SOMATIC COLIPHAGE PHIX174 INACTIVATION KINETICS, MECHANISMS AND MODELING IN SURFACE WATERS

-ROLE OF UVA/VISIBLE LIGHT, NOM, SALINITY AND MICROALGAE

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously."

Sun Chenxi

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Summary

Due to the prevalence of viral contaminants in surface waters and the inadequacy of current knowledge of virus fate in aquatic environments, a study was conducted to assess virus inactivation kinetics and mechanisms. Here, the kinetics and mechanisms of somatic coliphage (phiX174) sunlight inactivation were examined and used to develop mathematical models. The results were subsequently compared with field samples. The outcomes from this study contribute to the understanding of virus survival in aquatic environments and can be applied in quantitative microbial risk assessment and the management of water quality.

The results from this study showed that UVA and visible light wavelength spectrum of sunlight could result in virus inactivation where the inactivation followed pseudo-first order inactivation and could be described by the Chick-Watson equation. Compared with the results from previous studies where only the virucidal effects of UVB/UVC were studied and most of the studies were performed with RNA viruses, our study provides evidence for the direct damage on DNA caused by UVA/visible light.

Natural organic matter (NOM) would enhance virus inactivation at low concentrations through the generation of reactive oxygen species (ROS). Different ROS were observed to have different impacts on virus survival. At high concentrations, NOM contributed significantly to light attenuation in the water column and thus, resulted in decreased virus inactivation. Our study discovered that the impact of NOM on virus inactivation followed a sigmoidal

equation, which is unlike previous studies where only a linear relationship was considered.

Salinity was found not to directly affect virus inactivation as with temperature or sunlight. However, it indirectly affects virus survival by affecting its 'sensitivity', possibly by causing aggregation and increasing or decreasing ROS production under light. In our study where phiX174 was used as model virus, 15 ppt was observed to be a threshold value for salinity above which significant impacts on virus survival by sunlight were observed. In NOM free waters, higher salinity led to higher virus inactivation rates. However, in NOM rich waters, higher salinity led to lower virus inactivation rates.

In addition to the effects on indirect virus inactivation, salinity and NOM also affected the shape of the virus inactivation curve, indicating a possible differentiation of the virus population.

Synergistic effects were observed for NOM and light, and salinity and light on phiX174 inactivation. The coexistence of low concentration NOM (< 11pm) and sunlight were found to lead to higher phiX174 inactivation rate than that in the presence of sunlight but NOM free water or in the absence of NOM but NOM rich waters. Similar phenomenon was observed for salinity and sunlight. Increased salinity, especially higher than 15ppt, was found to cause higher phiX174 inactivation at the same surface sunlight intensity. In addition to the synergistic effects, a more complex interactive effect was observed for NOM, salinity and light. Increased salinity in NOM rich waters caused decreased phiX174 inactivation at the same surface sunlight intensity.

The alga, *Microcystis aeruginosa*, was not found to affect virus survival through either adsorption or altered indirect inactivation. When algae cells were lysed, NOM was formed which contributed to virus inactivation.

The virus inactivation coefficients towards sunlight, salinity and NOM were determined quantitatively and can be applied in further kinetics or modeling studies. In this study, a model based on the concept of the Weibull Model was formulated to predict the instantaneous inactivation of virus based on the combined effects of light, NOM and salinity.

$$\ln\left(\frac{N_t}{N_o}\right) = -\Big(\ln\Big(+\exp\Big(\mathrm{K_i} * \ln\left(1+\exp\big(\mathrm{K_s}*(\mathrm{S}-\mathrm{S_o})\big)*\mathrm{I}\right.$$

$$\left.*\exp(-\mathrm{j}*[\mathrm{TOC}])\Big)\Big)+\mathrm{K_{id}}*\mathrm{I}*\mathrm{S}*[\mathrm{TOC}]\Big)$$

$$*\,\mathrm{t}^{(\mathrm{n}*(1+\mathrm{K_1}*\mathrm{S})*(1+\mathrm{K_2}*[\mathrm{TOC}])}$$

The Weibull Model takes into consideration the differences among the effects of different environmental parameters on phiX174 inactivation and thus, could predict the instantaneous phiX174 inactivation rate with higher accuracy in this study. The model could potentially be used for virus inactivation estimation and microbial water quality management..

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List of Acronyms

NOM	Natural Organic Matter
ROS	Reactive Oxygen Species
TOC	Total Organic Carbon
PFU	Plaque Forming Unit
DAL	Double Agar Layer
FFA	Furfuryl Alcohol
TSB	Tryptic Soy Broth
TSA	Tryptic Soy Agar
HPLC	High Performance Liquid Chromatography
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
PCR	Polymerase Chain Reaction
SPSS	Statistical Package for the Social Sciences

1 IINTRODUCTION

1.1 Background

Human enteric viruses are viruses which are found in the human gastrointestinal tract and are capable of causing gastroenteritis in human (Wyn‐Jones and Sellwood 2001). The common enteric viruses include families *Adenoviridae* (adenovirus strains 2, 3, 7, 40, 41), *Caliciviridae* (norovirus, sapovirus), *Picornaviridae* (poliovirus, coxsackieviruses, enteroviruses, and hepatitis A viruses), and *Reoviridae* (reoviruses and rotaviruses). Enteric viruses have been found to be present widely in different water environments such as reservoirs, lakes, and beaches, etc and can transmit disease via water (Rose, Mullinax et al. 1987; Geldenhuys and Pretorius 1989; Nasser 1994; Taylor, Cox et al. 2001; Lee and Kim 2002; Lipp, Jarrell et al. 2002; Aw and Gin 2011). USEPA has included several enteric viruses including adenovirus, calicivirus, enterovirus and hepatitis A virus in their latest contaminant candidate list (USEPA 2009). Over the years, attempts have been made to understand the occurrence and survival of these viruses. However, recent studies have discovered that viruses are able to survive longer than traditional indicator microorganisms such as *E.coli* (Gerba, Goyal et al. 1979; Keswick, Gerba et al. 1982; Allwood, Malik et al. 2003), which makes the traditional indicator microorganism an inadequate predictor for viral contamination related microbial risk. The inadequacy of using traditional microbial indicators

to predict virus risk calls for an in depth study of the survival kinetics and mechanism of these viruses in different aquatic environments.

Surrogate viruses such as F^+ RNA coliphage and somatic coliphage have been used in many fate and survival studies as models of enteric viruses (Callahan, Taylor et al. 1995; Leclerc, Edberg et al. 2000; Allwood, Malik et al. 2003; Lee and Sobsey 2011) due to their similar structure, size and easy culture and quantification methods. Among these surrogate viruses, MS2 and phiX174 were two of the most commonly used and studied viruses.

Exposure to environmental parameters has been found to cause virus inactivation. These parameters include temperature, sunlight, predation, salinity, and pH (Ward 1982; Yates, Yates et al. 1987; Yates, Stetzenbach et al. 1990; Wommack, Hill et al. 1996; Bertrand, Schijven et al. 2012). The importance of these environmental factors differs depending on the virus type and aquatic environment. Many studies showed that temperature was found to be a key factor that causes virus inactivation (Bertrand, Schijven et al. 2012). Adverse effect of solar irradiation on virus survival has also been well documented (Love, Silverman et al. 2010) . However, the effect of a particular environmental parameter on different kinds of viruses could vary. For example, Sinton et al (2002) suggested that somatic coliphage was mainly inactivated by UVB, but F+RNA coliphage was inactivated by a broad spectrum from UVB to visible light. At the same time, somatic coliphage is more robust in seawater while F+RNA coliphage is more robust in freshwaters (Sinton, Hall et al. 2002). Salinity, pH and natural organic matter (NOM) (Stallknecht, Kearney et al. 1990; Šolić and Krstulović 1992; Kohn, Grandbois et al. 2007;

Silverman, Peterson et al. 2013) were also found to affect virus survival, but they were usually not as detrimental as temperature and irradiation. The studies on these environmental parameters were also less extensive and comprehensive. More specifically, compared with temperature and short wavelength spectrum (UVC and UVB), effects of long wavelength spectrum of sunlight (UVA and visible light) and environmental parameters such as NOM, algae and salinity on virus inactivation have not been thoroughly studied yet.

Water has always been considered a precious resource in Singapore for both drinking and recreational purposes. The potential risk caused by viral contamination in catchments, reservoirs and beaches has triggered an investigation into a better understanding of virus survival in local water systems. Temperature, even though it is known to be one of the most important factors affecting virus survival, is relatively constant throughout the year in Singapore. Thus, the impact of temperature fluctuation was considered minimal. Sunlight is high throughout the year, which makes it an important factor controlling virus survival. In addition, being an island state, seawater and estuary systems provide alternative environments to freshwater systems where viruses may survive. In addition, NOM and microalgae may act as photosensitizers (Zepp, Baughman et al. 1981; Marshall, Ross et al. 2005; Liu, Jing et al. 2010), affecting virus survival in aquatic ecosystems. In this study, we aim to investigate the sunlight mediated virus inactivation process in the simultaneous presence of different environmental parameters. In particular, we will investigate the roles of long wavelength spectrum of sunlight (UVA and

visible light), natural organic matter (NOM), salinity and microalgae (microcystis) on virus survival in the water column. The effects of these parameters on virus survival will be examined both individually and collectively. The virus inactivation processes due to these environmental factors will be studied both qualitatively and quantitatively. Efforts will also be made to establish a mathematical model based on multi-effects of different environmental factors to predict the virus inactivation pattern and rate. Previous surveillance study of Singapore waters showed a prevalence of somatic coliphage in Singapore surface waters (Aw and Gin 2010). Somatic coliphages have not been studied as thoroughly as F^+ RNA phages such as MS2. Therefore, somatic coliphage phiX174 was used as a model virus in this study. The results from this study can then be used as an example to estimate virus survival in tropical aquatic environments, facilitate microbial water quality management and provide viral contamination based warnings to general public.

1.2 Research Questions, Objective and Scope

This research aims to provide survival information for somatic coliphage (phiX174) in tropical surface waters, with a focus on virus inactivation kinetics and mechanisms influenced by factors that have not been thoroughly studied before.

In this research, the following research questions were asked,

a. What is the sunlight mediated inactivation pattern and inactivation rate of somatic coliphage, phiX174, in tropical water environments in the

presence of various environmental parameters such as NOM, salinity and algae?

b. How can we quantitatively assess the impact of each of the environmental parameters on phiX174 inactivation?
c. With known environmental parameters, can we predict the virus inactivation rate?

With these research questions, the objectives of this research are,

a. To determine the inactivation kinetics and mechanisms of phiX174 influenced by the long wavelength spectrum of sunlight (UVA and visible light), NOM, salinity and algae in water.
b. To establish a model based on multi-effects of different environmental factors on the inactivation of viruses.

This thesis is presented in seven chapters with the following organization:

a. Chapter 1 provides a general introduction and overview of the current research status of virus inactivation kinetics and mechanisms. The research questions and objectives are included in this chapter.
b. Chapter 2 is a comprehensive literature review summarizes the previous work on virus inactivation kinetics, mechanism and modeling based on multiple environmental parameters.
c. Chapter 3 covers the study on effects of natural organic matter (NOM) on light mediated phiX174 inactivation.

d. Chapter 4 covers the study on effects of salinity on light mediated phiX174 inactivation.
e. Chapter 5 covers the study on effects of microalgae (microcystis) on light mediated phiX174 inactivation.
f. Chapter 6 covers the development and validation of mathematical models to predict phiX174 inactivation based on multiple environmental parameters using both the Chick-Watson model and the Weibull model.
g. Chapter 7 summarizes the major conclusions in this study and suggests recommendations for future study

2 LITERATURE REVIEW

2.1 Overview

This chapter presents a detailed literature review on the significance of enteric and surrogate viruses in microbial water quality management, summarizing the major virus inactivation kinetics, mechanisms and models as well as the effects of different environmental parameters (temperature, sunlight, NOM, salinity and algae) on virus inactivation. Arising from this review, the knowledge gaps in the context of Singapore's water environment are identified to provide the research directions for this study.

