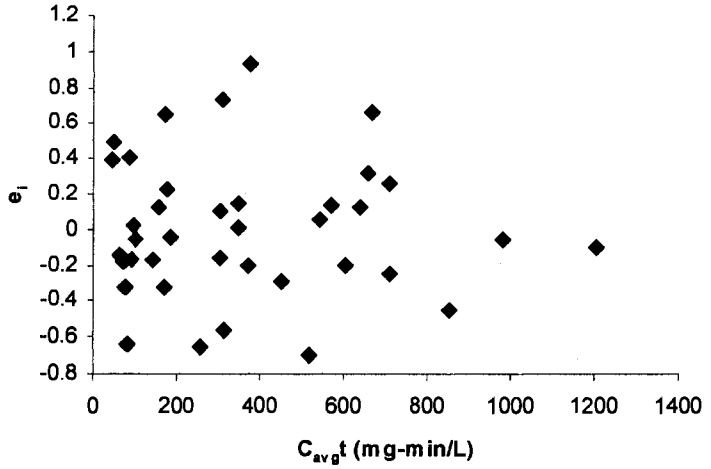


Figure 4.6(B) Hom Residual Error (e_i) versus Monochloramine $C_{avg}t$ Product

A linear model with a y-intercept was also fitted to the inactivation data using linear regression with Microsoft Excel Version 3.0 (Equation 4.9).

$$Log\frac{N}{N_o} = -k_L\left(C_{avg}t\right) - b \qquad \text{Eqn. 4.9}$$

where k_L is the inactivation constant (L/mg×min) and b (unitless) is the intercept. The model was significant (R^2 = 0.22, p-value = 0.003) with an inactivation constant of 0.00074 L/mg×min and an intercept of 0.952. The inactivation constant ranged from 0.00026 to 0.00122 at the 95% confidence interval. No patterns were observed in the residuals plots, and the residuals were distributed evenly about the x-axis (Figure 4.7). Using the linear model the estimated Ct products for 2, 3, and 4 log removal of Ad41 were 1400, 2800, and 4100 mg×min/L. It should be noted that the linear model was not adjusted for monochloramine decay rate, instead the cumulative monochloramines Ct was used in calculations.

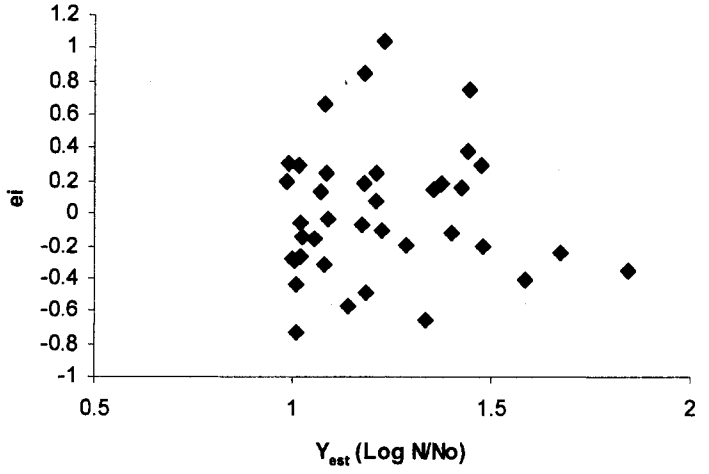


Figure 4.7(A) Linear Residual Error (e_i) versus Estimated Log Inactivation

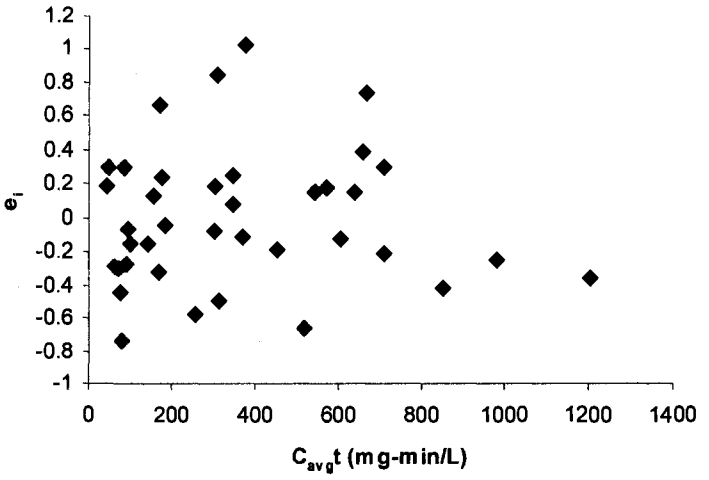


Figure 4.7 (B) Linear Residual Error (e_i) versus Monochloramine $C_{avg}t$ Product

The mean response and prediction intervals at the 95% confidence level were calculated on the Ad41 inactivation estimated with the linear model (Figure 4.8). There is a 95% certainty that the true mean is within the mean response interval. The mean response interval widens at higher $C_{avg}t$ values because fewer data points were placed in this range.

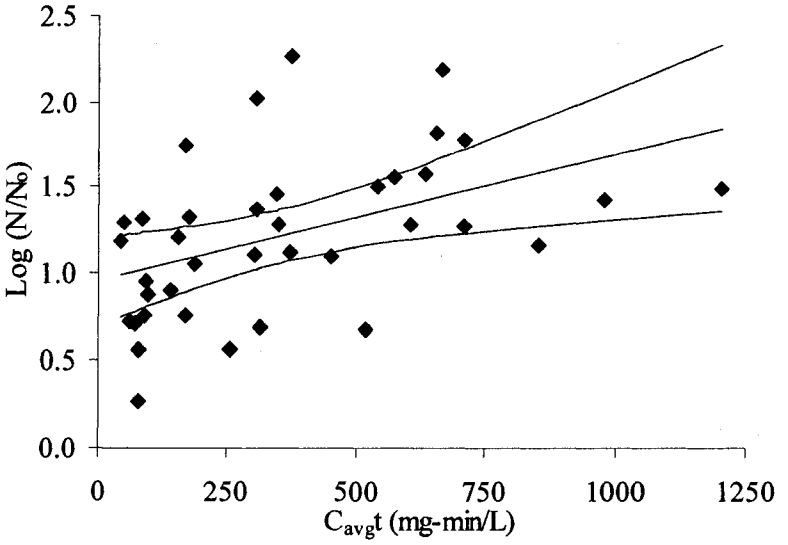


Figure 4.8 Confidence Intervals on Ad41 Log Inactivation Using ICC RT-PCR Method

The observed Ad41 inactivation and the predicted natural log inactivation of Ad41 using the Chick-Watson, linear, and Hom models are plotted on Figure 4.9. The Chick-Watson predictions have the most overlap with the actual data. The Hom predictions show a tailing at higher Ct values and accurately describe the initial curvature of the data. The linear predictions show the expected response to the monochloramine Ct but do not account for the initial non-linear nature of the inactivation. The inactivation results appeared to have a non-zero intercept in each of the three trial sets. An Ad41 log inactivation of greater than 1.0 was observed at monochloramine Ct products less than 100 mg×min/L. The cause of the non-zero intercept was unknown and could be an inactivation in the samples that was not normalized by the negative controls or the actual shape of the curve.

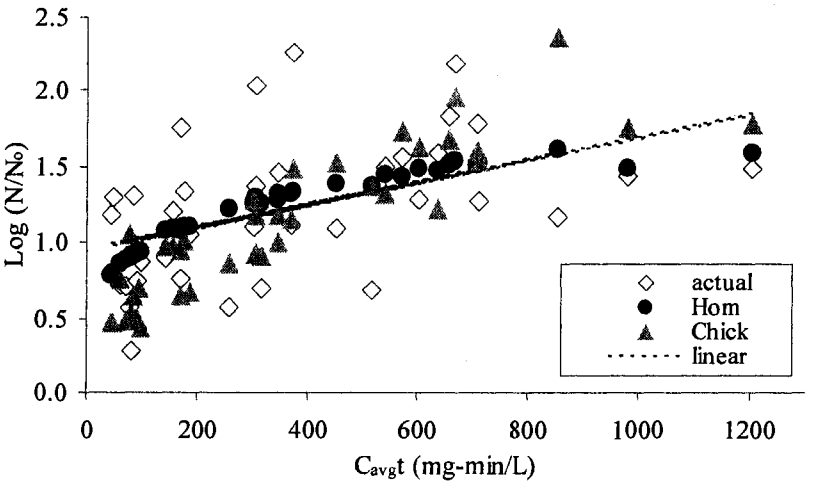


Figure 4.9 Monochloramine Inactivation Data (ICC RT-PCR) and Fitted Models

4.1.2. Fifty Percent Tissue Culture Infectious Dose Analysis ($TCID_{50}$)

From each monochloramine trial set one positive control sample, one negative control sample, and all samples collected from one reactor flask were randomly selected for $TCID_{50}$ analysis. CPE was not observed in any of the samples. The HEp-2 cell line became overgrown after approximately four days, so that CPE that was slow to appear could not be observed. DFA was required to confirm the presence or absence of Ad41

infectious particles. The Ad41 log inactivation [Log (N/N_o)] data as assayed by $TCID_{50}$ and DFA are displayed in Tables 4.3 and 4.4. All of the positive controls were negative for infectious Ad41, which indicated that the virus detected by ICC RT-PCR in the positive control samples were non-infectious. The presence of non-infectious RNA could have been caused by contamination from adenoviruses present in the laboratory environment.

Table 4.3 Monochloramine Negative Control Results Using TCID50

NH_2Cl Trial Set	**Sample #**	**Log (*No*)**
1	3	2.84
2	3	3.73
3	4	2.68

Table 4.4 Monochloramine Ad41 Log Inactivation Using TCID50

Trial Set 1		**Trial Set 2**		**Trial Set 3**	
$C_{avg}t$ (mg×min/L)	**Log (*N/No*)**	**$C_{avg}t$ (mg×min/L)**	**Log (*N/No*)**	**$C_{avg}t$ (mg×min/L)**	**Log (*N/No*)**
86	1.22	49	0.33	99	0.00
303	1.22	169	1.16	187	1.00
451	1.88	374	1.11	348	1.00
980	2.05	657	1.33	708	1.00
1205	1.88	-	-	-	-
Mean	1.65	-	0.98	-	0.75
Std Dev	0.40	-	0.45	-	0.50
COV	0.24	-	0.45	-	0.67

Notes: COV – coefficient of variation, std dev – standard deviation

Various statistical tests were applied to the monochloramine data to evaluate the reproducibility and precision of the disinfection and ICC RT-PCR methods. The means were compared using a two-sample t-test. The mean log inactivation results of each set of monochloramine trials were not statistically different at the 95% confidence level indicating that the method was reproducible. The COV is different in each set of trials showing that the precision varied between trial sets. A portion of the variation can be

attributed to the varied $C_{avg}t$ values for each sample. The Ct product can not be controlled as completely as the UV fluence.

A linear model with a slope (inactivation constant) and intercept of 0.0011 L/mg×min and 0.6733, respectively was fitted to the $TCID_{50}$ data (R^2 = 0.51, p-value = 0.006). The inactivation constant ranged from 0.0004 to 0.0019 at the 95% confidence interval, which included the ICC RT-PCR linear inactivation constant of 0.00074 L/mg×min. The $TCID_{50}$ data and fitted regression line are displayed on Figure 4.10. The monochloramine Ct required for 2, 3, and 4 log inactivation of Ad41 was approximately 1200, 2000, and 2900 mg×min/L, based on the linear model. No patterns were observed in the residuals plots, and the residuals were distributed evenly about the x-axis (Figure 4.11). The mean response interval at the 95% confidence level was calculated using the linear model inactivation estimates (Figure 4.12).