2.2 Significance of enteric viruses and application of surrogate viruses in microbial water quality management

Human enteric viruses are a collection of all the groups of viruses that may be present in the gastrointestinal tract, some of which may cause gastroenteritis, hepatitis, paralysis, fever or respiratory diseases. Such viruses would be present in sewage as they are shed in the feces of infected individuals. The common enteric viruses include *Picornaviridae* (such as poliovirus, enterovirus, coxsackievirus, hepatitis A virus and echovirus), *Adenoviridae* (such as adenovirus), *Caliciviridae* (such as norovirus, and sapovirus) and *Reoviridae* (such as rotavirus and reovirus). These enteric viruses are considered to be emerging waterborne pathogens. Among them, adenovirus, calicivirus, enterovirus and hepatitis A virus have been included in the USEPA Contaminant Candidate List 3 (USEPA 2009). Contaminants included in CCL 3 are currently not subject to any proposed or promulgated primary drinking

water regulations by the US government, but they are known or anticipated to occur in public water systems, and which may require regulation. The presence of these pathogenic viruses poses a risk for the safe use of water for both drinking and recreational purposes. For recreational waters, the public may potentially be exposed to these viral contaminants during activities such as boating, water skiing, fishing, swimming and diving. With enough contact time and pathogen concentration, the exposure might lead to gastroenteritis in the activity participants. Therefore, it is necessary to study the fate and survival of these viruses in aquatic systems. However, both the detection and quantification of viable enteric viruses are far from easy. The detection of viable enteric viruses from environmental samples mainly depends on cell culture based method, which requires delicate and tedious maintenance of cell lines. Molecular methods, such as the application of polymerase chain reaction (PCR), have become very popular in microbial detection and monitoring, but its inability of differentiating viable viruses from non viable virus particles often leads to an over estimation of microbial risks (Espinosa, Mazari-Hiriart et al. 2008; Liu, Hsiao et al. 2010). In addition, the presence of inhibitors in different water environments reduces the accuracy and reliability of this method (Alvarez, Buttner et al. 1995) .

Coliphages are a group of bacteriophages that infect *E.coli.* They are used as an indicator of fecal contamination as they are commonly found in animal and human feces (USEPA 2001) . Coliphages share some fundamental properties and features with enteric viruses which make them useful in monitoring the microbial quality of water, especially under circumstances

where traditional fecal indicators, such as *E.coli* are inadequate for predicting viral contamination (Grabow 2004). Coliphages have also been documented as surrogates for human enteric viruses because of their similar properties with enteric viruses in terms of size, morphology and mode of replication. Some coliphages are found to be more resistant to disinfection processes and replicate inside the host cell, similar to enteric viruses. Unlike enteric viruses which are hard to be cultivated in laboratories or are uncultivable, such as norovirus (Duizer, Schwab et al. 2004), coliphages can be easily and rapidly cultivated and quantified in laboratories (USEPA 2001). Due to these properties and characteristics, they have been widely used as model viruses for research purposes (Funderburg and Sorber 1985; Leclerc, Edberg et al. 2000; Lee and Sobsey 2011). Somatic coliphage and male-specific (F^+) coliphage are the two most recommended and studied surrogate viruses (Geldenhuys and Pretorius 1989; Leclerc, Edberg et al. 2000; Lee and Sobsey 2011).

2.3 Virus inactivation kinetics and mechanisms

Virus inactivation, similar to other microbial death, is defined as the failure to reproduce in suitable environmental conditions (Schmidt 1957). Microbial death is usually caused by 'lethal treatments'. Thus, the environmental parameters, depending on their effects on microbial activities, can be categorized into 'lethal agents' and 'non lethal agents'. Physical and chemical agents affecting microbial activities to such an extent as to deprive microbial particles of the expected reproductive capacity can be regarded as 'lethal agents'. The common physical lethal agents include heat and radiation, and the common chemical lethal agents compose a wide range of disinfectants such as

hydrogen peroxide and halogens (Casolari 1988). Factors that do not affect microbial activities thus should not be considered as 'lethal agents'.

Virus inactivation can be explained as a special case of general microbial inactivation. Two major microbial inactivation theories have been proposed, being 'Single Hit Theory' and 'Target Theory' (Casolari 1988). According to 'Singlet Hit Theory', the inactivation of a single molecule or 'site' inside a microorganism leads to the microbe's death and thus, the microbial inactivation rate is proportional to the number the remaining viable microorganisms and follows first order kinetics. An application of this theory is the well-know Chick-Watson equation. The 'Target theory', however, assumes differently. The 'target', considered a unit of biological function, must be 'hit' to result in microbial death (Nomiya 2013). Microbial death can result from 'multiple hits' or a 'single hit' on multiple 'targets'. Thus, the microbial survival or inactivation follows a probabilistic distribution and cannot be described by first order kinetics.

In the beginning of the 20th century, microbial inactivation was found to follow pseudo-first order reaction and was treated as an analog to chemical degradation according to Chick 's law (Chick 1908; Watson 1908). Until today, Chick's Law has been widely adopted and applied in many virus inactivation studies (Grant, List et al. 1993). This first order inactivation kinetics can be expressed by the Chick –Watson equation

$$\frac{dN}{dt} = -kN \qquad 2.1$$

Where N = virus concentration (PFU/ml)

k = pseudo first order inactivation rate constant

t = time

The solution to equation (2.1) is the exponential equation (2.2) which can be log transformed to give equation (2.3)

$$\frac{N_t}{N_o} = e^{-kt} \qquad 2.2$$

$$\ln\left(\frac{N_t}{N_o}\right) = -kt \qquad 2.3$$

According to first order kinetics, all microorganisms in the population have the same resistance to 'lethal treatment' (heat, radiation, chemicals) and thus, a log linear inactivation curve is expected (Schaffner and Labuza 1997). However, in the last few decades, significant deviation of microorganisms' survival from the Chick-Watson equation has been observed in many cases (Cerf 1977; Peleg and Cole 1998; van Boekel 2002). This deviation can be explained as a result of the presence of multiple microbial subpopulations, each of which has its own persistence, or by a probabilistic distribution of microbial resistance in the entire population. Being different from the first order kinetics, the probabilistic approach assumes a cumulative form of temporal distribution of 'lethality event' distribution among the microbial population (Cunha, Oliveira et al. 1998; Peleg and Cole 1998; Fernandez, Salmeron et al. 1999).

A number of new models have been proposed to better describe the non log linear microbial inactivation based on the probabilistic approach. Some of these new models include the Cerf Model (Cerf 1977), log logistic Model (Cole, Davies et al. 1993), Weibull Model (Peleg and Cole 1998), and Xiong

Model (Xiong, Xie et al. 1999). Compared with other models, the Weibull model does not involve too many parameters that complicate the model application and it is found to consistently produce better results (Couvert, Gaillard et al. 2005; Chen 2007). The Weibull Model was invoked to describe the time to failure in mechanical systems. Microbial inactivation can be treated as an analog to 'mechanical failure' and thus, the Weibull model can be applied to describe microbial inactivation. It is formulated based on the concept that the microbial inactivation events are considered as probabilities which follow a Weibull distribution. The microbial survival curve can then be treated as the cumulative form for a distribution of microbial inactivation events. This model is described by equation (2.4) (Peleg and Cole 1998)

$$\ln\left(\frac{N_t}{N_o}\right) = -b(L)t^{n(L)} \qquad 2.4$$

where L = concentration of 'lethal agent' that caused virus inactivation

b(L) = inactivation rate constant (scale factor)

n(L) = exponent (shape factor)

The exponent n(L) defines the shape of the survival curve. When n(L) < 1, the survival curve has an upward concavity, and when n(L) >1, the survival curve has a downward concavity. When n(L) =1, the survival curve appears to be linear in semi logarithmic coordinates, and has the same shape as the Chick-Watson equation. The rate constant b(L) defines the slope of the survival curve. The inactivation rate constant b(L) follows the log logistic equation and can be described by equation (2.5) (Campanella and Peleg 2001)

$$b(L) = ln\left(1 + exp\left(k_L * (L - L_o)\right)\right) \qquad 2.5$$

where L_o = initial 'L' concentration at which the inactivation rate starts to change

k_L = the approximate slope of b(L) vs. L when $L \gg L_o$.

The Weibull model has been successfully applied to describe thermal inactivation, radiation inactivation, pulsed electric field (PEF) inactivation, and pressure inactivation of bacteria, spores, and microbial vegetative cells (such as *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella enterica serovar Enteritidis, Salmonella enterica serovar Typhimurium*, *Staphylococcus aureus, Bacillus cereus, Bacillus pumilus, Bacillus sporothermodurans, and Clostridium botulinum*) in many studies (Fernández, Collado et al. 2002; Mafart, Couvert et al. 2002; Corradini, Normand et al. 2005; Couvert, Gaillard et al. 2005; Chen 2007).

Virus inactivation kinetics are dependent on both internal and external factors including the virus susceptibility or sensitivity to environmental factors, virucidal effects of environmental factors and the dose of the environmental factors (Girones, Jofre et al. 1989; Gantzer, Dubois et al. 1998; Thurston-Enriquez, Haas et al. 2003). A number of studies reported the effect of various physical and biological environmental factors that could affect virus inactivation including temperature (Yates, Yates et al. 1987), sunlight (Watts, Kong et al. 1995; Sinton, Finlay et al. 1999), salinity (Liltved, Hektoen et al. 1995), natural organic matter (NOM) (LaBelle and Gerba 1979) and indigenous microorganism (Yates, Stetzenbach et al. 1990). The effects of different environmental factors on virus inactivation are summarized in Table 2.1.

Table 2.1 Factors affecting virus survival in water

Environmental Factors	Effect on virus inactivation	Selected Studies
Temperature	most important factor affecting virus survival elevated, temperature accelerates virus inactivation	(Yates, Yates et al. 1987; Frerichs, Tweedie et al. 2000; Wells and Deming 2006; Bertrand, Schijven et al. 2012),
Sunlight irradiation	cause damage of nucleic acid ,endogenous or exogenous photooxidation of virus	(Meng and Gerba 1996; Davies-Colley, Donnison et al. 1997; Wilhelm, Weinbauer et al. 1998; Davies-Colley, Donnison et al. 1999; Fujioka and Yoneyama 2002; Silverman, Peterson et al. 2013)
pH	affect electrostatic behavior of viruses	(Stallknecht, Kearney et al. 1990; Šolić and Krstulović 1992; Frerichs, Tweedie et al. 2000; Gerba 2007)
Salt	Affects virus aggregation and stability	(Wells and Deming 2006; Brown, Goekjian et al. 2009; Mylon, Rinciog et al. 2009; Gutierrez 2010)
Organic Matter	affects light attenuation, photosensitizer, virus-NOM association	(Kohn, Grandbois et al. 2007; Mylon, Rinciog et al. 2009; Romero, Straub et al. 2011; Rosado-Lausell, Wang et al. 2013)

Indigenous microorganism	one of the most important factor affecting virus survival, grazing can significantly reduce virus concentration in aquatic environment, certain extra-cellular enzyme produced by microorganisms can cause degradation of viruses	(Fujioka, Loh et al. 1980; LaBelle and Gerba 1982; Ward 1982; Mylon, Rinciog et al. 2009)

Among these factors, temperature was found to be one of the most important in affecting virus inactivation (Yates, Yates et al. 1987; Bertrand, Schijven et al. 2012). A recent study (Bertrand, Schijven et al. 2012) reviewed over 500 previously published papers on how temperature governs enteric virus inactivation and proposed an empirical equation to correlate temperature and time required to reach first log reduction (TFL).

$$log_{10}TFL = \alpha_o + \alpha_1 T \qquad 2.6$$

where T is temperature in ^{o}C, and α_o, α_1 are coefficients empirically determined from experiments.

The mechanisms of virus inactivation due to temperature have been studied by many researchers. Ball and Olson (1957) provided the greatest insights into thermal inactivation of microorganisms through an analysis of both macroscopic and microscopic scales. In their work “Sterilization in food technology”, they pointed out that “macroscopic concepts of temperature and heat transfer break down and must be replaced by energy considerations involving molecules in the discrete, and not in the statistical sense. It is not something within the cell (such as temperature) which is the cause of death. The cause must be outside the cell. It must be in the medium, with one or more molecules in the surrounding medium having the greater mean velocity according to the velocity distribution curve" (Ball and Olson 1957; Casolari 1988).

In addition to heat, radiation is another major cause for microbial inactivation. Most of the studies carried out so far focused on the radiation inactivation of microorganisms by ionizing radiation or UV. The effectiveness

of radiation on microbial inactivation depends on the photon energy and absorbing material. This process usually involves a change in energy state or structure of atoms or molecules (Casolari 1988). Sunlight, which comprises of non-ionizing radiation, has also been found to affect the survival of enteric viruses in water systems.

2.4 Virus inactivation by sunlight

Sunlight has been shown to be a very important factor that affects virus inactivation in environmental waters (Wommack, Hill et al. 1996; Sinton, Finlay et al. 1999; Fujioka and Yoneyama 2002). The study from McLaren and Shugar pointed out that virus photoinactivation might involve both nucleic acid and protein damage. It was also found that the base sequences and the secondary structure of viral genomes played an important role in determining the virus sensitivity to irradiation (McLaren and Shugar 1964). Davies-Colley et al (1999) proposed a three pathways microbial inactivation mechanism due to irradiation that can be applied for sunlight mediated virus inactivation: (i) the photobiological inactivation which involves the direct absorbance of photons by viral nucleic acid that leads to structural damage of virus genome ; (ii) the endogenous photooxidation of virus that involves the absorbance of photons by materials inside virus and subsequent production of free radicals that lead to internal damage of the virus and (iii) exogenous photooxidation that involves the absorbance of photons by materials in the surrounding environment and subsequent production of free radicals that lead to external damage of the virus (Davies-Colley, Donnison et al. 1999). More recent studies in 2010 and 2011 confirmed direct viral protein damage upon

irradiation. This damage can occur both on the surface of the viral capsid involving the oxidation of protein residues (amino acid) and at specific sites of protein chains involving genome-mediated backbone cleavage (Rule Wigginton, Menin et al. 2010; Wigginton, Menin et al. 2012).