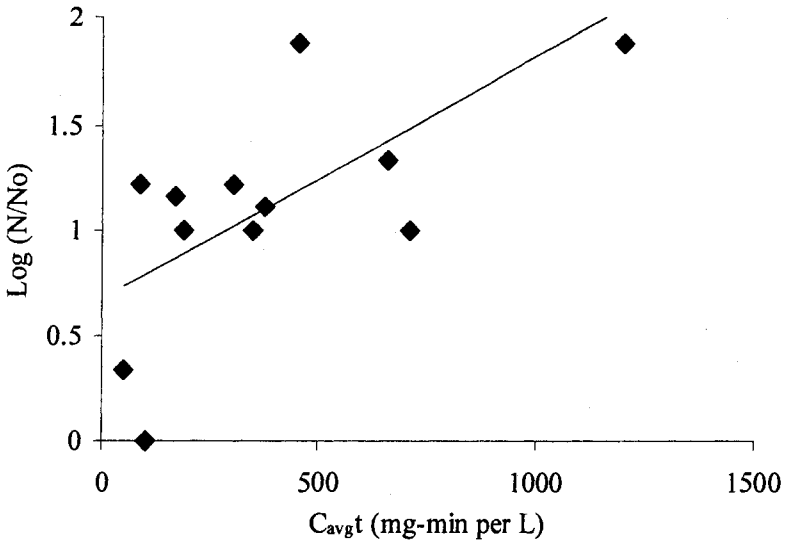


Figure 4.10 Monochloramine Log Inactivation of Ad41 Using $TCID_{50}$ Method and Linear Regression Line

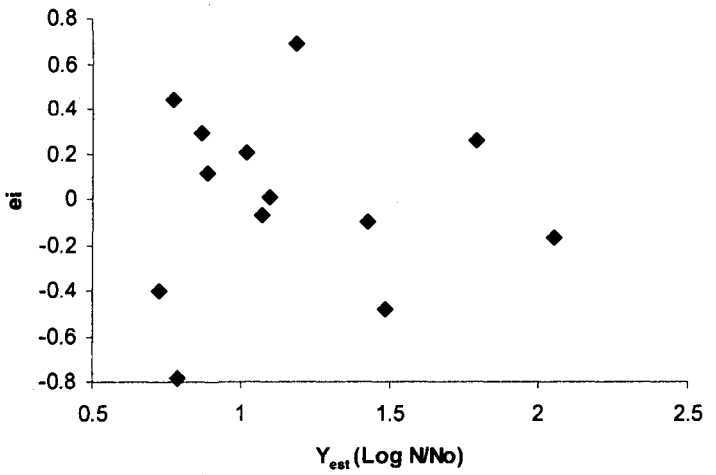


Figure 4.11(A) Residual Error (e_i) versus Estimated Ad41 Log Inactivation

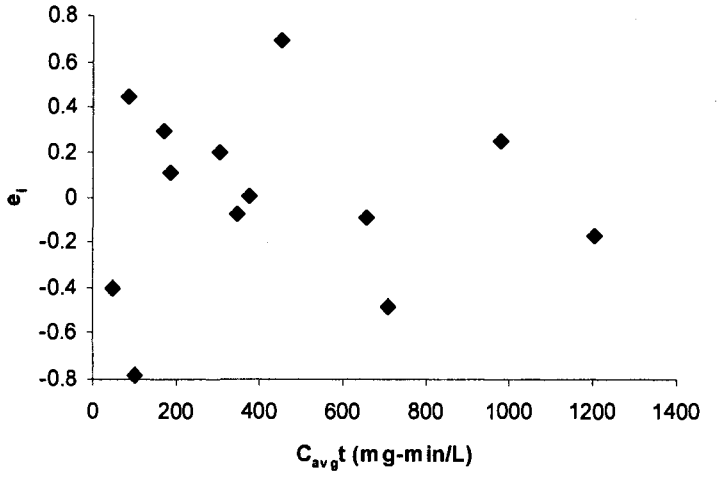


Figure 4.11(B) Residual Error (e_i) versus $C_{avg}t$ Product

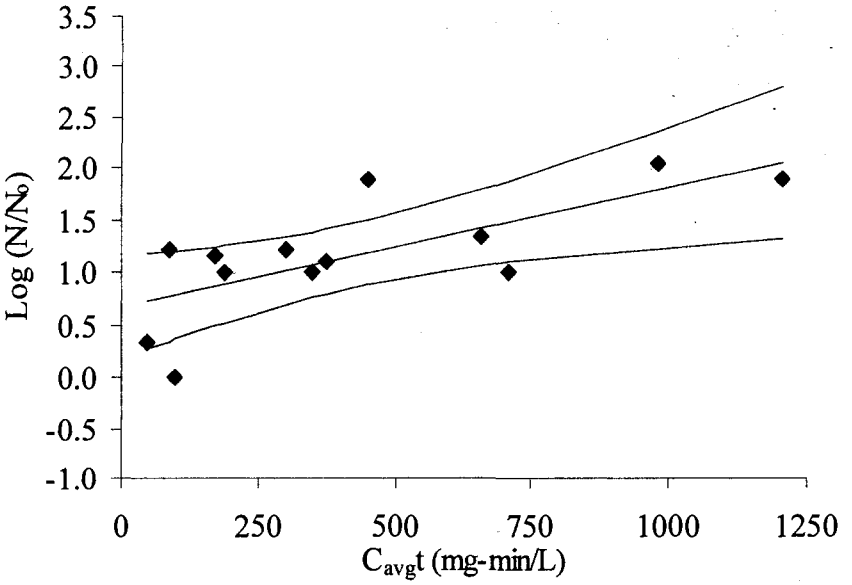


Figure 4.12 Confidence Intervals on Ad41 Log Inactivation Using TCID50 Method

4.2 Summary of Monochloramine Inactivation Experiments

Three monochloramine inactivation experiments were performed with Ad41 stock. The viral titre of the Ad41 stock before transport to the disinfection lab was determined to be on the order of 10^5 to 10^6 RNA copies per PCR reaction using RT-PCR. Monochloramine exposed samples were analyzed for Ad41 using ICC RT-PCR methods. Select samples were also analyzed with $TCID_{50}$ followed by DFA. The main conclusions drawn from the experiments are as follows:

1. No adenovirus was observed in the positive controls assayed by $TCID_{50}$ and DFA. The RNA detected in the positive controls using the ICC RT-PCR assay was therefore non-infectious. A potential cause of the RNA was contamination of the controls in the laboratory.
2. The variability observed in the ICC RT-PCR data is partly attributed to the large variability that was associated with the viral titre assayed in the negative controls.
3. The ICC RT-PCR data were fitted with the Chick-Watson and Hom models, which explicitly included disinfectant decay. The Hom model fit the data better than the Chick-Watson model based on the residuals plots.

4. A linear model was also fitted to the ICC RT-PCR and $TCID_{50}$ data with inactivation constants of 0.0007 and 0.0011 L/mg×min, respectively. The ICC RT-PCR and $TCID_{50}$ mean response intervals overlap indicating that the mean responses are not significantly different at the 95% confidence level (Figure 4.13). The inactivation constants were confirmed to be the same at the 95% confidence level using a t-test to compare the slopes of the inactivation models (p-value = 0.12) (Zar, 1984).

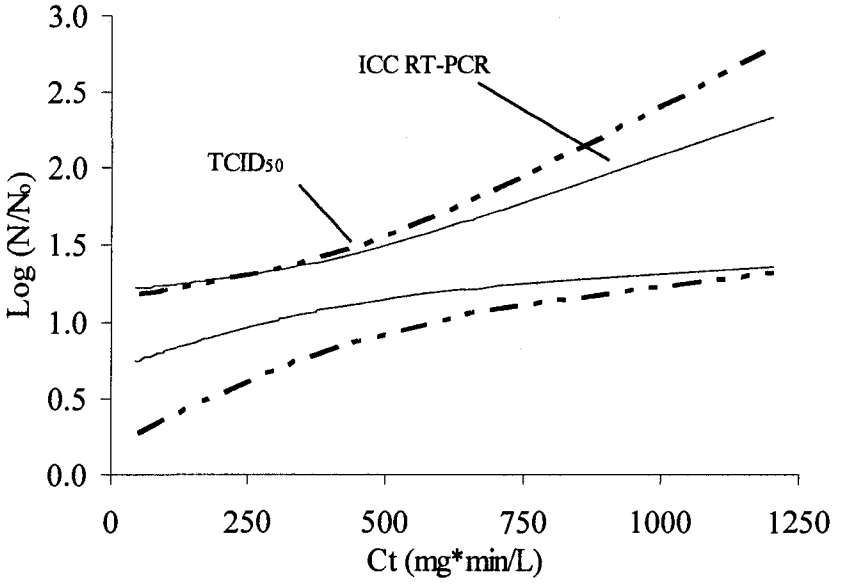


Figure 4.13 Comparison of 95% Confidence Intervals on Ad41 Log Inactivation Using TCID50 and ICC RT-PCR Assays

5. The required Ct product for 2, 3, and 4 log Ad41 inactivation was 1400, 2800, and 4100 mg×min/L based on the ICC RT-PCR linear model. The $TCID_{50}$ linear model predicted 1200, 2000, and 2900 mg×min/L would be required for 2, 3, and 4 log Ad41 inactivation, respectively.
6. A non-zero intercept (significant at a 95% confidence level) was observed for both the $TCID_{50}$ and ICC RT-PCR assayed results. The non-zero intercept indicates a loss of Ad41 viability in the reactors that was not observed in the negative control. The intercept could be a function of variability in the assays.

5.0 Sequential Inactivation of Adenovirus 41

5.1 Sequential Inactivation Results and Discussion

The potential for synergistic effects between UV and monochloramine was evaluated by applying UV at a fluence of 42 mJ/cm^2 followed by exposure to monochloramine Ct products ranging from 55 to 623 mg×min/L. The sequential trials were performed on April 16, April 30, and May 14, 2008 using Ad41 stock. The raw results of the monochloramines experiments are located in Appendix A, Table A.1. A new stock was used for each set of trials consisting of passage 4 for trial sets one and two, and pooled passages 4, 5, and 6 for trial set three. The viral titre of each Ad41 stock before transport to the disinfection lab was determined to be on the order of 10^5 to 10^6 RNA copies per PCR reaction (10^8 to 10^9 RNA copies per mL, see Appendix A for conversion details) using RT-PCR. The viral titre of the Ad41 stock was on the order of 10^4 when assayed by $TCID_{50}$. All samples were analyzed using real time, quantitative ICC RT-PCR and select samples were analyzed using $TCID_{50}$. The ICC RT-PCR and $TCID_{50}$ results cannot be compared directly due to the different units (copies per PCR reaction vs. IU/mL); however they can be compared when normalized as log inactivation.

5.1.1. Integrated Cell Culture RT-PCR Analysis

A PCR standard curve was created for each PCR run. The equation of the line fitted to the PCR standard curve (Appendix A, Figure A.3) was used to quantify the PCR *CP* results of the monochloramine exposed samples (Equation 5.1).

$$CP = -mLog(C) + b \qquad \text{Eqn. 5.1}$$

where m = slope, C = viral titre, b = intercept, and CP = crossing point. The RNA copies per PCR reaction for each sample (N) was calculated and divided by the averaged RNA copies per PCR reaction of the corresponding negative controls (N_o). The log inactivation [Log (N/N_o)] was then determined.

At the 95% confidence level an Ad41 log inactivation of 0.75 +/- 0.48 was expected from a UV fluence of 42 mJ/cm^2 based on the Chick-Watson model fitted to the UV data (Figure 3.2). The observed Ad41 log inactivation was 0.38, 0.36, and 0.13 in trial sets one, two, and three, respectively. Condensation build-up was occasionally noted on the quartz cover during UV exposure. The condensation may have reduced the UV transmission to the virus stock. The cause of the much lower inactivation in trial set three is unknown; no changes were made to the UV exposure method. The UV exposed virus stock, and the positive and negative controls were assayed by ICC RT-PCR only.

Positive and negative controls were conducted in all three sets of sequential trials (Table 5.1). The COV of all the negative controls was 6%, indicating that the disinfection and viral assay methods were precise. RNA was detected in two of four positive control samples from trial set one, and was not detected in any of the seven positive control samples from trial set two or three. The viral titre of the positive controls ranged from 1.23 to 2.04 RNA copies per PCR reaction. The viral titre of the samples collected from the reactor flasks after monochloramine exposure ranged from 1.53 to 3.87 RNA copies per PCR reaction. RNA detected in the positive control indicated that despite the biological amplification step, non-infectious virus could still be detected. The RNA of the heat inactivated virus was likely still detectable by RT-PCR. Bhattacharya et al. (2004) determined that viral RNA may still be synthesized by RT after heat inactivation depending on the locations of strand breakage, cDNA synthesis, and PCR amplification primers.