The three mechanisms of virus inactivation (Figure 2.1) proposed by Davies-Colley have been widely used in virus fate studies. The photobiological inactivation of viruses was observed to be mainly caused by solar UVB or UVC, and it has been widely applied in disinfection processes. On the other hand, the photooxidation processes can be induced by a wider range of wavelengths (including visible light) in the presence of photosensitizers (substances that initiate/catalyze photochemical reactions) such as natural organic matter (NOM), Fenton particles and algae (Kohn, Grandbois et al. 2007; Nieto-Juarez, Pierzchła et al. 2010). Compared with the effects from short wavelengths of sunlight (UVB (280-315 nm) and UVC (100-280 nm)), the effects of UVA (315 – 400 nm) and visible light on virus inactivation have not been studied as extensively (Shuval, Thompson et al. 1971; Jiang, Rabbi et al. 2009; Romero, Straub et al. 2011).

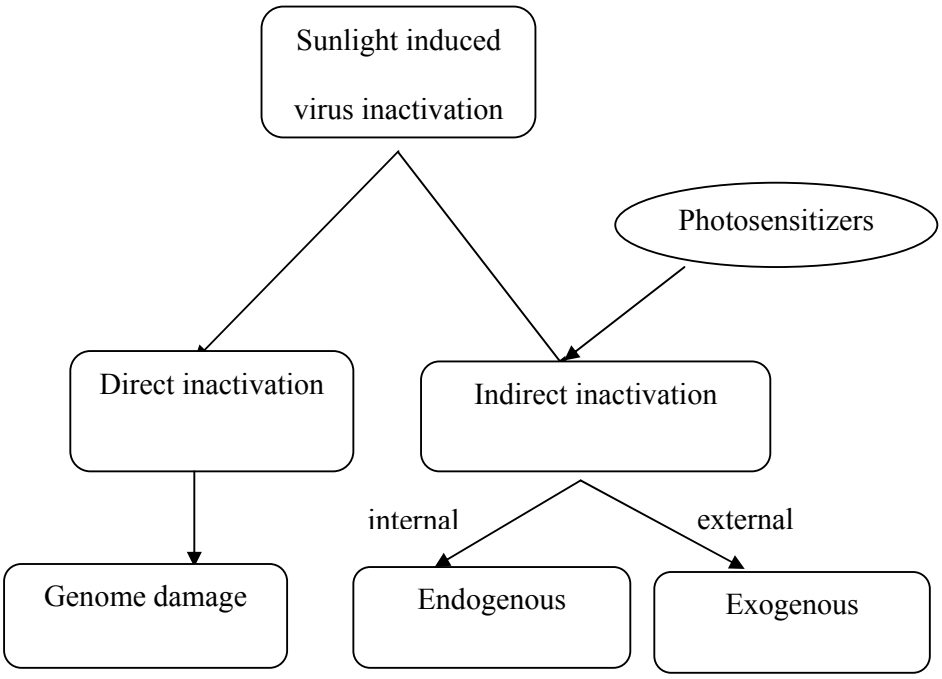


Figure 2.1 Sunlight induced virus inactivation mechanism

The sunlight mediated virus inactivation rate constant k can be expressed by equation (2.7) (Kowalski, Bahnfleth et al. 2009)

$$k = \frac{ln(S)}{D} \qquad 2.7$$

Where D= fluence (sunlight exposure dose) (J/m^2)

k = fluence based virus inactivation rate constant (m^2/J)

S = virus survival ratio (N_t/N_o)

The mean fluence based inactivation rate constant (m^2/J) of different viruses from some previous studies are summarized in Table 2.2. Results showed that even for the same virus, the inactivation rate constant could vary significantly

between different studies. This was mainly due to the different experiment conditions and measurement methods. Among these viruses, MS2 (k_{MS2}=0.0156 m^2/J) and adenovirus (k_{Adv}=0.027 m^2/J) were the two most resistant to irradiation (Havelaar, Meulemans et al. 1990; Wilson, Roessler et al. 1992; Battigelli, Sobsey et al. 1993; Meng and Gerba 1996; Sommer, Haider et al. 1998; Gerba, Gramos et al. 2002; Thompson, Jackson et al. 2003; Thurston-Enriquez, Haas et al. 2003; de Roda Husman, Bijkerk et al. 2004; Malley 2004; Ko, Cromeans et al. 2005; Mamane-Gravetz, Linden et al. 2005; Simonet and Gantzer 2006), ; T4 (k_{T4}=0.345 m^2/J) and phiX174 ($k_{phiX174}$=0.396 m^2/J) were the two least resistant to irradiation (Battigelli, Sobsey et al. 1993; Sommer, Haider et al. 1998; Sommer, Pribil et al. 2001; Otaki, Okuda et al. 2003).

Table 2.2 Fluence based virus inactivation rate constant

Virus	Average k(m^2/J)	Reference
MS2	0.015588	(Havelaar, Meulemans et al. 1990; Wilson, Roessler et al. 1992; Battigelli, Sobsey et al. 1993; Meng and Gerba 1996; Sommer, Haider et al. 1998; Thurston-Enriquez, Haas et al. 2003; de Roda Husman, Bijkerk et al. 2004; Ko, Cromeans et al. 2005; Mamane-Gravetz, Linden et al. 2005; Simonet and Gantzer 2006)
MS2	0.122	(Malley 2004)
Hepatitis A Virus	0.04066	(Wilson, Roessler et al. 1992; Battigelli, Sobsey et al. 1993; Wang, Mauser et al. 2004)
Hepatitis A Virus	0.0075	(Nuanualsuwan, Mariam et al. 2002)
coliphage T4	0.345	(Otaki, Okuda et al. 2003)
coliphage T4	0.1709	(Winkler, Johns et al. 1962; Harm 1968; Templeton, Andrews et al. 2006)
Adenovirus	0.027333	(Meng and Gerba 1996; Gerba, Gramos et al. 2002; Thompson, Jackson et al. 2003; Thurston-Enriquez, Haas et al. 2003; Malley 2004)
Rotavirus	0.128	(Wilson, Roessler et al. 1992; Battigelli, Sobsey et al. 1993; Malley 2004)
phiX174	0.396	(Battigelli, Sobsey et al. 1993; Sommer, Haider et al. 1998; Sommer, Pribil et al. 2001)
PRD1	0.128	(Meng and Gerba 1996)
PWH3a-PI	0.7-0.85 (h^{-1})	(Wilhelm, Weinbauer et al. 1998)
Adnovirus ST2	0.55-0.63 (h^{-1})	

MS2	0.41-0.45 (h^{-1})	
phiX174	1.81 (h^{-1})	
phiX174	0.65-0.90 (h^{-1})	(Kapuscinski and Mitchell 1983)

2.5 Effect of natural organic matter (NOM) on sunlight mediated virus inactivation

Natural organic matter (NOM) is a heterogeneous mixture of humic compounds, hydrophilic acids, proteins, lipids, carbohydrates, carboxylic acids, amino acids and hydrocarbons (Garcia 2011). NOM found in aquatic environments can be classified into two groups: autochthonous and allochthonous. The autochthonous NOM is formed in the aquatic environment from cellular constituents of indigenous aquatic organisms. The allochthonous NOM is brought into the aquatic environment mainly by run-off which originated from soil. The aquatic NOM concentration is thus influenced by the indigenous organisms, soil types, vegetation and rainfall events in the environment (Tan 2014). A strong relationship is found between the intensity of rainfall and aquatic NOM concentration. The run-off usually leads to a higher NOM discharge. Due to the complex and undefined composition of NOM, NOM concentration is usually measured through surrogate parameters such as total organic carbon (TOC), dissolved organic carbon (DOC) or UV absorbance.

Even though NOM is usually only present at low concentration in the aquatic environments, it can affect the fate of aquatic viral contaminants through either increasing or decreasing the inactivation rate in the presence of sunlight. NOM, as a light absorbing material, can reduce the effective light intensity in environmental waters both in the UV and the visible light wavelengths and thus, decrease the direct effect of photons on aquatic viruses, leading to decreased sunlight inactivation rates (Bricaud, Morel et al. 1981).

At the same time, NOM, as a photosensitizer (substances which can induce a chemical reaction in another compound through absorption of light), can generate reactive oxygen species (ROS) under sunlight, which can disrupt virus stability (Canonica, Jans et al. 1995) and thus, increase the virus inactivation (Kohn and Nelson 2007; Rosado-Lausell, Wang et al. 2013; Silverman, Peterson et al. 2013). Furthermore, a study performed with MS2 as the model virus found that the association of virus particles with NOM could increase sunlight inactivation rates (Kohn, Grandbois et al. 2007).

Previous studies discovered that different types of reactive oxygen species (ROS) could be generated under light irradiation in aquatic environments in the presence of NOM (Kohn and Nelson 2007; Nieto-Juarez, Pierzchła et al. 2010). The generated ROS include hydroxyl radical, singlet oxygen, superoxide, hydrogen peroxide and others. Among these ROS, singlet oxygen was found to be the most effective in inactivating MS2 (Kohn and Nelson 2007). The MS2 inactivation rate was found to be first order with respect to steady state $^1O_2$ concentration and could be written as a pseudo second order equation (2.8)

$$\frac{d[MS2]}{dt} = k[\ ^1O_2]_{ss}[MS2] \qquad 2.8$$

In addition to $^1O_2$, the triplet excited state DOM (*DOM) was also found to affect the survival of MS2 (Rosado-Lausell, Wang et al. 2013).

The characteristics of NOM determined the difference in their virucidal efficiency. For example, Fluka humic acid was found to be more efficient for MS2 inactivation than Suwannee river humic acid and waste stabilization

pond constituents under sunlight irradiation (Kohn and Nelson 2007). The intrinsic characteristics of viruses determined the difference in virus response to the same dose of sunlight irradiation and NOM. For example, the inactivation rates of viruses such as human adenovirus serotype 2 (HAdV2) , bacteriophage PRD1 and coliphage MS2 under full spectrum solar radiation were increased upon addition of NOM, but inactivation of poliovirus type 3 (PV3) was not affected similarly (Silverman, Peterson et al. 2013).

2.6 Effect of salinity on virus inactivation by sunlight

Salinity is an important parameter that affects water quality, microbial community and survival. Many studies have been performed to evaluate the effect of salinity on aquatic microorganisms including viruses. Survival of poliovirus and pancreatic necrosis virus were found to be unaffected by salinity (Le Guyader, Dincher et al. 1994; Gantzer, Dubois et al. 1998; Mortensen, Nilsen et al. 1998). While, survival of avian influenza virus (Stallknecht, Kearney et al. 1990), naturally occurring viruses in urban sewage (Bordalo, Onrassami et al. 2002), murine norovirus (Lee, Zoh et al. 2008), and bacteriophage 9A (Wells and Deming 2006) were found to experience greater infectivity loss at higher salinities. In addition, the effects of salinity on virus persistence seem to vary with virus characteristics. Somatic coliphage was found to be more persistent than F^+ RNA coliphage in seawater but less persistent in freshwaters (Sinton, Hall et al. 2002).

Interactive effects between salinity and other parameters such as pH (Stallknecht, Kearney et al. 1990), temperature (Wells and Deming 2006) and

sunlight (Šolić and Krstulović 1992; Bordalo, Onrassami et al. 2002) have also been observed for virus survival in aquatic environments. Sunlight and salinity were found to act synergistically on faecal indicators inactivation by Solic and Krstulovic (1992) and Bordalo (2002). Briefly, the presence of salinity and sunlight increased the detrimental effects of sunlight and salinity on faecal indicators (Šolić and Krstulović 1992; Bordalo, Onrassami et al. 2002). The synergistic effect may be caused by the osmotic pressure change at different salinities which can lead to variation in virus stability. In NOM enriched waters, higher ionic strength or salinity was found to increase virus-NOM binding affinity (Templeton, Andrews et al. 2008) and thus, increase virus inactivation rate. A greater sunlight inactivation rate of MS2 was observed with increased ionic strength in several studies (Sinton, Hall et al. 2002; Kohn, Grandbois et al. 2007). In addition, salinity can also affect aggregation (Wong, Mukherjee et al. 2012) of viruses in water environments and thus affect their survival (Brennecke 2009). Furthermore, the halides in aquatic environments can increase both the formation and quenching rates of ROS (Grebel, Pignatello et al. 2012) and thus, affect virus inactivation rate.

A summary of effects of salinity on virus and other microorganism survival is shown in Table 2.3.

Table 2.3 Effects of Salinity on microbial survival

Microorganism	Salinity	Result	Selected Studies
pancreatic necrosis virus	0-40ppt	No significant effect	(Mortensen, Nilsen et al. 1998)
2009 Pandemic Influenza A(H1N1)	0-35, 270 ppt	Decrease of persistence with increased salinity	(Dublineau, Batejat et al. 2011)
Avian Influenza Virus	0-30 ppt	Strong interactive effect between pH and salinity; greater infectivity loss at higher salinity with optimum pH tolerance shifted to more acidic values	(Stallknecht, Kearney et al. 1990)
Avian Influenza Virus	0-30ppt	Decreased survival at higher salinity, two response patterns-3 parameter Gaussian function, or log linear function	(Brown, Goekjian et al. 2009)
MS2	0-30ppt	Increased salinity increased MS2 sorption to sediments, DOC reduced MS sorption to sediment	(Cao, Tsai et al. 2010)

MS2		Inactivation rate not affected by salinity	(Silverman, Peterson et al. 2013)
poliovirus	14-33 ppt	salinity has no effect on poliovirus survival	(Gantzer, Dubois et al. 1998)
Poliovirus	14-33 g/L	Salinity has no significant effect on poliovirus survival	(Le Guyader, Dincher et al. 1994)
Bacteriophage 9A	21 - 161 psu	increased salinity greatly reduced 9A survival at environmentally relevant temperature; salinity had no effect on 9A survival at non environmentally relevant temperature; most of the infectivity loss involved viral protein instead of nucleic acid	(Wells and Deming 2006)
Bacteriophage ps1	4M NaCl	Osmotic shock could cause inactivation of bacteriophage, 99% inactivation observed	(Whitman and Marshall 1971)
MS2	0.01-1.0M	Li^+, Na^+, K^+ did not affect MS2 aggregation, increasing Ca^{2+} increases MS2 aggregation	(Mylon, Rinciog et al. 2009)

HadV	1, 100mM NaCl	higher NaCl concentration greatly increased aggregation at neutral to basic pH	(Wong, Mukherjee et al. 2012)
HAdV2		Increased inactivation rate with increased salinity	(Silverman, Peterson et al. 2013)
Feline Calicivirus	Seawater	inactivation had a tailing effect	(Slomka and Appleton 1998)
Murine Norovirus	0,0.5,1M NaCl	Increased MNV inactivation with increased salinity	(Lee, Zoh et al. 2008)
Faecal coliforms	6-40ppt; 10-35ppt	Reduced survival with increased salinity, inactivation model T(90)=aS^b ; the increase in temperature and salinity was more detrimental to faecal coliforms in the presence of sunlight, suggesting sunlight may have acted synergistically with temperature or salinity	(Šolić and Krstulović 1992)
Naturally occurring microorganism in urban sewage	0.7,1.9, 31psu	Increased inactivation at high salinities; light inactivation enhanced at higher salinities	(Bordalo, Onrassami et al. 2002)

2.7 Algae in water environment

Algal blooms refer to the rapid growth and accumulation of suspended algae or phytoplankton. They have been associated with water quality deterioration problems such as foul odor and tastes, deoxygenation of bottom water, toxicity and food web alteration (Paerl, Fulton et al. 2001). However, algae may also potentially affect the survival of viruses in several different ways. First, the presence of algae may increase the removal of viruses from the water column through adsorption due to their surface area and structure, but they may also act as a protective shield for sorbed viruses. Second, algal blooms can reduce sunlight penetration into the water column and thus, reduce the direct photoinactivation of viruses and prolonging the survival of viruses. Third, algae is also suspected to be a photosensitizer, producing several different kind of ROS (Zepp and Schlotzhauer 1983; Shimada, Akagi et al. 1991). The alteration in ambient ROS concentration may affect the exogenous photo-oxidative inactivation of viruses in aquatic environments. However, there have been no previous studies which have explored the potential effects of algae on the persistence of viruses in environmental waters.

2.8 Singapore surface water and knowledge gaps

2.8.1 Singapore surface water

As stated earlier, the composition of the water matrix plays an important role in virus inactivation. The tropical climate in Singapore leads to a higher water temperature and more intensive solar irradiation across the year than temperate countries where most previous virus survival studies were carried out. Urban

runoff and tropical aquatic ecosystem processes generate significant NOM in local water systems, as well as provide high levels of nutrients which would contribute to algae blooms. In Singapore, all waste water is channeled to water reclamation plants where the treated effluent is subsequently discharged to the sea. However, urban catchments are still prone to sewage leakage from compromised pipe and thus, there is interest to study virus survival for a range of salinities from freshwaters to marine environments.

2.8.2 Knowledge gaps

Due to the high levels of solar irradiation but relatively constant temperature, sunlight is expected to play a more important role in virus inactivation than temperature in Singapore. Even though many studies have been conducted previously on virus inactivation by irradiation, most of these studies focused on the disinfection process where high frequency UV wavelengths were used. Compared with UVC and UVB, the effect of UVA and visible light on virus survival, however, has not been well studied. Previous studies discovered the effects of NOM (such as Fluka Humic Acid, Waste stabilization pond constituents and Suwannee river humic acid) on reactive intermediates (such as $^1O_2$ and *DOM) generation and indirect virus inactivation (such as MS2, PRD1, PV3 and HAdV2), but the results were inconclusive for different viruses. In our study, we aim to provide a more comprehensive understanding on the role of NOM on virus inactivation in the presence of sunlight. Similar to NOM, even though many studies have been performed to examine the effect of salinity on virus survival, most of these studies were more qualitative than quantitative. The interaction between salinity and sunlight was observed

for faecal indicators study (Šolić and Krstulović 1992; Bordalo, Onrassami et al. 2002), but the interactive effect has not been well studied for viruses yet. The interaction of sunlight, salinity and NOM was also not thoroughly studied for viruses. In this study, we would like to examine the effect of salinity to obtain a more quantitative understanding as well as its interaction with sunlight and NOM on virus inactivation. We would also like to investigate the effect of algae on virus inactivation with a focus on microcystis, a common blue-green algae found in Singapore reservoir (Te and Gin 2011).

With the quantitative data obtained from the above, models will be developed based on the physicochemical parameters of water to provide an estimate of virus survival patterns in tropical urban environments.

3 PHIX174 INACTIVATION BY LONG WAVELENGTHS SUNLIGHT AND NOM

3.1 Abstract

In this study, the effect of natural organic matter (NOM) on the inactivation of viruses was investigated. Suwannee River Natural Organic Matter (SRNOM) was used as the model NOM, while the somatic coliphage phiX174 was used as the model virus. The relationship between phiX174 inactivation and SRNOM concentrations followed a sigmoidal curve. When SRNOM concentration increased from 0 to 11 ppm, the inactivation rate of phiX174 increased simultaneously. Thereafter, the inactivation rate decreased with increasing SRNOM concentration. When SRNOM concentration increased to 65ppm, the phiX174 inactivation rate decreased to below the inactivation rate of direct photoinactivation, and SRNOM started to show a protective effect on virus survival. The initial increase in phiX174 inactivation rate was believed to be due to indirect inactivation or exogenous photooxidation where OH▪ and $^1O_2$ were the effective reactive oxygen species (ROS) for this process. The observed phiX174 $\log_{10}$ based inactivation rate constant k_{obs} varied linearly with [OH▪], and could be expressed as $k_{obs} = 4\times10^{13}[OH▪] + 1.4004$ ($R^2 = 0.8527$). However, no correlation was found between k_{obs} and $[^1O_2]$. H_2O_2 did not appear to be effective in inactivating phiX174. These results show that the removal of phiX174 in aquatic systems when irradiated with sunlight can be expressed as a function of NOM content.

3.2 Introduction

Human enteric viruses infect humans via the gastrointestinal tract and cause acute gastroenteritis, hepatitis, and/or other adverse health effects (Wyn‐Jones and Sellwood 2001). It has been found that enteric viruses occur widely in various environmental waters, including drinking water sources (e.g., river, reservoir, lake, groundwater) and recreational water (Rose, Mullinax et al. 1987; Geldenhuys and Pretorius 1989; Cruz, Bartlett et al. 1992; Fong and Lipp 2005; Shimizu, Phan et al. 2007; Aw and Gin 2011). Recently, global attention has been drawn to understand the occurrence and survival of these viruses in environmental waters. In fact, several enteric viruses, namely adenovirus, calicivirus, enterovirus, and hepatitis A virus, have been included in the latest U.S. Environmental Protection Agency's Contaminant Candidate List (CCL3), a list of emerging contaminants that may pose a public health risk in water environments (USEPA 2009).

A number of studies have reported that the survival of these viruses in aquatic environments can be affected by various physical and biological factors, including sunlight (Watts, Kong et al. 1995; Sinton, Finlay et al. 1999), and natural organic matter (NOM) (LaBelle and Gerba 1979). Sunlight is an important factor that affects virus inactivation rate in environmental waters (Wommack, Hill et al. 1996; Sinton, Finlay et al. 1999; Fujioka and Yoneyama 2002). Sunlight-induced virus inactivation may involve three independent mechanisms: direct photobiological damage, exogenous photooxidation (capsid damage by exogenous chromophores), and endogenous photooxidation (Davies-Colley, Donnison et al. 1999; Sinton, Hall et al. 2002).

The photobiological damage on virus occurs when photons are absorbed by viral nucleic acid or proteins, leading to structural damage of the viral genome or protein (McLaren and Shugar 1964; Rule Wigginton, Menin et al. 2010; Wigginton, Menin et al. 2012). The photooxidation processes involve the absorbance of photons, transformation of energy and subsequent generation of reactive intermediates ($^1O_2$, ▪OH, *DOM, etc.) which can lead to virus inactivation (Kohn and Nelson 2007; Rosado-Lausell, Wang et al. 2013). This process is catalyzed by photosensitizers (substances that initiate/catalyze photochemical reaction). Natural Organic Matter (NOM) is a commonly found photosensitizer in surface waters with complex and undefined characteristics (Canonica, Jans et al. 1995). It has been found to either contribute to virus inactivation through generation of virucidal reactive intermediates or increase virus inactivation through association with virus particles (Kohn, Grandbois et al. 2007). An MS2 sunlight inactivation study carried out using waste stabilization pond water with a concentration of 15 mg/L total organic carbon found that the indirect inactivation process mediated by the organic matter was more dominant than the direct photo inactivation process (Kohn and Nelson 2007). In addition, as a light scattering and absorbing substance, the presence of NOM reduces the effective sunlight intensity penetrating into the water column both in the UV and visible wavelengths (Bricaud, Morel et al. 1981; Morris, Zagarese et al. 1995), and decreases the photobiological damages on the virus, leading to a decreased virus inactivation. Under full spectrum sunlight exposure, the presence of Suwannee River Natural Organic Matter (SRNOM) was found to decrease the overall sunlight inactivation rates of

porcine rotavirus and coliphage MS2 due to attenuation of lower wavelengths (Romero, Straub et al. 2011).Therefore, the overall effects of NOM on virus inactivation by sunlight are a combination of all processes and could be highly dependent on its composition and concentration.

For this reason, the present study aims to provide a more comprehensive understanding on the role of NOM on virus inactivation by sunlight. Specifically, we investigated the effects of NOM (ranging from 0 to 65 ppm TOC which covers most surface water NOM concentrations) on inactivation kinetics of virus using the somatic coliphage phiX174 as a surrogate for human enteric viruses. The results from the study provides information on virus survival influenced by NOM and answers the question of whether the presence of NOM increases or decreases the persistence of virus in the aquatic environments. The data was also used to conduct a mathematical model for the prediction of virus attenuation by sunlight irradiation in the presence of NOM. Such models can be used to estimate virus decay rates in water environments and thus, assess microbial water quality.

3.3 Methods

Suwannee River NOM was obtained from the International Humic Substances Society (IHSS). Somatic coliphage phiX174 (ATCC 13706-B1) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All experiments were conducted in 1.0 mM $NaHCO_3$ (Sigma S6014) solution. A sunlight simulator (Atlas SUNTEST CPS+) was used to generate sunlight (UVA and visible light) with constant adjusted intensity (450 W/m^2).

Temperature was maintained constant at 30 ^{o}C by circulating water from a chiller (Shelton SAE-AC1). All experiments were run in duplicate with duplicate measurements.

3.3.1 Coliphage and Host Bacteria Preparation

The somatic coliphage, phiX174 (ATCC 13706-B1), and its host bacteria, *E. coli* CN-13 (ATCC 700609), were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). PhiX174 was propagated in *E. coli* using the agar overlay method as described by ATCC for *E.coli* phage (ATCC 13706-B1) with adjustment for lab conditions. Briefly, 0.7% tryptic soy agar (TSA; Sigma-Aldrich) mixed with log phase *E. coli* and somatic coliphage phiX174 was overlaid on 1.5% TSA plates. Each plate contained 5ml of 0.7% TSA with 200 µL *E.coli* and 500µL phiX174 stock. The total number of plates was determined by the total volume of 0.7% TSA. The inoculated plates were then incubated for 16-24 h before the soft agar layers from different plates were scraped off the surfaces and centrifuged at 1000 rpm for 25 minutes to sediment the host cellular debris and agar. The supernatant was then collected and filtered through a 0.22 µm membrane (Merck Millipore). The filtrate was then subjected to purification process.