Table 5.1 Results of Sequential Controls Using ICC RT-PCR

Control	Trial Set 1		Trial Set 2		Trial Set 3	
	$C_{avg}t$ (mg×min/L)	**Log (*N*)**	**$C_{avg}t$ (mg×min/L)**	**Log (*N*)**	**$C_{avg}t$ (mg×min/L)**	**Log (*N*)**
positive	92	1.23	57	ND	47	ND
	258	2.04	144	ND	137	ND
	420	ND	418	ND	110	ND
	463	ND	-	-	-	ND
negative	-	5.40	-	4.85	-	4.75
	-	5.33	-	4.69	-	4.62
	-	5.15	-	4.70	-	4.94
	-	5.29	-	5.42	-	4.76
Mean	-	5.29	-	4.91	-	4.77
Std Dev	-	0.11	-	0.34	-	0.13
COV (%)	-	2	-	7	-	3

Notes: the negative controls were not exposed to monochloramines, ND – not detected, COV – coefficient of variation, std dev – standard deviation, '-' intentionally left blank

The sequential log inactivation (Log (N/N_o)) as measured by ICC RT-PCR is provided in Table 5.2. Various statistical tests were applied to the monochloramine data to evaluate the precision of the disinfection and ICC RT-PCR methods. The mean of all three datasets was compared using a two-sample t-test. The mean log inactivation results of each sequential monochloramine trial set were statistically different at the 95% confidence level, indicating that the method had a low level of reproducibility. No changes were made to the monochloramine exposure methods; however the initial monochloramine concentration in all reactor flasks was lower during trial set three than in trial sets one and two. The COV for each sequential trial set was similar, and the COVs were lower than those observed in the non-sequential monochloramine trials. The COV of all the monochloramine exposed samples was 25%. The relatively small COV of the negative controls indicates the disinfection and viral analysis methods were precise, and that the variability in the monochloramine exposed samples was likely due to actual differences in log inactivation due to the Ct values.

Table 5.2 Sequential Ad41 Log Inactivation Using ICC RT-PCR

Trial Set 1		Trial Set 2		Trial Set 3	
$C_{avg}t$ (mg×min/L)	Log (N/N_o)	$C_{avg}t$ (mg×min/L)	Log (N/N_o)	$C_{avg}t$ (mg×min/L)	Log (N/N_o)
90	1.42	76	1.76	55	1.27
268	1.98	225	2.94	127	1.54
459	2.25	427	2.87	235	1.63
571	2.17	503	1.96	283	1.51
155	1.76	151	2.11	56	1.27
241	1.75	256	1.93	138	1.77
418	2.05	431	2.50	243	1.45
516	2.23	568	2.44	304	1.76
102	1.65	103	1.64	103	1.32
264	1.91	233	2.29	233	1.42
481	2.21	469	2.25	469	1.64
617	2.40	623	3.39	623	1.76
Mean	1.98	-	2.34	-	1.53
Std Dev	0.29	-	0.52	-	0.19
COV	0.15	-	0.22	-	0.12

Notes: ND – not detected, '–' intentionally left blank, COV – coefficient of variation, std dev – standard deviation

The inactivation measured in trial set two was higher than that measured in trial set three, and there was overlap of the inactivation measured in trial set one and that measured in trial sets two and three (Figure 5.1). Linear regression of each set of sequential trials was performed individually and the slopes (inactivation constants) were compared using a t-test. The inactivation constants of set one and two were equal, and the inactivation constant of set three was different from that of both sets one and two.

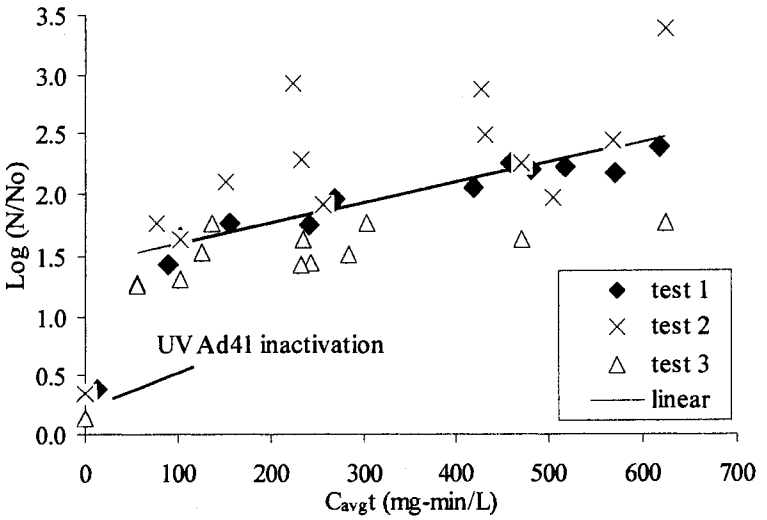


Figure 5.1 UV followed by Monochloramine Ad41 Inactivation Results Assayed by ICC RT-PCR and Linear Regression Predicted Inactivation

The sequential inactivation was corrected by subtracting the observed UV inactivation from the inactivation observed after sequentially applied UV and monochloramine in order to facilitate evaluation of synergistic effects. The inactivation data from trial sets one, two, and three were corrected by 0.38, 0.36, and 0.13, respectively. Linear regression of the sequential monochloramine inactivation data was performed using Microsoft Excel Version 3.0. The model was significant ($R^2 = 0.40$, p-value = 2.97×10^{-5}) with an inactivation constant of 0.0015 L/mg×min and an intercept of 1.20.

A funneling pattern was observed in the plot of residuals versus inactivation estimates indicating non-constant variance (Figure 5.2). The residuals were distributed evenly about the x-axis. The mean response interval at the 95% confidence level was calculated on the Ad41 inactivation estimated with the corrected linear model (Figure 5.3). Using the linear model the estimated $C_{avg}t$ values for 2, 3, and 4 log inactivation of Ad41 are 500, 1200, and 1900 mg×min/L. The required Ct products calculated using the sequential model were lower than those calculated using the monochloramines only model (1400, 2800, and 4100 mg×min/L). The Hom model was also fitted to the corrected sequential Ad41 inactivation data, but patterns observed in the residuals (not shown) indicated that the model did not fit the data.

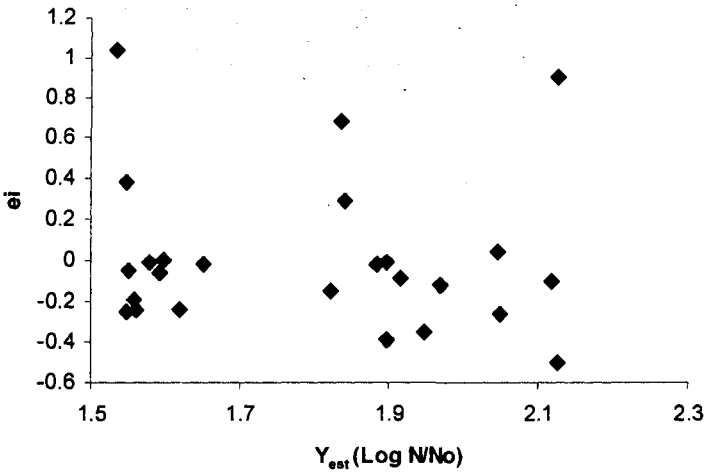


Figure 5.2(A) Linear Model Residual Error (e_i) for Corrected Sequential Inactivation versus Estimated Ad41 Log Inactivation

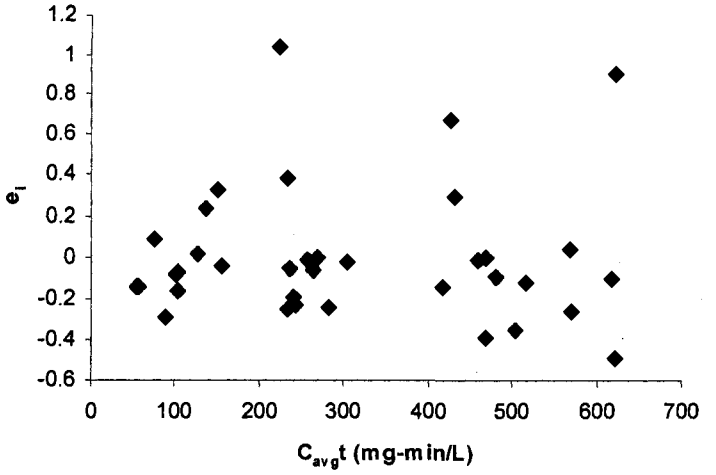


Figure 5.2(B) Linear Model Residual Error (e_i) for Monochloramine $C_{avg}t$ Product

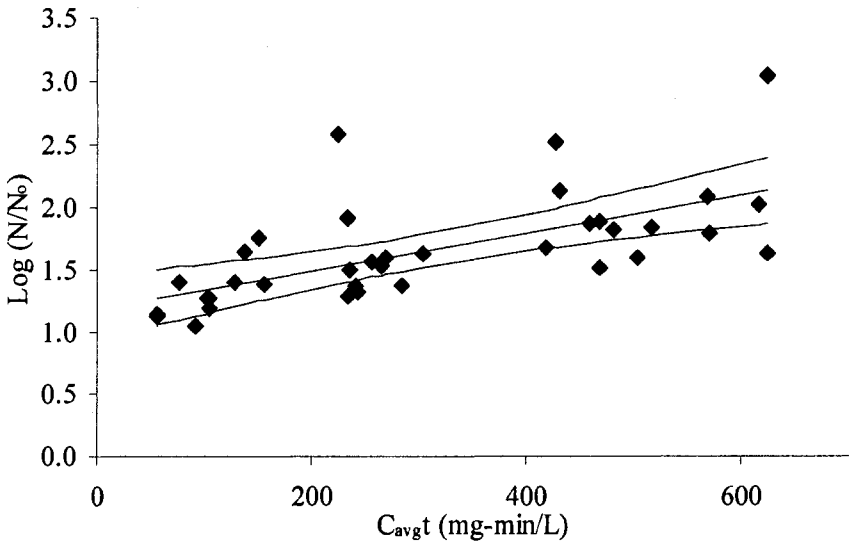


Figure 5.3 Confidence Intervals on Corrected Sequential Ad41 Log Inactivation Using ICC RT-PCR Method and Linear Model Estimates

5.1.2. Fifty Percent Tissue Culture Infectious Dose Analysis ($TCID_{50}$)

From each monochloramine set of trials the samples collected from one reactor flask, one positive control sample, and one negative control sample were randomly selected for $TCID_{50}$ analysis. CPE was not observed in any of the samples. The HEp-2 cell line became overgrown after approximately four days, so CPE that was slow to appear could not be observed. DFA was required to confirm the presence or absence of Ad41 infectious particles. The Ad41 log inactivation [Log (N/N_o)] data as assayed by $TCID_{50}$ and DFA are displayed in Tables 5.3 and 5.4. All of the positive controls were negative for infectious Ad41, which indicated that the virus detected by ICC RT-PCR in the positive control samples was non-infectious.

Table 5.3 Sequential Negative Control Results Using $TCID_{50}$

NH_2Cl Test	Sample #	Log (N_o)
1	3	2.85
2	3	2.68
3	4	3.51

Table 5.4 Sequential Ad41 Log Inactivation Using $TCID_{50}$

Trial Set 1		Trial Set 2		Trial Set 3	
$C_{avg}t$ (mg×min/L)	Log (N/N_o)	$C_{avg}t$ (mg×min/L)	Log (N/N_o)	$C_{avg}t$ (mg×min/L)	Log (N/N_o)
155	0.17	56	1.00	55	1.83
241	1.00	138	1.00	127	1.83
418	0.17	243	1.17	235	1.78
516	1.34	304	1.17	283	1.83
Mean	0.67	-	1.09	-	1.82
Std Dev	0.59	-	0.10	-	0.03
COV (%)	89	-	9	-	1

Notes: COV – coefficient of variation, std dev – standard deviation

Various statistical tests were applied to the monochloramine data to evaluate the reproducibility and precision of the disinfection and ICC RT-PCR methods. The means were compared using a two-sample t-test. The mean log inactivation results of sequential trial sets one and two were not statistically different at the 95% confidence level, and the mean results of trial set three were statistically different from trial sets one and two. The means were compared using a two-sample t-test. Sequential trial sets two and three had much lower standard deviations and COVs than trial set one, which had a larger amount of variation.

The sequential Ad41 inactivation assayed by $TCID_{50}$ did not exhibit the expected trend (Figure 5.4). A linear model was fitted to the $TCID_{50}$ data, but was found to be not significant at the 95% confidence level (p-value = 0.56). The UV and monochloramines $TCID_{50}$ assayed results confirm that the methodology was sound. The random appearance of the data is not attributed to the sequential application of disinfectants. The

HEp-2 cells appeared unhealthy at the end of May and the beginning of June and the poor results may have been due to unhealthy host cells. The results of the negative controls could not be used to evaluate the cell health because only one negative control was assayed by $TCID_{50}$ for each set of trials. No further analysis was performed on the sequential $TCID_{50}$ data.