The phiX174 stock was concentrated and purified with polyethylene glycol (PEG) precipitation method, as described previously (Lewis and Metcalf 1988) with modification. Briefly, PEG 8000 (Sigma P5413) and NaCl (Sigma S3014) were added to the phiX174 raw stock to form a final concentration of 8% (wt/vol) and 0.5 M, respectively. The resulting suspension was then stirred for

2 h at 4 ^{o}C and centrifuged at 14,000 rpm for 60 min. The PEG containing supernatant was discarded. The pellet was then re-suspended in sterile 1.0 mM $NaHCO_3$ solution, sonicated for 30 s, shaken for 20 min at 250rpm, and re-centrifuged at 10,000 rpm for 20 min. The supernatant was collected andthe titer of phiX174 in the purified stock was determined to be 3×10^9 PFU/ml. The purified stock was kept at 4 ^{o}C before experiments were conducted.

3.3.2 Coliphage Enumeration

The concentration of phiX174 was determined by the double-agar layer plaque assay (DAL), as described in USEPA method 1602 (USEPA 2001). The samples were diluted as needed, and all dilutions were assayed in duplicate.

3.3.3 Sunlight Inactivation Experiment

The purified phiX174 stock was spiked into 10 ml of each water sample to a final coliphage concentration of approx. 1.0×10^6 PFU/ml. The samples were irradiated by UVA and visible light from a sunlight simulator (Atlas SUNTEST CPS+) at 450 W/m^2 for 2 hours each. The irradiation spectrum is shown in Figure 3.1. The temperature for the experiment was maintained by circulating water from a chiller at 30 °C. A 200-μL sample was taken from each reactor at 30 minute intervals and kept at 4°C in the dark until samples were subjected to coliphage titration.

3.3.4 Quencher Experiment and reactive oxygen species (ROS) measurement

To evaluate the indirect inactivation (exogenous photooxidation) of coliphage, the effects of hydroxyl radical, singlet oxygen, peroxide, superoxide, and

*NOM were determined using chemicals that suppressed a certain type of ROS (quenchers). The quenchers used for the selected ROS and their concentrations are shown in Table 3.1 as described in earlier studies (Kohn and Nelson 2007; Grebel, Pignatello et al. 2011). In the experiment, two control samples were used. One was a 1.0 mM $NaHCO_3$ solution, which was used to provide a baseline for direct photo-inactivation of somatic coliphage. The second control was 1.0 mM $NaHCO_3$ with 10 ppm Suwannee River NOM (IHSS).

The measurement of steady state concentrations of OH▪ and $^1O_2$ followed the methods described by Kohn and Nelson (2007) (Kohn and Nelson 2007). Briefly, probe chemicals (i.e., phenol for OH▪, furfuryl alcohol (FFA) for $^1O_2$) were added into samples and irradiated with sunlight at the same intensity and temperature for 2 hours each. Samples were taken at fixed intervals and centrifuged at 12,000 rpm for 30 min at 4°C to remove suspended particles. The treated samples were then measured using high performance liquid chromatography (HPLC; Agilent1200) to determine the change in probe chemical concentration. The steady state concentrations of OH▪ and $^1O_2$ were calculated with known quenching rate constants, being $K_{qFFA} = 1.2 \times 10^8$ $M^{-1}S^{-1}$ for FFA (Furfuryl Alcohol) (Haag and Hoigne 1986) and $K_{qphenol}=1.4\times10^{10}$ $M^{-1}S^{-1}$ for phenol (Kochany and Bolton 1991). The concentration of peroxide was determined colorimetrically using PeroxiDetect Kit (Sigma-Aldrich PD1).

3.3.5 Effects of NOM

To study the overall effect of NOM under constant sunlight intensity, the inactivation of somatic coliphage at different NOM concentrations was determined. The NOM concentrations used covered most natural surface water conditions (0, 3, 5, 7, 11, 19, 30, 65 ppm) .

3.3.6 Data Analysis

The phiX174 inactivation rate constant (k, [h^{-1}]) was calculated as the slope of a linear regression curve of the ln transformed survival ratio (N_t/N_o) versus time t (h). Non-linear regression was performed with Statistical Package for the Social Sciences (SPSS) (IBM).

3.3.7 Inactivation Model Description

The effects of NOM on phiX174 can be analyzed as follows.

PhiX174 inactivation follows pseudo-first order reaction, which can be written as equation (3.1) and equation (3.2),

$$\frac{dN}{dt} = -k_1 N \qquad 3.1$$

$$\ln\left(\frac{Nt}{No}\right) = -k_1 t \qquad 3.2$$

$$k_1 = k_i + k_d \qquad 3.3$$

Where k_1 = the pseudo-first order inactivation rate constant (h^{-1})

k_i = the indirect inactivation rate constant

k_d = the direct inactivation rate constant

For indirect inactivation, k_i, the indirect inactivation rate constant, is assumed to follow equation (3.4),

$$k_i = k_r[ROS]^a \quad 3.4$$

where k_r = virus inactivation rate constant due to ROS

a= order of ROS induced virus inactivation (assuming a=1 for this study)

The value of a is determined from experiment, and the steady state concentration of ROS can be expressed as equation (3.5), assuming the steady state [ROS] is proportional to [NOM] and surface sunlight intensity (I)

$$[ROS] = b[NOM]I \quad 3.5$$

Light attenuation is caused by water, and light absorbing and scattering substances which can be expressed as a function of water depth and concentration of impurities (Lee and Rast 1997). When NOM is the only light absorbing and scattering substance other than water, light attenuation can be written as a function of NOM concentration and water depth, which is expressed as follows,

$$I = I_o/(Z^2) \times e^{-(\mu(NOM)\times[NOM]+\mu(w))\times Z} \quad 3.6$$

where μ(NOM) = the light attenuation coefficient due to NOM,

[NOM] = concentration of NOM

μ(w) = the light attenuation coefficient due to pure water

Z = water depth

In our study, the water depth Z in the reactors was minimized to avoid the influence of water on virus inactivation rate, and thus, equation (3.6) can be simplified as a function of NOM concentration only, as in equation (3.7),

$$I = I_o \times e^{-(\mu(NOM)\times[NOM])} \quad 3.7$$

For equation (3.4), (3.5) and (3.7), and substituting [ROS] and I,

$$k_i = k_{ir}[NOM] \times I_o \times e^{-(\mu(NOM)\times[NOM])} \quad 3.8$$

where I_o = 450 W/m^2 for the study

k_{ir} = virus inactivation rate constant due to NOM and surface light

$$k_i = k_m[NOM] \times e^{-(\mu(NOM)\times[NOM])} \quad 3.9$$

where $k_m = k_{ir} \times I_o$

For direct inactivation

$$k_d = k_e I \quad 3.10$$

where k_e = direct virus inactivation rate constant due to surface sunlight

Substituting I with equation (3.7)

$$k_d = k_e I_o \times e^{-(\mu(NOM)\times[NOM])} \quad 3.11$$

Substituting I_o= 450 W/m^2

$$k_d = k_n \times e^{-(\mu(NOM)\times[NOM])} \quad 3.12$$

where $k_n = k_e \times I_o$

Therefore,

$$k_1 = k_m[NOM]e^{-(\mu(NOM)\times[NOM])} + k_n e^{-(\mu(NOM)\times[NOM])} \quad 3.13$$

The unknown parameters are k_m, k_n, and μ(NOM) and these will be determined from non-linear regression of experimental data using SPSS (IBM). Statistical analysis was performed with Excel (Microsoft).

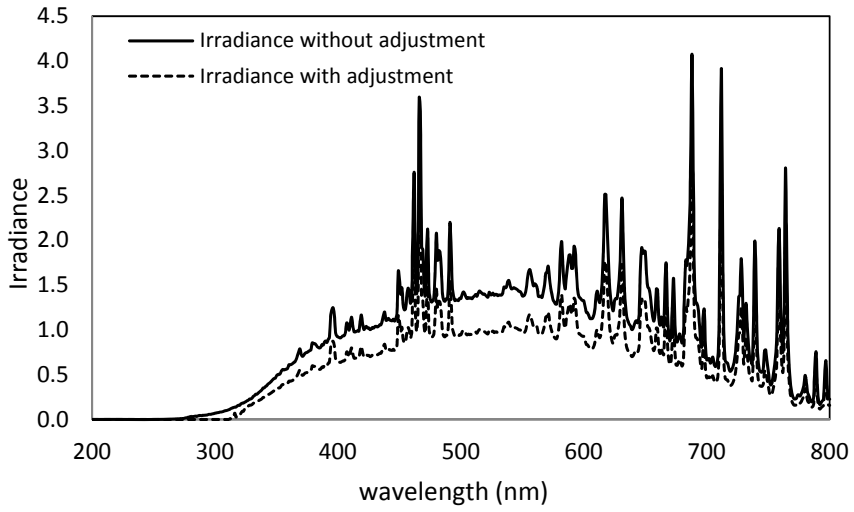


Figure 3.1 Irradiation spectrum of sunlight simulator

3.4 Results

3.4.1 Synergistic Effects of sunlight and NOM

In order to investigate the effects of sunlight and NOM on coliphage inactivation, we determined the survival of phiX174 under light and dark conditions in the presence (5 ppm) and absence of NOM. The results are shown in Figure 3.2. Under dark conditions, no significant inactivation of phiX174 was observed over 2 hours, regardless of the presence or absence of NOM. When the samples without NOM were irradiated with simulated sunlight at 450 W/m^2 for 2 hours, 1.5 log reduction was observed. In the

presence of 5 ppm NOM, a 2.7 log reduction for samples was observed. Thus, it was considered that the inactivation of phiX174 was enhanced in the presence of 5 ppm NOM.

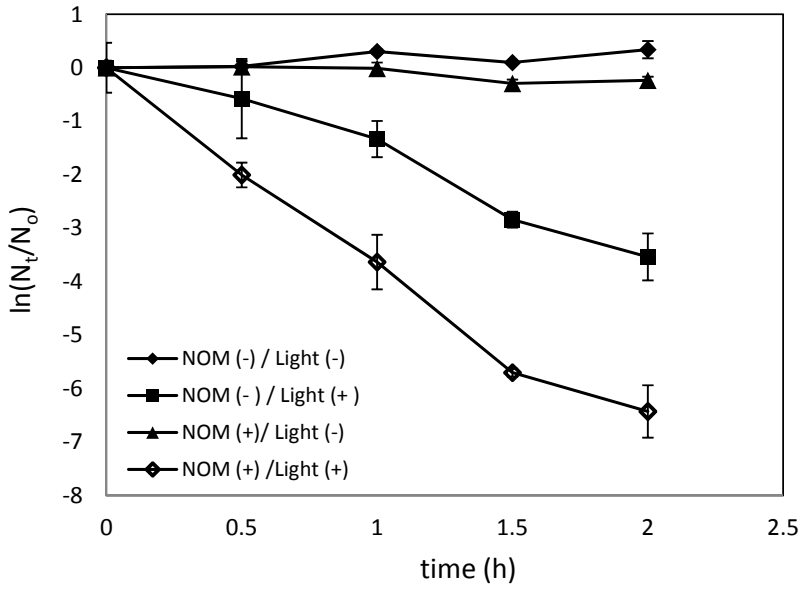


Figure 3.2 Effects of UVA/visible light and 5 ppm SRNOM on the inactivation of the somatic coliphage phiX174

3.4.2 Quencher Experiments

In order to differentiate and investigate the effects of individual ROS on virus inactivation, the survival of phiX174 was determined in samples containing different quenchers. The quenching chemicals and respective ROS are summarized in Table 3.1, while the results are shown in Figure 3.3. Compared with the control sample containing 10ppm NOM without any quenching chemicals, the phiX174 concentration in samples with 2,4-hexadienoic acid, catalase and SOD did not show any significant reduction after 2 h, indicating

that *NOM, Peroxide, and Superoxide generated by sunlight, if any, did not substantially contribute to coliphage inactivation. In contrast, the addition of sodium formate and L-histidine caused a reduction in coliphage inactivation compared with the control sample containing 10ppm NOM. In addition, the resulting inactivation was also similar to the control sample with only 1mM $NaHCO_3$, suggesting that the corresponding ROS, i.e. ▪OH and $^1O_2$, could be responsible for the exogenous inactivation of phiX174. A summary of the inactivation of phiX174 after 2 hours in the presence of different quenchers is shown in Table 3.2.