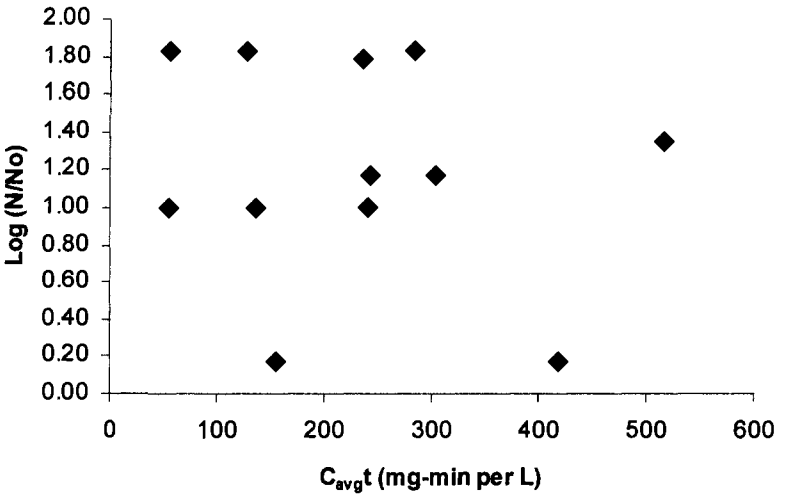


Figure 5.4 Sequential Monochloramine Results Using $TCID_{50}$

5.2 Synergy in Sequential Inactivation Experiments

Analysis of the sequential data for synergy was performed using only the ICC RT-PCR data. The corrected sequential Ad41 inactivation was consistently higher than the monochloramine Ad41 inactivation with some overlap in the data (Figure 5.5).

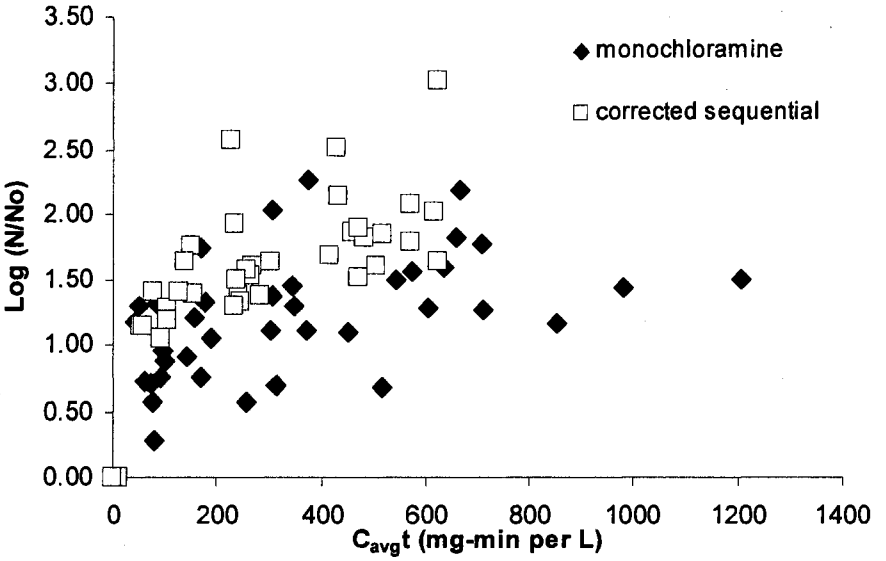


Figure 5.5 Sequential (Corrected) and Monochloramine Ad41 Inactivation

The mean response intervals at the 95% confidence level were calculated on the corrected sequential and monochloramines Ad41 inactivation (Figure 5.6). There is a 95% certainty that the true mean is within the mean response interval. There is only a slight overlap of the mean response intervals at low Ct products indicating that the mean inactivation was higher with sequential disinfection, and increased in difference with increasing Ct products. A t-test was also performed to compare the slopes (inactivation constants) of the corrected sequential and monochloramine only linear models. The inactivation constant of the sequential data was significantly higher than that of the monochloramine data (p-value = 0.001).

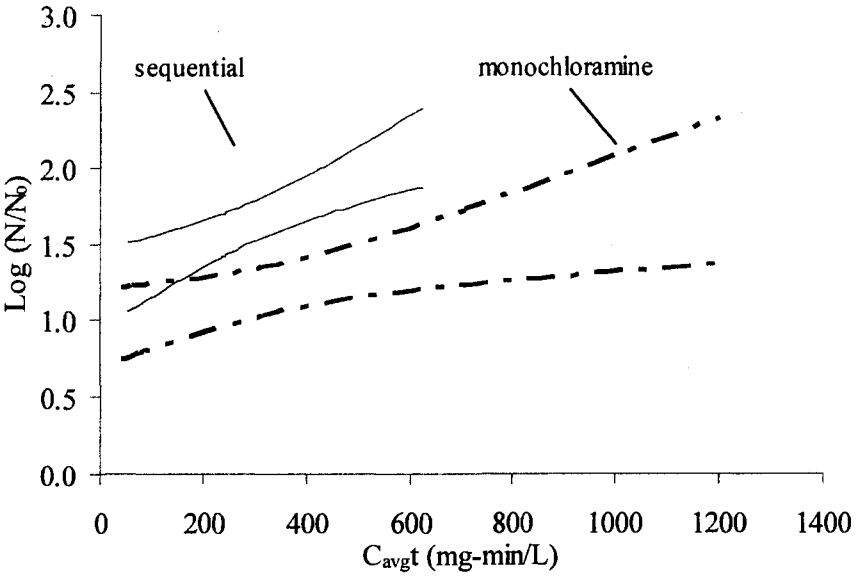


Figure 5.6 Mean Response Intervals on Corrected Sequential and Monochloramine Ad41 Log Inactivation Assayed by ICC RT-PCR

The linear models fitted to the monochloramine and corrected sequential ICC RT-PCR data were used to predict the Ad41 log inactivation for cumulative *Ct* products ranging from 100 to 600 mg×min/L. The *Ct* products selected were within the range of the *Ct* products tested in the laboratory in order to reduce the uncertainty associated with extrapolating data. The difference in log inactivation ranged from 0.3 to 0.7 log, and increased with increasing *Ct* products. Higher monochloramine *Ct* products may cause increased synergistic effects.

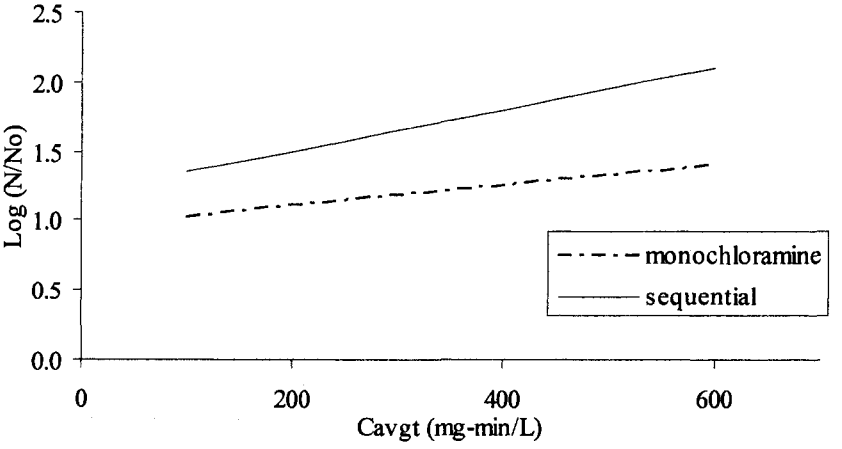


Figure 5.7 Monochloramine and Sequential Linear Model Predictions

5.3 Summary of Sequential Inactivation Experiments

The ICC RT-PCR data was less variable than the $TCID_{50}$ data. A linear model fitted to the ICC RT-PCR data was significant. The $TCID_{50}$ data did not show the expected response to monochloramine exposure; the Ct product did not significantly impact the Ad41 log inactivation. The poor results from the $TCID_{50}$ assay were attributed to unhealthy host cells used in the infectivity assay. The sequential application of UV followed by monochloramine appeared to have a synergistic effect when compared to the inactivation provided by monochloramine alone. The feasibility of using UV and monochloramine to inactivate enteric adenovirus in water treatment can be evaluated using the models fitted to the disinfection results. The Chick-Watson UV model predicts that a 50 mJ/cm^2 UV fluence will achieve 0.90 log inactivation of Ad41. The remaining 3.1 log inactivation would require approximately 16 hours of monochloramine exposure at a dose of 2 mg/L, based on the linear sequential model. A 16 hour contact time is long. The use of sequential UV and monochloramine likely would not be more cost effective than installing a large UV system unless the treatment facility has a sufficient storage capacity.

6.0 Summary of Results

6.1 Inactivation Models

Ad41 was exposed to UV, monochloramine, and sequentially applied UV and monochloramine. Ad41 inactivation was assayed using both ICC RT-PCR and $TCID_{50}$ combined with DFA. The $TCID_{50}$ assay was only applied to select samples due to time and materials constraints. A Chick-Watson model and a linear model with a non-zero intercept were fitted to the UV inactivation data, and the Chick-Watson model was found to have the best fit. A Chick-Watson, Hom, and linear model were fitted to the monochloramine and sequential data, and the linear model was found to have the best fit. The Ad41 log inactivation predicted by the $TCID_{50}$ model was higher than that predicted by the ICC RT-PCR model, but was not significantly different at the 95% confidence level.

Table 6.1 Summary of Ad41 Inactivation Kinetics for UV, Monochloramine, and Sequentially Applied UV and Monochloramine

Disinfectant	**Assay**	**Model**	**p-value**	***k* or k_L****	**Predicted Exposure for Log Inactivation**		
					2	**3**	**4**
UV (mJ/cm^2)	ICC RT-PCR	Chick-Watson	1×10^{-7}	0.041	60	110	170
	$TCID_{50}$		0.014	0.014	40	80	120
Monochloramine (mg×min/L)	ICC RT-PCR	Linear	0.003	0.0007	1400	2800	4100
	$TCID_{50}$		0.006	0.0011	1200	2000	2900
Sequential* (mg×min/L)	ICC RT-PCR	Linear	2.97×10^{-5}	0.0015	500	1200	1900
	$TCID_{50}$		-	-	-	-	-

Notes: '-' indicates data was not calculated, ** - units are the inverse of the disinfectant units and k_L applies to the monochloramines and sequential results, * - sequential includes 42 mJ/cm^2 UV fluence preceding monochloramine exposure

6.2 Comparison to Previous Studies

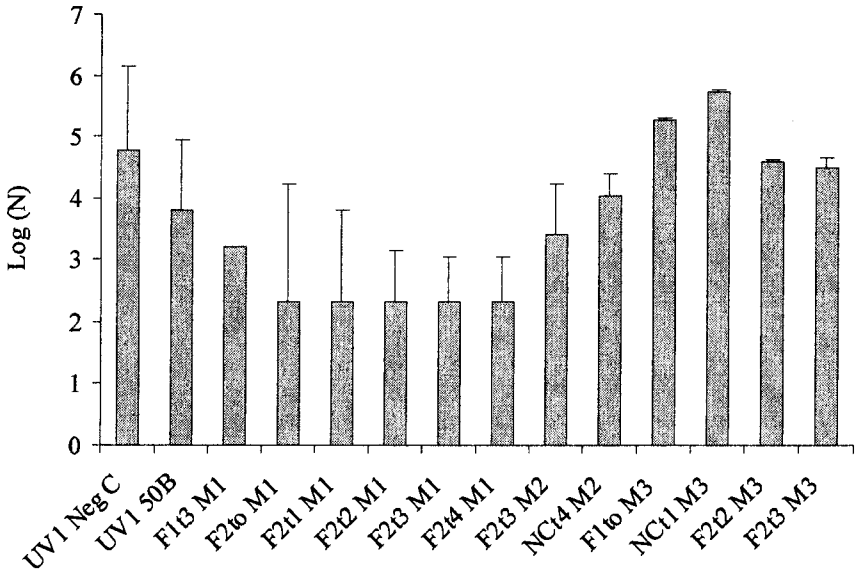
The results of five previous studies that evaluated the effect of UV on Ad41 and Ad40 were discussed in Chapter One. The UV fluence required for 4 log inactivation of Ad41 as assayed by ICC RT-PCR in the present study was 220 mJ/cm^2, which agrees with the observations of Ko, Cromeans and Sobsey (2005). The results are also similar to the observations of Jacangelo *et al.* (2002) for Ad40; however Meng and Gerba (1996) found that Ad40 is more UV resistant than Ad41. The 160 mJ/cm^2 UV fluence required for 4 log inactivation as assayed by $TCID_{50}$ was in the middle of the range observed by previous studies. The study by Ko, Cromeans and Sobsey (2005) was the only one to use an ICC RT-PCR assay; however conventional PCR was used whereas real-time PCR was used in the present study.

Few studies using monochloramine for inactivation of enteric adenovirus are available for comparison. Baxter *et al.* (2007) found that only 300 mg×min/L was required to achieve 2.5 log inactivation of Ad5 and Ad41 at a pH of 8 and a temperature of 5°C. Jacangelo *et al.* (2003) found that a Ct of greater than 360 mg×min/L was required for 2 log inactivation of Ad40 at a pH of 7 and temperature of 5 to 6 °C. In the present study, the Ct required for 2, 3, and 4 log inactivation was 1400, 2800, and 4100 mg×min/L, respectively with a pH of 7 to 8 and a temperature of 11 °C. The required Ct product observed in this study was higher than those observed in previously published studies. A possible explanation is that the virus stock used in previous studies was purified, but the virus stock was not purified in the present study. The presence of cell debris and insoluble proteins remaining in the virus stock likely exerted an oxidant demand that consumed a portion of the monochloramines added to the reactors.

No studies regarding the synergistic effect of UV and monochloramines on enteric adenovirus have been published. Ballester and Malley (2004) applied UV and monochloramine sequentially to Ad2 and found that the effect was greater than the expected additive effect, but a full analysis for synergy was not presented. In contrast, Baxter et al. (2007) found that there was no synergistic effect when UV and monochloramine were applied sequentially to Ad2. In this study synergistic effects were observed. The sequential monochloramine mean response was higher than the monochloramine only response.

6.3 ICC RT-PCR Method Variability

Experiments were conducted to evaluate the variability associated with the ICC RT-PCR method. The variability observed in the UV, monochloramine, and sequential monochloramine trials could then be compared to the viral assay variability. Fourteen samples, including samples exposed to UV or monochloramines and non-exposed controls, were assayed in duplicate with the ICC RT-PCR method. No sequential monochloramines samples were analyzed in duplicate. The mean and standard deviation of the sample replicates was calculated (Figure 6.1). The pooled standard deviation of the replicates was 0.98. The standard deviation observed within the UV, monochloramines, and sequential monochloramines disinfection experiments ranged from 0.19 to 0.52. The error observed in the experiments can therefore be largely attributed to inherent variability in the ICC RT-PCR assay.



Notes: Each bar represents the mean of duplicate samples and the error bar represents one standard deviation; M# indicates the monochloramine trial set number, UV# indicates the UV trial set number, F#t# indicates the flask and time, and NC or Neg indicates a negative control

Figure 6.1 ICC RT-PCR Method Variability Replicates

7.0 Conclusion and Recommendations

7.1 Conclusions

The purpose of this project was to compare the $TCID_{50}$ and ICC RT-PCR viral assay methods and to evaluate the ultraviolet and monochloramine inactivation kinetics of the enteric virus Adenovirus 41. Real time, quantitative PCR was used as opposed to the more common endpoint detection PCR. The following conclusions were drawn:

1. The UV inactivation kinetics were best described by the Chick-Watson model. The monochloramine inactivation kinetics were described well by both a Hom and a linear model. The sequential inactivation kinetics were best described by a linear model.
2. A UV fluence of 220 mJ/cm^2 was required to achieve four log inactivation of Ad41 based on the ICC RT-PCR results. The results were similar to one previous study that also used the integrated cell culture and molecular methods. The previous study used conventional, end-point PCR as opposed to the use of real time PCR in the present study. Other published studies observed a higher Ad41 inactivation using CPE based assay methods. A major difference between the current and previously published studies is the use of purification and a higher titre virus stock in the previous studies. The UV fluence required by the USEPA for four log inactivation of viruses based on enteric adenovirus is 186 mJ/cm^2.
3. A monochloramine *Ct* product of 4100 mg×min/L was required to achieve four log inactivation of Ad41 based on the ICC RT-PCR results. The Ad41 inactivation was lower than in published studies, though only one study with Ad41 has been published to date.
4. A synergistic mean response was observed when monochloramine was applied after UV exposure. No published studies have evaluated the potential for synergistic effects between UV and monochloramines on enteric adenovirus.
5. The UV Ad41 log inactivation predicted by the $TCID_{50}$ model was significantly higher (p-value = 0.02) than that predicted by the ICC RT-PCR model using a t-test for comparison of two slopes. The monochloramine Ad41 log inactivation predicted

by the $TCID_{50}$ model was the same as that predicted by the ICC RT-PCR model using a t-test for comparison of two slopes. The ICC RT-PCR method required a 5 day incubation while the $TCID_{50}$ method required a 10 – 14 day incubation. The capital cost of the ICC RT-PCR method is high, but the materials and labour costs are lower than the $TCID_{50}$ assay. The $TCID_{50}$ assay requires a number of serial dilutions for each sample and at least four replicate cell infections for CPE observation. The $TCID_{50}$ method is based on the subjective observation of CPE, while ICC RT-PCR does not. The ICC RT-PCR assay provided a faster, cost-effective method for determining the viral titre of samples.

6. The ICC RT-PCR method included the use of real time, quantitative PCR. Previous disinfection studies used conventional endpoint detection PCR. Real time PCR is more precise and has a higher sample throughput.

7.2 Recommendations

A number of changes are recommended to improve the results of future research. The use of a cell line that shows CPE quickly would remove the need for DFA so that more samples could be analyzed for method comparison. Appearance of CPE would also allow a visual confirmation before RT-PCR. A virus stock with a higher titre should be used in the disinfection experiments to decrease the variability of the viral assay inactivation results. The non-viable virus used for positive controls was inactivated with heat. A more appropriate method of inactivation would be high fluences of UV that would destroy inactive RNA that was detected by PCR. The observed monochloramine demand could have been reduced by purifying the virus stock prior to disinfection. A lower monochloramine demand could reduce the inactivation variability. The impact of different cell culture periods on the ICC RT-PCR assay was not evaluated and may have an important effect on the results. The inactivation at a variety of temperature and pH conditions must be evaluated in order to determine the complete monochloramine inactivation kinetics.

There are many knowledge gaps in the inactivation kinetics of Ad41. Few studies have been published and the results are conflicting. In particular, few studies have examined the inactivation of enteric viruses by monochloramine or sequential UV and

monochloramine. Each study used different methods for virus propagation and assay. Standard methods for the propagation, purification, and assay of enteric adenoviruses should be developed so that studies can be compared directly. The ICC RT-PCR assay is precise and fast, and it is not material or labour intensive. PCR is sensitive to small changes in RNA, and CPE is not. The ICC RT-PCR method provides a result in 7 days, which is half the amount of time required for a $TCID_{50}$ result. The amount of time, materials, and labour required by the ICC RT-PCR method does not increase substantially as the number of samples increases due to the automated RT-PCR system. Each additional sample substantially increases the materials and labour required for the $TCID_{50}$ assay.

Inactivation of enteric adenoviruses using only UV would require large UV systems compared to what is required for inactivation of other microorganisms. It was hypothesized that a combination of UV and monochloramines could provide sufficient enteric adenovirus inactivation. The Chick-Watson UV model developed in the present study predicted that a 50 mJ/cm^2 UV fluence would achieve 0.90 log inactivation of Ad41. The remaining 3.1 log inactivation would require approximately sixteen hours of monochloramines exposure at a dose of 2 mg/L, based on the linear sequential model. A sixteen hour contact time is long, and may not be cost effective unless the treatment facility has sufficient reservoir capacity. Free chlorine is a stronger disinfectant than monochloramines, but DPBs are formed when chlorine is used in the presence of organic compounds. The use of free chlorine to provide additional enteric adenovirus inactivation is likely the most efficient method of adenovirus inactivation for the majority of water treatment facilities.

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APPENDICES

A. Raw Results of Viral Analyses and Sample Calculations

The following three figures display a sample of the PCR output obtained from the ABI Prism 7500 with a real time Taqman probe. The CP corresponding to each control, viral sample or standard dilution is shown on Figure A.1. The intersection of each amplification plot and the horizontal line on Figure A.2 indicates the crossing point (CP) for the corresponding sample. The Ad2 standard curve created was used to quantify the Ad41 CP results (Figure A.3).