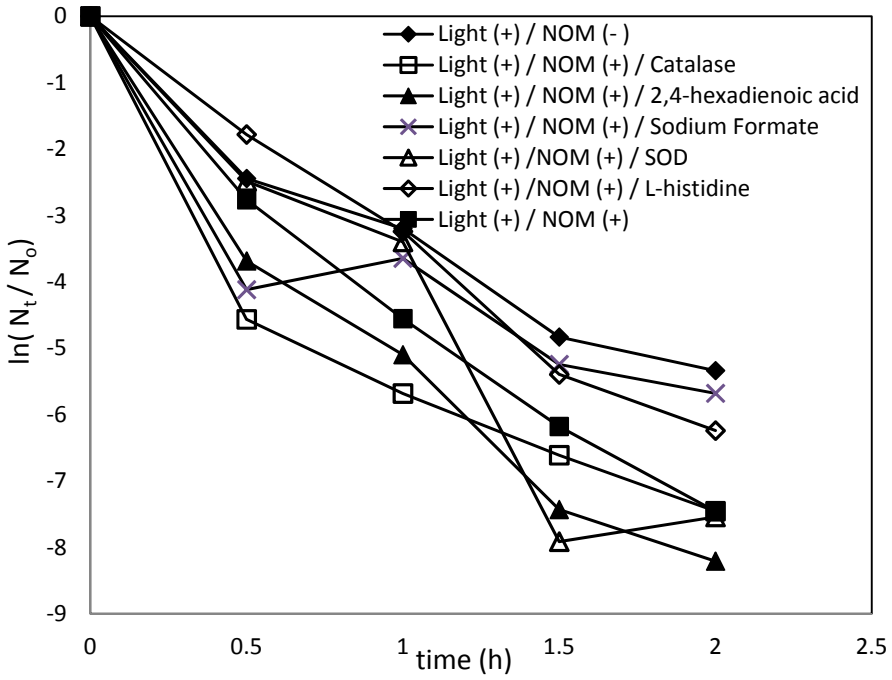


Figure 3.3 Effects of different ROS quenching chemicals on the inactivation of phiX174

Table 3.1 Quenching chemicals and respective ROS

Quenching chemical	2,4-hexadienoic acid	Catalase	SOD (Superoxide Dismutase)	Sodium Formate	L-Histidine
Concentration	50mM	200U/ml	2U/ml	50mM	20mM
ROS	*NOM	O_2^{2-}	O_2^-	OH▪	$^1O_2$

Table 3.2 Effects of different quenching chemicals on phiX174 inactivation after 2 hours

Samples	NOM -	NOM +	NOM +	NOM +	NOM +	NOM +	NOM +
	Quencher -	Quencher -	2,4-hexadienoic acid +	SOD +	Catalase +	L-Histidine +	Sodium Formate +
$Ln(N_t/N_o)$	-5.34	-7.46	-8.21	-7.54	-7.46	-6.24	-5.68

3.4.3 Effects of ▪OH and $^1O_2$ and H_2O_2

To confirm the results obtained from the quencher experiment, we analyzed the relationship between the phiX174 inactivation rate constant and each of the effective radicals. The correlation between the phiX174 inactivation rate constant k (h^{-1}) as slope of ln transformed (N_t/N_o) with time t (h) and concentrations of ▪OH and $^1O_2$ are shown in Figure 3.4 and Figure 3.5, respectively. The results showed that the phiX174 inactivation rate constant was linearly correlated with the steady state ▪OH concentration ($R^2 = 0.8527$). However, no obvious correlation was found between phiX174 inactivation and steady state $^1O_2$ concentrations ($R^2 = 0.00189$; Figure 3.5).

In order to determine the effect of H_2O_2 on phiX174 inactivation, H_2O_2 was added at various concentrations (0, 0.5, 2, 4, 8, 12 μM) to the 1mM $NaHCO_3$ solution containing phiX174. The experiment was performed under dark conditions at 30 ^oC to avoid photolysis of H_2O_2 and potential production of OH▪. The entire experiment was 4 hours and duplicate samples were taken every hour. The ln transformed N_t/N_o value was calculated and plotted in Figure 3.6.

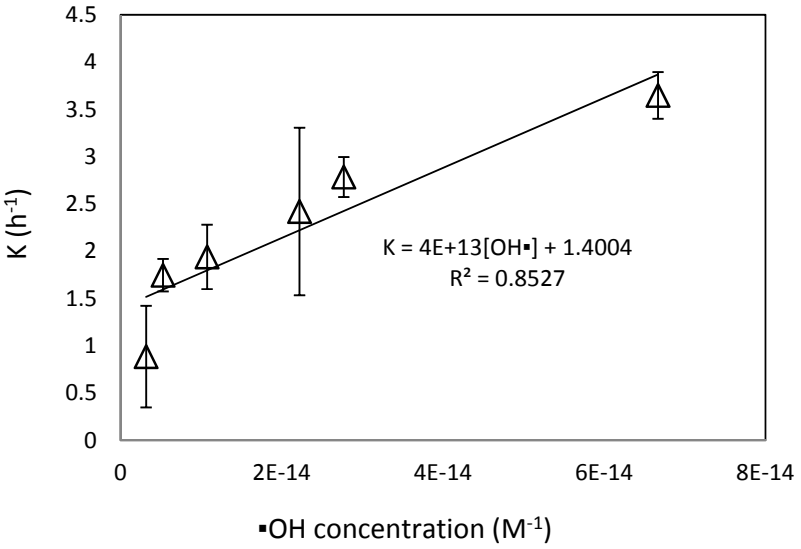


Figure 3.4 Correlation of phiX174 Inactivation rate constant and OH▪ concentration

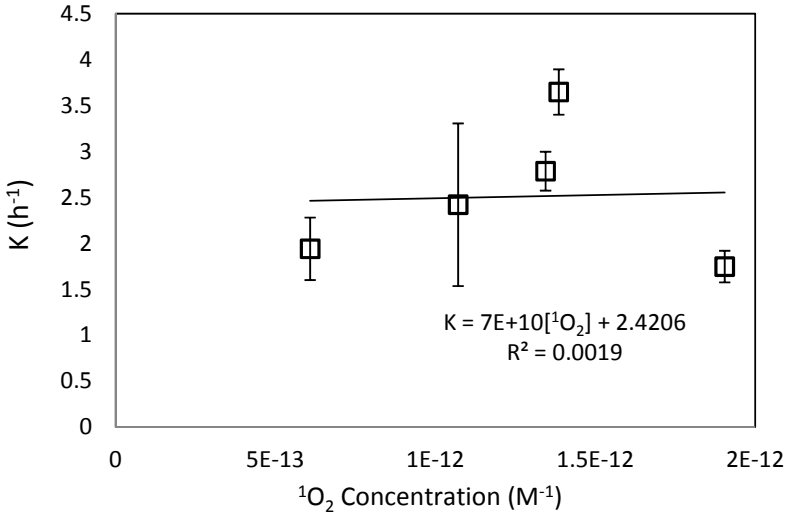


Figure 3.5 Correlation of phiX174 inactivation rate constant and $^1O_2$ concentration

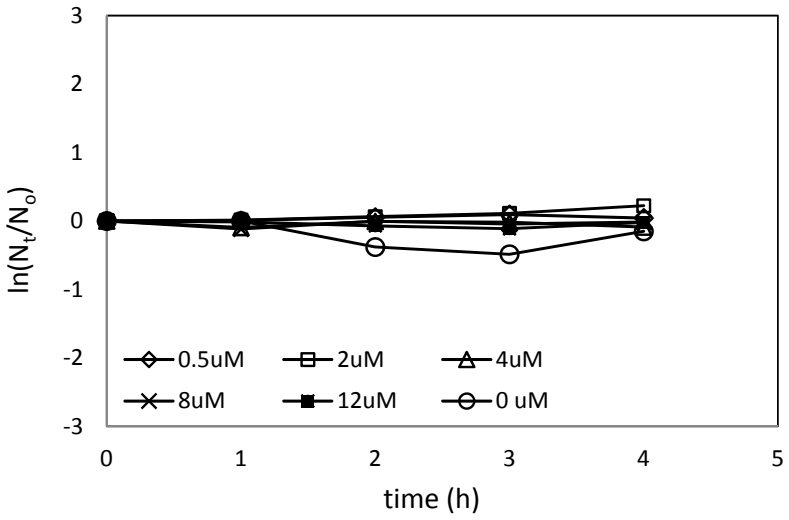


Figure 3.6 Inactivation of phiX174 at different H_2O_2 concentrations

Results from Figure 3.6 showed that no significant inactivation of phiX174 was observed over 4 hours in the presence of externally added H_2O_2 for a concentration ranging from 0- 12 μM. The results showed that in the absence of light, H_2O_2 molecules did not cause noticable inactivation of phiX174 at 30 ^oC.

3.4.4 Effects of Different NOM concentrations

In order to examine the effects of NOM on the inactivation of phiX174 in natural waters, we performed phiX174 inactivation experiments with NOM at different concentrations determined as TOC (ppm). The ln transformed (N_t/N_o) values at different NOM (ppm) concentrations with respect to time (h) are shown in Figure 3.7. The results showed that for NOM concentrations that were close to 0 and for concentrations that were significantly high (>19 ppm),

the enhanced inactivation was smaller than for samples with NOM concentration at 11ppm.

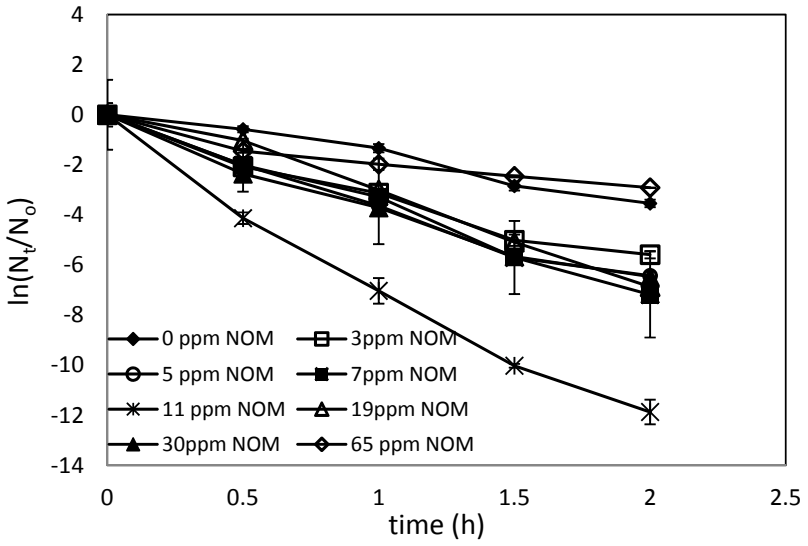


Figure 3.7 Effects of different NOM concentrations on phiX174 inactivation

The inactivation rate constants k (h^{-1}), obtained from Figure 3.7 as the slope of $\ln(N_t/N_o)$ with respect to t (h) , was plotted in Figure 3.8 as a function of TOC (ppm) concentration. From this figure, a sigmoidal graph was obtained where it can be seen that for low concentrations of TOC (<11ppm), phiX174 inactivation rate constant increased with TOC until an optimum was reached at about 11 ppm. However, for higher TOC concentrations, the coliphage inactivation rate constant decreased with TOC.

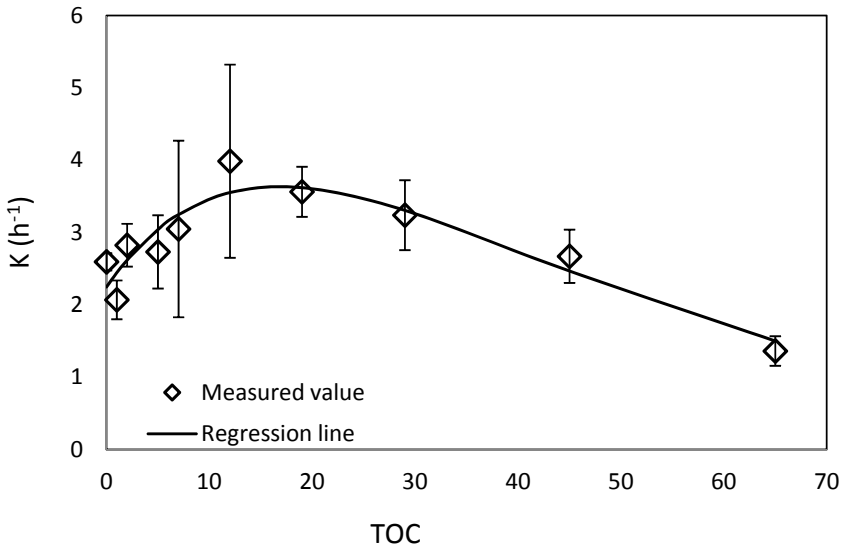


Figure 3.8 Non linear regression of $\log_{10}$ based inactivation rate constant K_2 vs [TOC]

Earlier, we derived the total light mediated inactivation rate constant, k_1, for coliphage,

$$k_1 = k_m[NOM]e^{-(\mu(NOM)\times[NOM])} + k_n e^{-(\mu(NOM)\times[NOM])} \qquad (3.13)$$

Here, the unknown parameters, k_m, k_n and μ(NOM), were obtained by using non-linear regression (SPSS) from the experimental data. The results are shown in Table 3.3, where k is in h^{-1}, and NOM is in ppm.

Table 3.3 Parameter estimates for non linear regression of equation (3.13)

Parameter	Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
k_m	0.279	0.051	.177	.417
k_n	2.246	0.199	1.775	2.717
μ(NOM)	.041	.004	.031	.050

To estimate the ln based phiX174 inactivation rate as a function of virus concentration and TOC content (ppm) for a surface sunlight intensity of 450 W/m^2, we have

$$\frac{dN}{dt} = f(N, NOM)$$

and

$$\frac{dN}{dt} = -\left\{k_m[NOM]e^{-(\mu(NOM)\times[NOM])} + k_n e^{-(\mu(NOM)\times[NOM])}\right\}N \qquad 3.14$$

$$\ln\left(\frac{N_t}{N_o}\right) = -\left\{k_m[NOM]e^{-(\mu(NOM)\times[NOM])} + k_n e^{-(\mu(NOM)\times[NOM])}\right\}\mathrm{t} \qquad 3.15$$

When it is necessary to consider the depth of water (Z), equation (3.6) should be applied to equation (3.15) to give equation (3.16) as follows

$$ln\left(\frac{N_t}{N_o}\right) = -\frac{\left\{k_m[NOM]e^{-(\mu(NOM)\times[NOM]+\mu(w))^{\mathrm{Z}}} + k_n e^{-(\mu(NOM)\times[NOM]+\mu(w))^{\mathrm{Z}}}\right\}}{Z^2}\times \mathrm{t} \qquad 3.16$$

The correlation between fluence (exposure dose) based virus inactivation rate constant and UV irradiation has been reported as by Kowalski et al. (2009) (Kowalski, Bahnfleth et al. 2009) as,

$$k_D = -\ln\left(\frac{N_t}{N_o}\right)/D \qquad 3.17$$

where D = UV exposure dose (fluence), J/m^2

k_D= fluence based virus inactivation rate constant (m^2/J)

N = Virus count

We extended the application of the above equation to UVA and visible light used in this study, and rewrote D (UV exposure dose) as a product of light

intensity (W/m^2) and time (h). The correlation between the fluence based virus inactivation rate constant and light intensity can then be expressed as

$$k_D = -\ln\left(\frac{N_t}{N_o}\right)/(I \times t) \quad 3.18$$

or

$$ln\left(\frac{N_t}{N_o}\right) = -k_D \times I \times t = -k \times t \quad 3.19$$

where k = the virus inactivation rate constant obtained from experiments in this study (h^{-1}) = k_D×I

where

k_D*I =k,

I = light intensity (W/m^2)

t = time (h)

Therefore, k is linearly correlated to I by a constant k_D, which is dependent on virus characteristics.

The phiX174 survival ratio after time t based on virus concentration, depth in water and NOM content at surface sunlight intensities other than 450 W/m^2 can then be expressed as,

$$ln\left(\frac{N_t}{N_o}\right) = -\frac{\left\{k_m[NOM]e^{-(\mu(NOM)\times[NOM]+\mu(w))^Z} + k_n e^{-(\mu(NOM)\times[NOM]+\mu(w))^Z}\right\}\times I}{450*Z^2} \times t \quad 3.20$$

where, I = the surface irradiation intensity (W/m^2)

N = virus count

NOM = NOM content (ppm TOC)

Z = depth (m),

t= time (h)

3.5 Discussion

The goal of this study was to investigate phiX174 inactivation as a surrogate for enteric viruses by sunlight in environmental waters containing NOM. We conducted batch-scale experiments and determined the inactivation rate constant under various combinations of environmental factors, namely, the presence and absence of long wavelength sunlight, NOM, and different types of ROS. The main conclusions to be drawn from this study is that NOM, which can work as a photosensitizer, can either increase or decrease virus inactivation depending on the concentration, through the interference with sunlight.

3.5.1 Direct phiX174 inactivation by long wavelengths of sunlight (UVA and visible light)

In a preliminary experiment, we compared phiX174 inactivation in the dark and in sunlight with NOM free water and NOM containing (5ppm) water. We observed significant increase in phiX174 inactivation for both water samples when they were subjected to long wavelength sunlight (UVA and visible light) irradiation compared to that in the dark. Results in NOM free water proved that UVA and visible light could cause rapid inactivation of phiX174, a single stranded DNA virus. This result is different from some previous studies where UVA and visible light was only found to cause slow inactivation of RNA

viruses such as MS2, murine norovirus and rotavirus (Davies-Colley, Donnison et al. 1999; Kohn and Nelson 2007; Romero, Straub et al. 2011; Lee and Ko 2013) and negligible inactivation of FDNA phage (Davies-Colley, Donnison et al. 1997). The explanation for previous observations was that DNA mainly absorbs shorter wavelengths (i.e. UVB) and thus the longer wavelengths such as UVA and visible light could not cause significant direct virus inactivation. However, the direct absorption of UVA by DNA, which was found to be a function of GC content in the DNA, even though much weaker than shorter wavelengths, has been discovered (Sutherland and Griffin 1981). A study performed recently discovered the direct damage of DNA caused by UVA in 2009 (Jiang, Rabbi et al. 2009) through the direct generation of abasic sites, single strand break (SSB), double strand break (DSB) and pyrimidine dimmers. The observation in our study of the phiX174 inactivation by UVA and visible light may serve as an evidence for direct DNA damage caused by long wavelength sunlight (UVA and visible light).

3.5.2 Effect of NOM on phiX174 inactivation by sunlight

NOM is widely present in natural surface waters, and it has been shown in previous studies to act as a photosensitizer. A preliminary experiment in this study was carried out to compare phiX174 inactivation in NOM free water and NOM containing water (5 ppm) under both light and dark conditions. The results showed that during the experiment period (2h), phiX174 inactivation showed no significant difference in the absence or presence of NOM in the dark. This result suggests that the addition of NOM cannot rapidly remove

phiX174 from the water column through adsorption, inactivation or aggregation in the absence of sunlight.

However, the addition of NOM (5ppm) in sunlight was found to increase total phiX174 inactivation. NOM has been found to influence virus inactivation differently in previous studies. As a light absorbing and scattering substance, NOM can reduce effective light intensity in the water column and thus, reduce virus inactivation (Romero, Straub et al. 2011). However, the virus- NOM association was also observed to enhance virus inactivation (Kohn, Grandbois et al. 2007). As a photosensitizer, its role in exogenous virus inactivation has been discovered (Kohn and Nelson 2007; Rosado-Lausell, Wang et al. 2013). Therefore, the overall effect of NOM on virus inactivation is a result of the combination of at least these above mentioned processes. The increase in phiX174 inactivation in the presence of 5ppm NOM observed in this study showed that the presence of NOM may not always prolong virus survival, and it could potentially increase virus inactivation and thus reduce virus survival in aquatic systems.

3.5.3 Indirect phiX174 inactivation by sunlight and NOM

The indirect virus inactivation by sunlight and photosensitizers has been documented in previous studies. Briefly, the energy from photons is transferred by the photosensitizer to form reactive intermediates (radicals, $^1O_2$, *NOM), and the reactive intermediates will subsequently inactivate viruses (Davies-Colley, Donnison et al. 1999). Therefore, this process is also referred to as a photooxidation process. Results from our experiments showed that the addition of NOM caused changes in phiX174 inactivation rate. To confirm the

effects of NOM on photooxidation of phiX174, we conducted quencher experiments to identify the effective reactive intermediates (i.e. measurements of OH▪ and $^1O_2$ concentrations), and phiX174 inactivation with externally added H_2O_2. Results from the quencher experiments showed that the suppression of OH▪ and $^1O_2$ resulted in decreased phiX174 inactivation, suggesting the virucidal effects of these two reactive oxygen species (ROS). This result is consistent with previously reported studies. $^1O_2$ has been found in many studies to be the major ROS that causes MS2 inactivation (Kohn and Nelson 2007; Rosado-Lausell, Wang et al. 2013). The effect of OH▪ on microorganisms inactivation has not been proved directly by as many studies but has been confirmed indirectly by many studies on *E.coli*, MS2, astrovirus, feline calicivirus and murine norovirus through the increased microbial inactivation rate upon addition of TiO_2 under irradiation (Sjogren and Sierka 1994; Cho, Chung et al. 2004; Sang, Phan et al. 2006; Lee and Ko 2013). However, most of the previous studies were performed with RNA virus or bacteria; our experiment results suggest that OH▪ can also cause inactivation of DNA virus. In our experiment, the $[OH▪]_{ss}$ under different conditions showed good correlation with the phiX174 inactivation rate constant (R^2= 0.8527), which confirmed the virucidal effect of OH▪ from the quencher experiments. However, in our experiment, no correlation between $[^1O_2]_{ss}$ was observed with phiX174 inactivation rate constant (R^2= 0.0019). To further verify whether H_2O_2 might play a role in phiX174 inactivation, we carried out an experiment in the dark with externally added H_2O_2 at 6 concentrations (0, 0.5, 2, 4, 8, 12 μM). No virus inactivation was observed after 4 hours at all

concentrations, which proved that H_2O_2 did not contribute to phiX174 inactivation.

3.5.4 PhiX174 survival in NOM containing waters

We found that the presence of NOM in aquatic systems under sunlight irradiation could either prolong or shorten phiX174 survival, depending on the NOM concentration. The phiX174 inactivation rate constant with respect to NOM concentration exhibited a sigmoidal shape. In our study, at fixed light irradiation intensity (450W/m^2), the initial increase in NOM concentration (0-11ppm) was found to increase the phiX174 inactivation. As stated earlier, the effect of NOM on phiX174 inactivation might be a result of the influence on light attenuation, ROS generation and virus-NOM association. At relatively low NOM concentrations, it is believed that even though the increase in NOM concentration increased the light attenuation and reduced the effective light intensity (Bricaud, Morel et al. 1981) causing direct phiX174 inactivation, the increased NOM concentration resulted in higher steady state ROS (i.e. OH▪, $^1O_2$) concentrations and thus, significantly increased the photooxidation of phiX174, which compensated the inactivation rate loss from the direct inactivation process. In addition, phiX174 may be associated with added NOM, and this process could have enhanced the light mediated phiX174 inactivation as previously reported for MS2 (Kohn, Grandbois et al. 2007). As a result of these possibilities, the addition of low concentration of NOM led to increasing phiX174 inactivation. The maximum phiX174 inactivation rate constant was observed at an NOM concentration of 11 ppm in our study. When NOM concentration increased beyond this point, the overall phiX174 inactivation

showed a decreasing trend. In this range, it is reasonable to assume that the decrease in phiX174 inactivation rate due to light attenuation at higher NOM concentrations could no longer be compensated by the photooxidation process or virus-NOM association mediated by NOM. At higher NOM concentrations, the photooxidation process may also become weaker as less irradiation energy is available for the generation of reactive intermediates when the effective light intensity is reduced. At the same time, the increased concentration of NOM, which is a radical scavenger (Westerhoff, Aiken et al. 1999), may increase the quenching of reactive intermediates and lead to decreased steady state concentrations of reactive intermediates. Therefore, the overall effect of NOM on phiX174 survival in the presence of sunlight irradiation depends on the relative importance of different processes. Our observations are consistent with previous studies. For example, Girones et al. (1989) reported that moderately polluted water samples showed more antiviral activity than more heavily polluted or unpolluted samples (Girones, Jofre et al. 1989). Cantwell et al. (2008) reported that the presence of 50 and 120 ppm of humic acid and NOM significantly reduced the inactivation rate of *E. coli* and *B. subtilis* by UV irradiation. It is believed that when light attenuation dominates over generation of reactive intermediates (usually at high NOM concentration), the presence of NOM will decrease virus inactivation and show a protective effect. However, when photooxidation of virus dominates over light attenuation (usually at low NOM concentration), the presence of NOM would increase the phiX174 inactivation and lead to faster removal of viruses from aquatic systems.

To quantitatively understand the effects of NOM on both direct and indirect phiX174 inactivation, we propose a mathematical model that is based on NOM concentration in the presence of sunlight. The results show that the direct phiX174 inactivation rate constant (k_n=2.246 h^{-1}) is about 8 times that of the indirect inactivation rate constant (k_m=0.279 h^{-1}), which suggests that the direct damage on phiX174 by irradiation is more rapid than the photooxidation process. However, NOM, which acts as a light absorbing/ scattering substance and photosensitizer, can affect contributions from both processes.