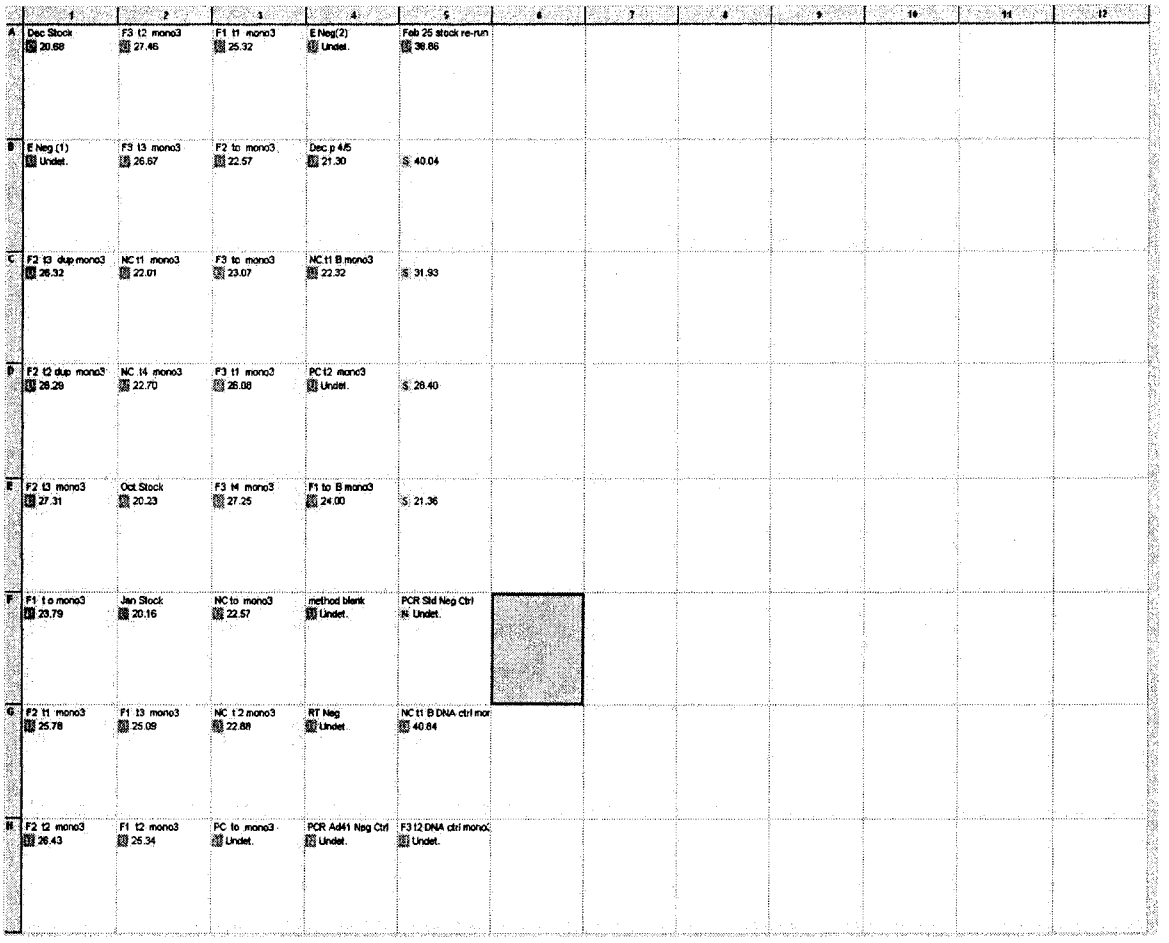


Figure A.1 RT-PCR Crossing Point (*CP*) Results

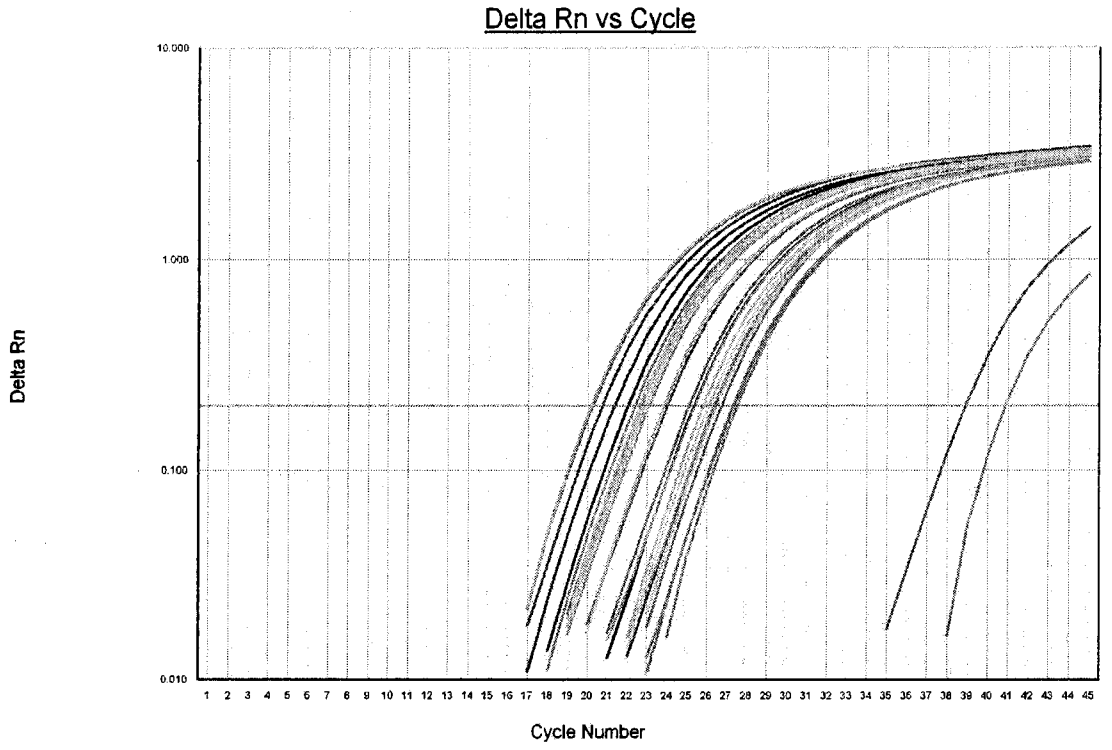
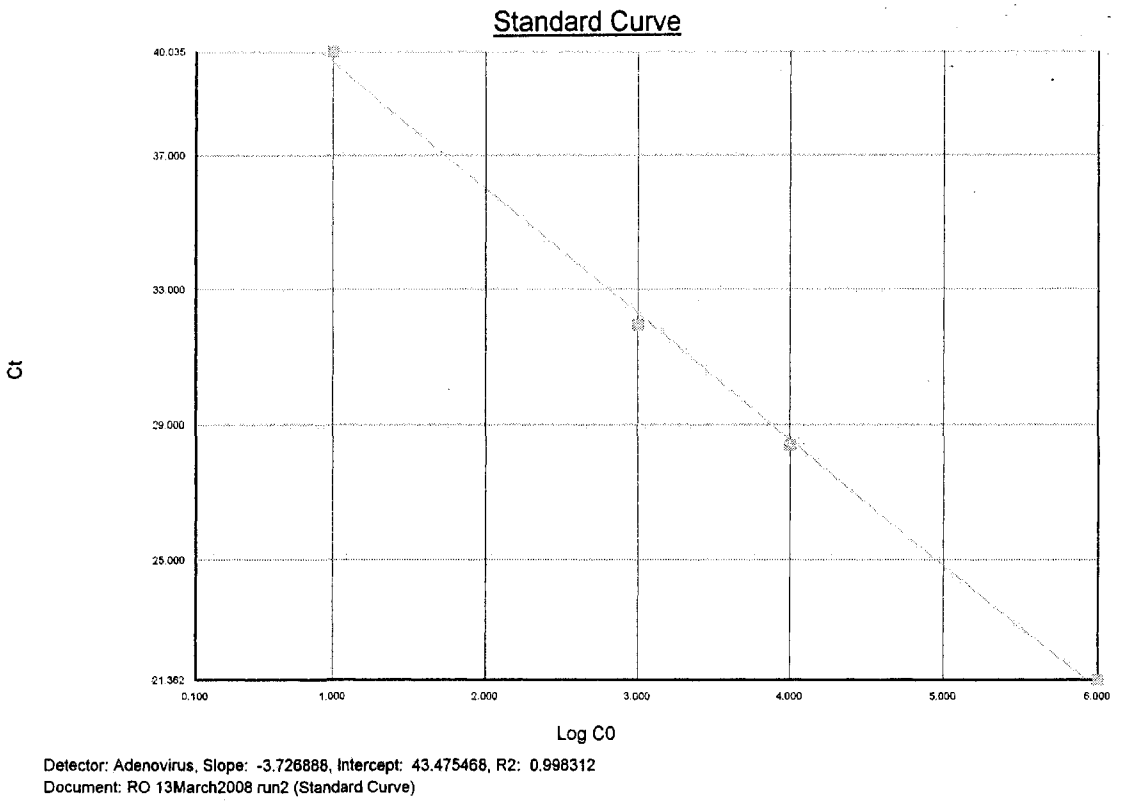


Figure A.2 RT-PCR Amplification Plot of Increased Fluorescence versus PCR Cycle Number with Crossing Point (*CP*) Line



Notes: The y-axis label (Ct) is equivalent to the crossing point (*CP*), the x-axis label (Log C0) indicates the RNA copies (Log N_o)

Figure A.3 Example Results of Adenovirus 2 Standard Curve for Quantification of Adenovirus 41 Results

Conversion of PCR Units

The PCR results were expressed in copies per PCR reaction throughout the text. The results can be converted to copies per mL by accounting for the concentration and dilution that occurs during the ICC RT-PCR assay (Table A.1). A 200 µL aliquot of harvested virus is used for assay of the disinfection sample viral titre.

Table A.1 PCR Conversion Calculations

	Cell harvest	Nucleic Acid Extract	DNA digest	RT	PCR Reaction
Volume (μL)	200	100	50 in 555	5 in 20	5 in 25
Dilution or Concentration Factor	1	concentrated 2X	Diluted by 11.1	Diluted by 4	5 μL of sample added

$$\text{ICC RT-PCR Cnversion Factor} = \left(\frac{1}{2}\right)x(11.1)x(4)x\left(\frac{1}{5}\right)x\left(\frac{1000\mu L}{mL}\right) = 4{,}440$$

The RNA copies per PCR reaction can be converted to RNA copies per mL by multiplying by the conversion factor as follows:

$$\left(\frac{10^6 copies}{PCR}\right)x4{,}440 = 4.44x10^9\,\frac{copies}{mL}$$

Table A.2 ICC RT-PCR Raw Results and Computed Log RNA Copies

Trial Set	Sample Name	CP	Standard Curve		log RNA Copies
			Slope	Intercept	
UV Trial Set 1 ICC RT-PCR	27A	25.84	-3.14	42.032	5.16
	27B	28.08	-3.14	42.032	4.44
	27C	27.17	-3.14	42.032	4.73
	53A	27.99	-3.14	42.032	4.47
	53B	27.56	-3.14	42.032	4.61
	53C	30.10	-3.14	42.032	3.80
	72A	30.67	-3.14	42.032	3.62
	72B	30.12	-3.14	42.032	3.79
	72C	28.21	-3.14	42.032	4.40
	96A	29.06	-3.14	42.032	4.13
	94B	31.34	-3.14	42.032	3.41
	94C	29.11	-3.14	42.032	4.12
	posA	ND	-3.14	42.032	ND
	posB	ND	-3.14	42.032	ND
	posC	ND	-3.14	42.032	ND
	negA	23.89	-3.14	42.032	5.78
	negB	26.13	-3.14	42.032	5.06
	negC	24.01	-3.14	42.032	5.74
UV Trial Set 1 Direct	27A	29.32	-3.14	42.032	4.05
	27B	27.91	-3.14	42.032	4.50
	27C	28.19	-3.14	42.032	4.41
	53A	27.23	-3.14	42.032	4.71
	53B	28.59	-3.14	42.032	4.28
	53C	28.40	-3.14	42.032	4.34
	72A	28.64	-3.14	42.032	4.26
	72B	29.08	-3.14	42.032	4.12
	72C	ND	-3.14	42.032	ND
	96A	28.68	-3.14	42.032	4.25
	94B	28.99	-3.14	42.032	4.15
	94C	ND	-3.14	42.032	ND
	posA	ND	-3.14	42.032	ND
	posB	ND	-3.14	42.032	ND
	posC	ND	-3.14	42.032	ND
	negA	28.02	-3.14	42.032	4.46
	negB	27.62	-3.14	42.032	4.59
	negC	28.22	-3.14	42.032	4.40
UV Trial Set 2 ICC RT-PCR	25A	27.95	-3.14	42.032	4.48
	25B	27.05	-3.14	42.032	4.77
	25C	25.95	-3.14	42.032	5.12
	50A	29.05	-3.14	42.032	4.13
	50B	29.17	-3.14	42.032	4.10
	50C	29.09	-3.14	42.032	4.12
	75A	ND	-3.14	42.032	ND

Trial Set	Sample Name	CP	Standard Curve		log RNA Copies
			Slope	Intercept	
	75B	29.57	-3.14	42.032	3.97
	75C	30.25	-3.14	42.032	3.75
	100A	31.33	-3.14	42.032	3.41
	100B	30.25	-3.14	42.032	3.75
	100C	34.21	-3.14	42.032	2.49
	posA	ND	-3.14	42.032	ND
	posB	ND	-3.14	42.032	ND
	posC	ND	-3.14	42.032	ND
	negA	26.51	-3.14	42.032	4.94
	negB	26.19	-3.14	42.032	5.05
	negC	26.19	-3.14	42.032	5.05
UV Trial Set 3 ICC RT-PCR	25A	27.08	-3.14	42.032	4.76
	25B	26.76	-3.14	42.032	4.86
	25C	ND	-3.14	42.032	ND
	50A	27.56	-3.14	42.032	4.61
	50B	27.91	-3.14	42.032	4.50
	50C	28.64	-3.14	42.032	4.26
	75A	31.05	-3.14	42.032	3.50
	75B	30.79	-3.14	42.032	3.58
	75C	30.34	-3.14	42.032	3.72
	100A	30.07	-3.14	42.032	3.81
	100B	ND	-3.14	42.032	ND
	100C	31.31	-3.14	42.032	3.41
	posA	ND	-3.14	42.032	ND
	posB	ND	-3.14	42.032	ND
	posC	ND	-3.14	42.032	ND
	negA	ND	-3.14	42.032	ND
	negB	26.4	-3.14	42.032	4.98
	negC	ND	-3.14	42.032	ND
Monochloramine Trial Set 1 ICC RT-PCR	flask1 to	27.09	-3.56	42.43	4.31
	flask1 t1	30.08	-3.56	42.43	3.47
	flask1 t2	30.16	-3.56	42.43	3.45
	flask1 t3	31.07	-3.56	42.43	3.19
	flask1 t4	30.44	-3.56	42.43	3.37
	flask2 to	30.31	-3.68	43.79	3.66
	flask2 t1	31.45	-3.68	43.79	3.35
	flask2 t2	33.11	-3.68	43.79	2.90
	flask2 t3	35.02	-3.68	43.79	2.38
	flask2 t4	33.4	-3.68	43.79	2.82
	flask3 to	26.04	-3.56	42.43	4.60
	flask3 t1	28.45	-3.56	42.43	3.93
	flask3 t2	29.10	-3.56	42.43	3.74
	flask3 t3	33.11	-3.56	42.43	2.62
	flask3 t4	31.23	-3.56	42.43	3.15
	neg ctrl to	25.63	-3.56	42.43	4.72
	neg ctrl t1	25.59	-3.56	42.43	4.73
	neg ctrl t2	25.77	-3.56	42.43	4.68

Trial Set	Sample Name	CP	Standard Curve		log RNA Copies
			Slope	Intercept	
	neg ctrl t3	26.13	-3.56	42.43	4.58
	neg ctrl t4	26.47	-3.56	42.43	4.48
	pos ctrl to	33.15	-3.56	42.43	2.61
	pos ctrl t1	36.17	-3.56	42.43	1.76
	pos ctrl t2	35.38	-3.56	42.43	1.98
	pos ctrl t3	32.81	-3.56	42.43	2.70
Monochloramine Trial Set 2 ICC RT-PCR	flask1 to	27.39	-3.61	43.74	4.53
	flask1 t1	27	-3.61	43.74	4.64
	flask1 t2	26.47	-3.61	43.74	4.78
	flask1 t3	26.89	-3.61	43.74	4.67
	flask2 to	25.09	-3.61	43.74	5.17
	flask2 t1	25.13	-3.68	43.79	5.07
	flask2 t2	29.37	-3.61	43.74	3.98
	flask2 t3	30.06	-3.61	43.74	3.79
	flask3 to	26.14	-3.61	43.74	4.88
	flask3 t1	29.17	-3.61	43.74	4.04
	flask3 t2	28.41	-3.61	43.74	4.25
	flask3 t3	28.39	-3.61	43.74	4.25
	flask3 t4	29.59	-3.61	43.74	3.92
	flask3 t5	29.81	-3.61	43.74	3.86
	flask4 to	28.18	-3.61	43.74	4.31
	flask4 t1	26.5	-3.61	43.74	4.78
	flask4 t2	32.33	-3.61	43.74	3.16
	flask4 t3	28.63	-3.61	43.74	4.19
	neg ctrl to	22.55	-3.61	43.74	5.87
	neg ctrl t1	24.27	-3.61	43.74	5.39
	neg ctrl t2	27.57	-3.61	43.74	4.48
	neg ctrl t3	26.28	-3.61	43.74	4.84
	neg ctrl t4	27.54	-3.61	43.74	4.49
	pos ctrl to	ND	-3.61	43.74	ND
	pos ctrl t1	ND	-3.61	43.74	ND
	pos ctrl t2	ND	-3.61	43.74	ND
	pos ctrl t3	38.63	-3.61	43.74	1.42
	pos ctrl t4	ND	-3.61	43.74	ND
Monochloramine Trial Set 3 ICC RT-PCR	flask1 to	23.79	-3.73	43.48	5.28
	flask1 t1	25.32	-3.73	43.48	4.87
	flask1 t2	25.34	-3.73	43.48	4.86
	flask1 t3	25.09	-3.73	43.48	4.93
	flask1 t5	28.93	-3.73	43.48	3.90
	flask2 to	22.57	-3.73	43.48	5.61
	flask2 t1	25.78	-3.73	43.48	4.75
	flask2 t2	26.43	-3.73	43.48	4.57
	flask2 t3	27.31	-3.73	43.48	4.34
	flask2 t4	29.63	-3.73	43.48	3.71
	flask3 to	23.07	-3.73	43.48	5.47
	flask3 t1	26.08	-3.73	43.48	4.66
	flask3 t2	27.46	-3.73	43.48	4.29

Trial Set	Sample Name	CP	Standard Curve		log RNA Copies
			Slope	Intercept	
	flask3 t3	26.67	-3.73	43.48	4.51
	flask3 t4	27.25	-3.73	43.48	4.35
	neg ctrl t1	22.57	-3.73	43.48	5.61
	neg ctrl t2	22.01	-3.73	43.48	5.76
	neg ctrl t3	22.88	-3.73	43.48	5.52
	neg ctrl t4	22.7	-3.73	43.48	5.57
	pos ctrl to	ND	-3.73	43.48	ND
	pos ctrl t1	ND	-3.73	43.48	ND
Sequential Trial Set 1 ICC RT-PCR	flask1 to	28.99	-3.44	42.29	3.87
	flask1 t1	30.89	-3.44	42.29	3.32
	flask1 t2	31.82	-3.44	42.29	3.04
	flask1 t3	31.55	-3.44	42.29	3.12
	flask2 to	30.16	-3.44	42.29	3.53
	flask2 t1	30.11	-3.44	42.29	3.54
	flask2 t2	31.16	-3.44	42.29	3.24
	flask2 t3	31.75	-3.44	42.29	3.07
	flask3 to	29.76	-3.44	42.29	3.64
	flask3 t1	30.67	-3.44	42.29	3.38
	flask3 t2	31.68	-3.44	42.29	3.09
	flask3 t3	32.33	-3.44	42.29	2.90
	neg ctrl to	23.72	-3.44	42.29	5.40
	neg ctrl t1	23.98	-3.44	42.29	5.33
	neg ctrl t2	24.59	-3.44	42.29	5.15
	neg ctrl t3	24.1	-3.44	42.29	5.29
	pos ctrl to	38.07	-3.44	42.29	1.23
	pos ctrl t1	35.29	-3.44	42.29	2.04
	pos ctrl t2	ND	-3.44	42.29	ND
	pos ctrl t3	ND	-3.44	42.29	ND
Sequential Trial Set 2 ICC RT-PCR	flask1 to	31.85	-3.41	42.59	3.15
	flask1 t1	35.85	-3.41	42.59	1.98
	flask1 t2	35.63	-3.41	42.59	2.04
	flask1 t3	32.53	-3.41	42.59	2.95
	flask2 to	33.04	-3.41	42.59	2.80
	flask2 t1	32.41	-3.41	42.59	2.99
	flask2 t2	34.35	-3.41	42.59	2.42
	flask2 t3	34.17	-3.41	42.59	2.47
	flask3 to	31.42	-3.41	42.59	3.28
	flask3 t1	33.64	-3.41	42.59	2.63
	flask3 t2	33.52	-3.41	42.59	2.66
	flask3 t3	37.38	-3.41	42.59	1.53
	neg ctrl to	26.07	-3.41	42.59	4.85
	neg ctrl t1	26.60	-3.41	42.59	4.69
	neg ctrl t2	26.59	-3.41	42.59	4.70
	neg ctrl t3	24.14	-3.41	42.59	5.42
	pos ctrl to	ND	-3.41	42.59	ND
	pos ctrl t1	ND	-3.41	42.59	ND
	pos ctrl t2	ND	-3.41	42.59	ND

Trial Set	Sample Name	CP	Standard Curve		log RNA Copies
			Slope	Intercept	
Sequential Trial Set 3 ICC RT-PCR	flask1 to	30.3	-3.51	42.57	3.50
	flask1 t1	31.23	-3.51	42.57	3.23
	flask1 t2	31.55	-3.51	42.57	3.14
	flask1 t3	31.13	-3.51	42.57	3.26
	flask2 to	30.28	-3.51	42.57	3.50
	flask2 t1	32.05	-3.51	42.57	3.00
	flask2 t2	30.94	-3.51	42.57	3.32
	flask2 t3	32.01	-3.51	42.57	3.01
	flask3 to	30.47	-3.51	42.57	3.45
	flask3 t1	30.83	-3.51	42.57	3.35
	flask3 t2	31.59	-3.51	42.57	3.13
	flask3 t3	32.02	-3.51	42.57	3.01
	neg ctrl to	25.9	-3.51	42.57	4.75
	neg ctrl t1	26.36	-3.51	42.57	4.62
	neg ctrl t2	25.23	-3.51	42.57	4.94
	neg ctrl t3	25.86	-3.51	42.57	4.76
	pos ctrl to	ND	-3.51	42.57	ND
	pos ctrl t1	ND	-3.51	42.57	ND
	pos ctrl t2	ND	-3.51	42.57	ND

Notes: ND – not detected, neg ctrl - negative control, pos ctrl – positive control, t'#' - indicates sample time, log RNA copies are per PCR reaction

PCR Sample Calculation (Sequential Trial Set 3, Flask 1, Sample t2)

Log RNA copies = {(CP – intercept)/ slope} = {(31.55 – 42.57)/-3.51} = 3.14

Log (N/N_0) = Log (exposed sample RNA copies/negative control averaged RNA copies)

$= \text{Log}\{(10^{3.14})/(10^{4.77})\}$

$= -1.63$

Table A.3 DFA Raw Data for Disinfectant Exposed Samples

Trial Set	Sample Name	Dilution	Well				Log (IU/mL)
			A	B	C	D	
UV Trial Set 1 $TCID_{50}$	25A	10^0	+	+	+	+	1.32
		10^-1	+	+	+	+	
		10^-2	+	-	+	-	
		10^-3	-	-	-	-	
	50B	10^0	+	+	+	+	1.37
		10^-1	+	+	-	+	
		10^-2	+	-	-	-	
	75C	10^0	+	+	+	+	0.11
		10^-1	-	+	-	+	
		10^-2	+	-	-	-	
		10^-3	-	-	-	-	
	100C	10^0	+	-	-	-	-0.18
		10^-1	-	-	-	-	
		10^-2	-	-	-	-	
	neg ctrl C	10^-2	+	+	+	+	2.49
		10^-3	-	+	-	-	
		10^-4	+	-	-	-	
UV Trial Set 2 $TCID_{50}$	25B	10^-1	+	+	+	-	1.32
		10^-2	-	-	-	-	
		10^-3	-	-	-	-	
	50B	10^0	+	+	+	-	0.37
		10^-1	-	-	+	-	
		10^-2	-	-	-	-	
	75B	10^0	-	+	+	+	0.37
		10^-1	-	+	-	+	
		10^-2	-	-	-	-	
	100C	10^0	+	+	+	+	0.32
		10^-1	-	-	-	-	
		10^-2	-	-	-	-	
	neg ctrl C	10^-1	+	+	+	+	1.49
		10^-2	+	-	-	-	
UV Trial Set 3 $TCID_{50}$	25B	10^-1	+	+	+	-	1.57
		10^-2	-	+	-	-	
		10^-3	-	-	-	-	
	50B	10^0	+	+	+	-	0.32
		10^-1	+	-	+	-	
		10^-2	-	-	-	-	
	75B	10^0	-	+	-	-	-1.18
		10^-1	-	-	-	-	
		10^-2	-	-	-	-	
	100C	10^0	+	+	-	-	0.11
		10^-1	-	+	-	-	
		10^-2	-	-	-	-	
	neg ctrl C	10^-2	-	+	+	+	2.16
		10^-3	-	-	-	-	

Trial Set	Sample Name	Dilution	Well				Log (IU/mL)
			A	B	C	D	
Trial Set 1		10^-3	-	-	-	-	
$TCID_{50}$	flask2 t2	10^-1	+	+	+	+	1.32
		10^-2	-	-	-	-	
	flask2 t3	10^-1	-	+	+	+	1.37
		10^-2	+	-	-	-	
	flask2 t4	10^-1	+	+	+	-	1.15
		10^-2	-	-	-	-	
	neg ctrl t3	10^-2	+	+	+	+	2.48
		10^-3	-	+	-	-	
Monochloramine	flask3 t1	10^-2	+	-	+	+	2.15
Trial Set 2		10^-3	-	-	-	-	
$TCID_{50}$	flask3 t2	10^-2	+	+	-	+	2.15
		10^-3	-	-	-	-	
	flask3 t3	10^-1	+	+	+	+	1.49
		10^-2	-	+	-	-	
	flask3 t4	10^-1	+	+	+	+	1.32
		10^-2	-	-	-	-	
	flask3 t4	10^-1	+	+	+	+	1.49
		10^-2	-	-	-	-	
	neg ctrl t4	10^-3	-	+	+	+	3.37
		10^-4	+	-	-	-	
Monochloramine	flask2 t1	10^-2	+	+	+	+	2.32
Trial Set 3		10^-3	-	-	-	-	
$TCID_{50}$	flask2 t2	10^-1	+	+	+	+	1.32
		10^-2	-	-	+	+	
		10^-3	-	-	-	-	
	flask2 t3	10^-1	+	+	+	+	1.32
		10^-2	+	-	+	-	
		10^-3	-	-	-	-	
	flask2 t4	10^-1	+	+	+	+	1.32
		10^-2	-	-	+	+	
		10^-3	-	-	-	-	
	neg ctrl t4	10^-2	+	+	+	+	2.32
		10^-3	+	-	+	-	
		10^-4	-	-	-	-	
Sequential	flask2 t1	10^-2	+	+	+	+	2.32
Trial Set 1		10^-3	-	-	-	-	
$TCID_{50}$	flask2 t2	10^-1	+	+	+	+	1.49
		10^-2	-	+	-	-	
	flask2 t3	10^-2	+	+	+	+	2.32
		10^-3	-	+	-	-	
	flask2 t4	10^-1	+	+	+	-	1.15
		10^-2	-	-	-	-	
	neg ctrl t1	10^-2	+	+	+	+	2.49
		10^-3	+	+	-	-	
		10^-4	-	-	-	-	

Trial Set	Sample Name	Dilution	Well				Log (IU/mL)
			A	B	C	D	
Sequential Trial Set 2 $TCID_{50}$	flask2 t1	10^-1	+	-	-	+	1.32
		10^-2	-	-	-	-	
	flask2 t2	10^-1	-	+	+	-	1.32
		10^-2	-	-	-	-	
	flask2 t3	10^-1	+	+	-	+	1.15
		10^-2	-	-	-	-	
	flask2 t4	10^-1	+	+	-	+	1.15
		10^-2	-	-	-	-	
	neg ctrl t3	10^-2	+	+	+	+	2.32
		10^-3	-	-	-	-	
Sequential Trial Set 3 $TCID_{50}$	flask1 t1	10^-1	+	+	+	+	1.32
		10^-2	+	-	+	-	
	flask1 t2	10^-1	+	+	+	+	1.32
		10^-2	+	-	-	+	
	flask1 t3	10^-1	+	+	+	-	1.37
		10^-2	-	-	-	+	
	flask1 t4	10^-1	+	+	+	+	1.32
		10^-2	-	+	-	+	
	neg ctrl t3	10^-2	+	+	+	+	3.15
		10^-3	+	-	+	+	

Notes: + indicates positive for DFA, - indicates negative for antibodies, neg ctrl – negative control, positive controls were all negative, t'#' - indicates sample time

Reed-Muench Sample Calculation (UV Trial Set 1, Sample 25A)

1. Tabulate results of DFA test.

Sample	A	B	C	D (%)	E	F	G (%)
25A	10^-1	4	0	100	6	0	100
	10^-2	2	2	50	2	2	50
	10^-3	0	4	0	0	6	0

The columns are defined as follows:

A: dilution factor;

B: total wells showing CPE;

C: total wells not showing CPE;

D: percent of wells showing CPE {B/(B+C)};

E: sum of column B from the bottom up (cumulative number showing CPE);

F: sum of column C from the top down (cumulative number not showing CPE; and
G: cumulative percent of wells showing CPE {(E/E+F)}.

2. Calculate proportionate distance (PD)

$$PD = \frac{(G_{>50\%}) - 50\%}{(G_{>50\%}) - (D_{<50\%})} = \frac{(100\%) - 50\%}{(100) - (0)} = 0.5 \qquad \text{Eqn. A.1}$$

where >50 indicates next above 50 and < 50 indicates next below 50.

3. Correct PD for dilution factor (DF)

$$PDxDF = 0.5x(-1) = -0.5 \qquad \text{Eqn. A.2}$$

4. Calculate 50% endpoint dilution factor by adding corrected dilution factor to negative log of dilution next above 50% exhibiting DFA.

$$TCID_{50} = 10\text{^}(\log(\text{dilution above } 50\%) - PD) = 10\text{^}\{\log(-1) - 0.5)\} = 10^{-0.5} \qquad \text{Eqn. A.3}$$

5. Calculate the infectious units per millilitre (IU/mL).

$$\frac{1}{V_{well}} x \frac{1}{TCID_{50}} = \frac{1}{2.5} x \frac{1}{10^{-0.5}} = 2.11x10^{1} \frac{IU}{mL} \qquad \text{Eqn. A.4}$$

B. ANOVA Calculations

The following tables display the calculations made in fitting models to the results of the disinfection experiments. ANOVA calculations using Microsoft Excel Version 3.0 Solver and the Microsoft Excel Version 3.0 linear regression tool were used.

Table B.1 Chick-Watson Model ANOVA of UV ICC RT-PCR Results

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.764481
R Square	0.584432
Adjusted R Square	0.553182
Standard Error	0.898273
Observations	33

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	36.31269	36.31269	45.00299	1.66E-07
Residual	32	25.82064	0.806895		
Total	33	62.13333			

Model	*Coefficients*	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0					
X Variable 1	0.04128	0.002325	17.75702	3.95E-18	0.036545	0.046016

Table B.2 Chick-Watson Model ANOVA of UV $TCID_{50}$ Results

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.593654
R Square	0.352425
Adjusted R Square	0.261516
Standard Error	1.688859
Observations	12

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	17.07481	17.07481	5.986451	0.03445
Residual	11	31.37467	2.852243		
Total	12	48.44949			

Model	*Coefficients*	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0					
X Variable 1	0.055752	0.007185	7.759277	8.72E-06	0.039937	0.071566

Table B.3 Linear Regression of Monochloramine ICC RT-PCR Results

SUMMARY OUTPUT	
Regression Statistics	
Multiple R	0.46
R Square	0.22
Adjusted R Square	0.19
Standard Error	0.41
Observations	38

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1.68	1.68	9.92	3.28E-03
Residual	36	6.10	0.17		
Total	37	7.79			

Model	*Coefficients*	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.95	0.11	8.80	0.00	0.73	1.17
X Variable 1	7.40E-04	2.35E-04	3.15E+00	3.28E-03	2.64E-04	1.22E-03

Table B.4 Linear Regression of Monochloramine $TCID_{50}$ Results

SUMMARY OUTPUT	
Regression Statistics	
Multiple R	0.72
R Square	0.51
Adjusted R Square	0.47
Standard Error	0.42
Observations	13

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2.04	2.04	11.58	5.89E-03
Residual	11	1.94	0.18		
Total	12	3.98			

Model	*Coefficients*	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.67	0.19	3.62	0.00	0.26	1.08
X Variable 1	1.14E-03	3.36E-04	3.40E+00	0.00589	0.000404	0.00188

Table B.5 Linear Regression of Sequential ICC RT-PCR Results

SUMMARY OUTPUT	
Regression Statistics	
Multiple R	0.636839
R Square	0.405563
Adjusted R Square	0.38808
Standard Error	0.333458
Observations	36

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2.579376	2.579376	23.19703	2.9685E-05
Residual	34	3.780604	0.111194		
Total	35	6.35998			

Model	*Coefficients*	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	1.199306	0.110525	10.851	1.38E-12	0.97469217	1.423919
X Variable 1	0.00149	0.000309	4.816329	2.97E-05	0.00086117	0.002118

Table B.6 Linear Regression of Sequential $TCID_{50}$ Results

SUMMARY OUTPUT	
Regression Statistics	
Multiple R	0.185365
R Square	0.03436
Adjusted R Square	-0.0622
Standard Error	0.604904
Observations	12

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.1302	0.1302	0.355826	0.564092
Residual	10	3.659092	0.365909		
Total	11	3.789292			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	1.371979	0.350301	3.916574	0.002882	0.59146	2.152498
X Variable 1	-0.00078	0.001315	-0.59651	0.564092	-0.00371	0.002146

C. Raw Data from Disinfection Experiments

The monochloramine concentration for the monochloramine and sequential experiments is contained in Table C.1. The decay constant calculated by minimizing the SS_E using Microsoft Excel Version 3.0 Solver is also presented.

Table C.1 Monochloramine Concentration Data and Decay Constant Calculations

monochloramine trial set 1, flask 1				monochloramine trial set 2, flask 1			
time (min)	conc (mg/L)	yest	(yi-yest)^2	time (min)	conc (mg/L)	yest	(yi-yest)^2
0	3.34	3.34	0.00	0	2.83	2.83	0.00
14	2.87	3.21	0.11	30	1.89	2.36	0.22
57	2.38	2.84	0.21	150	1.22	1.15	0.00
140	2.19	2.23	0.00	465	0.44	0.17	0.07
270	1.78	1.53	0.06				
		SUM	0.39			SUM	0.30
k_d	2.88E-03			k_d	6.02E-03		

monochloramine trial set 1, flask 2				monochloramine trial set 2, flask 2			
time (min)	conc (mg/L)	yest	(yi-yest)^2	time (min)	conc (mg/L)	yest	(yi-yest)^2
0	3.46	3.46	0.00	0	3.17	3.17	0.00
15	3.06	3.34	0.07	30	2.20	2.69	0.24
57	2.68	3.02	0.12	150	1.53	1.39	0.02
140	2.25	2.47	0.05	430	0.38	0.30	0.01
270	2.11	1.81	0.09				
		SUM	0.33			SUM	0.27
k_d	2.41E-03			k_d	5.48E-03		

monochloramine trial set 1, flask 3				monochloramine trial set 2, flask 3			
time (min)	conc (mg/L)	yest	(yi-yest)^2	time (min)	conc (mg/L)	yest	(yi-yest)^2
0	3.33	3.33	0.00	0	6.18	6.18	0.00
22	2.38	3.07	0.48	15	5.26	5.86	0.36
57	2.12	2.70	0.33	60	4.39	4.99	0.36
140	1.88	1.98	0.01	95	4.07	4.40	0.11
270	1.72	1.23	0.25	135	3.76	3.81	0.00
		SUM	1.07	195	3.73	3.08	0.43
						SUM	1.26
k_d	3.69E-03			k_d	3.58E-03		

monochloramine trial set 2, flask 4			
time (min)	conc (mg/L)	yest	(yi-yest)^2
0	5.93	5.93	0.00
15	4.49	5.63	1.28
165	3.35	3.34	0.00
225	2.87	2.72	0.02
		SUM	1.31
k_d	3.47E-03		

monochloramine trial set 3, flask 1			
time (min)	conc (mg/L)	yest	(yi-yest)^2
0	6.60	6.60	0.00
15	5.46	6.14	0.45
30	5.13	5.70	0.33
60	4.56	4.93	0.14
90	4.39	4.26	0.02
135	3.90	3.42	0.23
		SUM	1.17
k_d	4.87E-03		

monochloramine trial set 3, flask 2			
time (min)	conc (mg/L)	yest	(yi-yest)^2
0	7.02	7.02	0.00
15	6.15	6.53	0.15
30	5.67	6.08	0.17
60	5.05	5.27	0.05
140	3.95	3.59	0.13
		SUM	0.48
k_d	4.78E-03		

monochloramine trial set 3, flask 3			
time (min)	conc (mg/L)	yest	(yi-yest)^2
0	6.90	6.90	0.00
15	5.74	6.48	0.55
30	5.24	6.09	0.73
68	5.02	5.21	0.04
140	4.41	3.86	0.30
		SUM	1.62
k_d	4.14E-03		

sequential trial set 1, flask 1			
time (min)	conc (mg/L)	yest	(yi-yest)^2
0	6.86	6.86	0.00
15	5.14	6.35	1.46
50	5.05	5.31	0.07
90	4.48	4.32	0.03
120	3.98	3.70	0.08
			1.64
k_d	5.14E-03		

sequential trial set 1, flask 2			
time (min)	conc (mg/L)	yest	(yi-yest)^2
0	7.23	7.23	0.00
25	5.16	5.98	0.66
45	3.45	5.14	2.85
90	4.41	3.65	0.58
120	3.66	2.91	0.56
			4.66
k_d	7.58E-03		

sequential trial set 1, flask 3			
time (min)	conc (mg/L)	yest	(yi-yest)^2
0	7.91	7.91	0.00
15	5.67	7.19	2.31
45	5.16	5.94	0.61
90	4.48	4.46	0.00
120	4.50	3.68	0.67
			3.59
k_d	6.37E-03		

sequential trial set 2, flask 1			
time (min)	conc (mg/L)	yest	(yi-yest)^2
0	6.30	6.30	0.00
15	3.87	5.92	4.20
50	4.63	5.12	0.23
90	5.48	4.33	1.31
120	3.52	3.83	0.09
		SUM	5.84
k_d	4.15E-03		

sequential trial set 2, flask 2			
time (min)	conc (mg/L)	yest	(yi-yest)^2
0	7.51	7.51	0.00
25	4.56	6.34	3.17
50	3.87	5.36	2.22
90	4.85	4.09	0.59
120	4.34	3.34	1.00
		SUM	6.98
k_d	6.75E-03		

sequential trial set 2, flask 3			
time (min)	conc (mg/L)	yest	(yi-yest)^2
0	8.01	8.01	0.00
17	4.14	7.27	9.81
45	5.16	6.19	1.06
90	5.32	4.78	0.29
120	4.95	4.03	0.84
		SUM	12.00
k_d	5.73E-03		

sequential trial set 3, flask 1			
time (min)	conc (mg/L)	yest	(yi-yest)^2
0	4.48	4.48	0.00
15	2.90	3.89	0.97
45	1.86	2.93	1.13
98	2.24	1.77	0.22
120	2.12	1.44	0.46
		SUM	2.78
k_d	9.46E-03		

sequential trial set 3, flask 2			
time (min)	conc (mg/L)	yest	(yi-yest)^2
0	4.51	4.51	0.00
15	2.94	3.94	1.00
45	2.50	3.02	0.26
90	2.18	2.02	0.03
120	2.08	1.55	0.29
		SUM	1.58
k_d	8.91E-03		

sequential trial set 3, flask 3			
time (min)	conc (mg/L)	yest	(yi-yest)^2
0	4.25	4.25	0.00
29	2.42	3.24	0.68
62	2.15	2.39	0.06
90	1.97	1.84	0.02
120	1.94	1.39	0.30
		SUM	1.06
k_d	9.28E-03		