3.5.5 Model application and limitations

The model we propose to describe phiX174 inactivation (equation (3.15)) considers the effects of NOM under long wavelength sunlight at different concentrations. It can quantitatively describe phiX174 survival in the presence of NOM and sunlight, and also demonstrate the relative efficiency of both direct and indirect inactivation processes. The inactivation rate constants measured (k_m, k_n) can be used to compare with other virus inactivation rate constants obtained under similar or different environmental conditions. The correlation obtained between inactivation rate constants of different viruses should make it possible to make a rough estimation of the survival patterns of other viruses in the presence of NOM and sunlight based on the value of phiX174.

However, since this model only considers two parameters (NOM and sunlight) and ignores the influence of other environmental factors (total suspended solids, pH, microbial activity, temperature, etc.) it is only suitable to give an approximate estimate of phiX174 survival. The 95% confidence

interval obtained for equation (3.15) also suggests that noticeable variation might be observed. Therefore, in field tests, the results should be adjusted accordingly. Note also that, equation (3.15) was developed for phiX174, and its application to other viruses would need modification based on the relative 'sensitivity' of different viruses.

3.6 Conclusion

In this study, the influence of NOM and long wavelengths sunlight on the inactivation of the somatic coliphage phiX174 was investigated. The effect of NOM on phiX174 inactivation varied depending on its concentration in water. The results indicated that for fixed sunlight intensity, the presence of moderate concentrations of NOM could enhance virus inactivation and reduce their persistence in water. Too low or too high a concentration of NOM would both decrease virus inactivation and prolong virus survival. The optimum concentration of NOM is also expected to be influenced by irradiation intensity, NOM characteristics and viral species. By analyzing the role of different ROS for phiX174 inactivation, we showed the importance of OH▪ and $^1O_2$ in indirect virus inactivation. This study shows the importance of considering the interactive effects of NOM and sunlight in estimating viral persistence.

4 PHIX174 INACTIVATION WITH VARYING SALINITY

4.1 Abstract

This study aimed to quantitatively determine the influence of long wavelengths spectrum (UVA and visible light), salinity on somatic coliphage survival in NOM-free and NOM-rich waters. In the study, a sunlight simulator (Atlas SUNTEST CPS+) was used to generate UVA and visible light at different intensities. NaCl (Sigma S3014) was used to adjust the salinity from 0 to 30 ppt. Somatic coliphage phiX174 was used as the model virus. Significant inactivation of phiX174 was observed in NOM-free water under long wavelengths spectrum (UVA and visible light) irradiation with intensities ranging from 175 W/m^2 to 525 W/m^2 over a period of 4 hours, which proved the ability of UVA and visible light causing direct genome damage on virus. The inactivation rate constant k_d was linearly related with irradiation intensity, which agreed with previous studies on virus inactivation by UV (Kowalski, Bahnfleth et al. 2009). The presence of salinity in NOM free water resulted in increased virus inactivation rates under long wavelengths spectrum irradiation. However, the presence of salinity in NOM rich water resulted in decreasing virus inactivation rates. The results suggested strong interactive effects among sunlight, salinity and NOM. The experiment results also found that salinity had a negative effect for steady state concentrations of $^1O_2$ and OH▪ under fixed sunlight intensity in NOM-rich water.

4.2 Introduction

Sunlight has been shown to be a very important factor that controls the persistence of viruses (Kapuscinski and Mitchell 1983; Sinton, Finlay et al. 1999; Fujioka and Yoneyama 2002) both in water treatment processes and in natural water systems. It can cause both direct and indirect virus inactivation by damaging the virus genome and capsid (Davies-Colley, Donnison et al. 1997) and thus, reducing viral contamination. UV, which has been widely used in water disinfection processes, has been proved by many studies to be very effective in removing viruses from water (Hijnen, Beerendonk et al. 2006). In aquatic environments, sunlight is considered an important factor that causes virus inactivation (Love, Silverman et al. 2010; Silverman, Peterson et al. 2013). Compared with UVB and UVC , which have been extensively studied before (Hijnen, Beerendonk et al. 2006; Lee, Zoh et al. 2008), the effects of UVA and visible light on virus inactivation have not been investigated as thoroughly. In this study, we would like examine the effect of UVA and visible light on phiX174 inactivation in aquatic environments.

Salinity is an important parameter that also affects water quality, microbial community structure and survival. Many studies have been performed to evaluate the effect of salinity on pathogens. Survival of poliovirus and pancreatic necrosis virus were found to be unaffected by salinity (Le Guyader, Dincher et al. 1994; Gantzer, Dubois et al. 1998; Mortensen, Nilsen et al. 1998). However, the avian influenza virus (Stallknecht, Kearney et al. 1990), naturally occurring viruses in urban sewage (Bordalo, Onrassami et al. 2002), murine norovirus (Lee, Zoh et al. 2008), faecal coliform (Šolić and Krstulović

1992) and bacteriophage 9A (Wells and Deming 2006) were found to experience greater infectivity loss at higher salinities. Salinity was also often found to have an interactive effect on virus survival with other parameters such as pH (Stallknecht, Kearney et al. 1990), temperature (Wells and Deming 2006) and sunlight (Šolić and Krstulović 1992; Bordalo, Onrassami et al. 2002) in aquatic environments. Synergistic effects were observed between sunlight and salinity on faecal indicators inactivation by Solic and Krstulovic (1992) and Bordalo (2002). Briefly, the presence of salinity and sunlight increased the detrimental effects of sunlight and salinity on faecal indicators respectively (Šolić and Krstulović 1992; Bordalo, Onrassami et al. 2002). Similar results were observed in several studies using MS2 (Sinton, Hall et al. 2002; Kohn, Grandbois et al. 2007). Salinity was also found to increase virus inactivation in NOM rich waters by increasing virus-NOM binding affinity (Templeton, Andrews et al. 2008), which led to increased virus inactivation rates. In addition, higher salinity could also affect the aggregation (Wong, Mukherjee et al. 2012) of viruses in aquatic environments and thus, affect their survival (Brennecke 2009). Furthermore, the halides in the aquatic environments can increase both formation and quenching rates of ROS (Grebel, Pignatello et al. 2012) and thus, affect virus inactivation rates.

In this study, we would like to examine the effects of long wavelengths spectrum on phiX174 (a common surrogate virus for human enteric viruses) inactivation at various salinities in both NOM free and NOM rich water. Specifically, we will investigate the change of phiX174 inactivation rates under UVA and visible light irradiation at various intensities with 0 salinity

and the effects of salinity on phiX174 inactivation under constant UVA and visible light irradiation in both NOM free and NOM rich waters. In addition, the effects of salinity on steady state concentrations of selected reactive oxygen species (ROS), OH▪ and $^1O_2$, and aggregation of viruses will also be studied.

4.3 Experiments

In this study, NaCl (Sigma S3014) was used to conduct artificial seawater microcosms for the experiment. Suwannee River Natural Organic Matter purchased from International Humic Substances Society (IHSS) was used in the experiments to prepare NOM rich water. Somatic coliphage phiX174 (ATCC 13706-B1) was used as the model virus. All experiments were performed in 1.0 mM $NaHCO_3$ buffer solution. A sunlight simulator (Atlas SUNTEST CPS+) was used for the light source where the irradiation profile shown in Figure 4.1. A filter was used to remove UVB (wavelength<320nm) for the experiment. Temperature was maintained constant at 30 ^oC by circulating water from a chiller (Shelton SAE-AC1). The measurement of $^1O_2$ and OH▪ followed methods described previously (Kohn and Nelson 2007) with modifications for lab conditions using HPLC (Agilent 1200). Particle sizes were measured using a Malvern Nano ZetaSizer Analyzer. All experiments were run in triplicate with duplicate measurements.

4.3.1 Coliphage and Host Bacteria Preparation

Somatic coliphage, phiX174 (ATCC 13706-B1), and its host bacteria, *E. coli* CN-13 (ATCC 700609), were obtained from American Type Culture

Collection (ATCC, Manassas, VA, USA). PhiX174 was propagated in *E. coli* using the agar overlay method as described by the ATCC product sheet for Escherichia coli phage (ATCC 13706-B1) with adjustment for lab conditions. Briefly, 0.7% tryptic soy agar (TSA; Sigma-Aldrich) mixed with log phase *E. coli* and somatic coliphage phiX174 was overlaid on 1.5% TSA plates. Each plate contained 5ml of 0.7% TSA with 200 μL *E.coli* and 500μL phiX174 stock. The total number of plates was determined by the total volume of 0.7% TSA. The inoculated plates were then incubated for 16-24 h before the soft agar layers from different plates were scraped off the surfaces and centrifuged at 1000 rpm for 25 minutes to sediment the host cellular debris and agar. The supernatant was then collected and filtered through a 0.22 μm Millipore membrane. The filtrate was subjected to purification.

The somatic coliphage phiX174 stock was concentrated and purified with polyethylene glycol (PEG) precipitation method, as described previously (Lewis and Metcalf 1988) with modification for lab conditions. Briefly, PEG 8000 (Sigma P5413) and NaCl (Sigma S3014) were added into phiX174 raw stock to form a final concentration of 8 % (wt/vol) and 0.5M respectively. The resulting suspension was then stirred for 2 h at 4 ^{o}C and centrifuged at 14,000 rpm for 60 min. The PEG containing supernatant was discarded. The pellet was then re-suspended in sterile 1.0 mM $NaHCO_3$ solution, sonicated for 30 s, shaken for 20 min at 250rpm, and re-centrifuged at 10,000 rpm for 20 min. The supernatant was then collected and the pH was adjusted to near neutral. The titer of somatic coliphage phiX174 in the purified stock was determined to be 3×10^9 PFU/ml. The purified stock was kept at 4 ^{o}C for experiments.

4.3.2 Coliphage Enumeration

The concentration of somatic coliphage phiX174 was determined by double-agar layer plaque assay (DAL) as described in USEPA method 1602 (USEPA 2001). Somatic coliphage phiX174 concentration was determined with duplicates measurements for triplicate samples.

4.3.3 Sunlight Inactivation Experiment

For the direct photolysis experiment, the purified somatic coliphage phiX174 stock was spiked into 10 ml of each water sample with zero salinity to be a final coliphage concentration of approx. 1.0×10^6 PFU/ml. The samples were irradiated by UVA and visible light from a sunlight simulator (Atlas SUNTEST CPS+) at different light intensities for 4 hours. The temperature for the experiment was maintained constant with circulating water at 30 °C by a chiller. A 200-μL sample was taken from each reactor every 30 minutes and kept at 4°C in dark until the samples were subjected to coliphage titration.

To determine the effect of salinity with sunlight in NOM free water samples and NOM rich water samples, purified somatic coliphage phiX174 stock was spiked into 10ml of each buffered water sample and water sample with 15 ppm NOM respectively with salinities varying from 0 to 30 ppt (0, 5, 10, 15, 20, 30) to be a final coliphage concentration of approximately 1.0×10^6 PFU/ml. The microcosms were irradiated and sampled as described above.

The matrix of tests is shown in Table 4.1.

Table 4.1 Experimental conditions to evaluate the effects of UVA/visible light, salinity on virus inactivation in NOM free and NOM rich waters

Effects of UVA/visible light	**Intensity (W/m^2)**	**175**	**245**	**385**	**455**	**525**		
	Salinity (ppt)	0	0	0	0	0		
	NOM (ppm)	0	0	0	0	0		
Effects of salinity in NOM free water	Salinity (ppt)	0	5	10	15	20	30	
	Intensity (W/m^2)	315	315	315	315	315	315	
	NOM (ppm)	0	0	0	0	0	0	
Effects of salinity in NOM rich water	Salinity (ppt)	0	5	10	15	20	25	30
	Intensity (W/m^2)	315	315	315	315	315	315	315
	NOM (ppm)	15	15	15	15	15	15	15

4.3.4 ROS Measurement

The steady state concentrations of OH▪ and $^1O_2$ were determined for NOM rich water samples and compared for the different salinities. The measurement was done by high performance liquid chromatography (HPLC; Agilent 1200.) as previously described by Kohn and Nelson (2007) (Kohn and Nelson 2007). Briefly, probe chemicals (i.e., phenol for OH▪, furfuryl alcohol (FFA) for $^1O_2$) were added into different samples and irradiated with sunlight at the same intensity and temperature for a sufficiently long period of time. Samples were taken at fixed time intervals and centrifuged at 12,000 rpm for 30 min at 4°C to remove suspended particles. The treated samples were then measured by HPLC to determine the change of probe chemical concentrations. The steady state concentrations of OH▪ and $^1O_2$ were calculated with known quenching rate constants, being $K_{qFFA} = 1.2 \times 10^8\ M^{-1}S^{-1}$ for FFA (Furfuryl Alcohol) (Haag and Hoigne 1986) and $K_{qphenol}=1.4\times10^{10}\ M^{-1}S^{-1}$ for phenol (Kochany and Bolton 1991).

4.3.5 Aggregation Experiment

The aggregation of phiX174 due to salinity was determined using a Malvern Nano ZetaSizer Analyzer. The distribution of particles of different sizes indicates the degree of phiX174 aggregation.

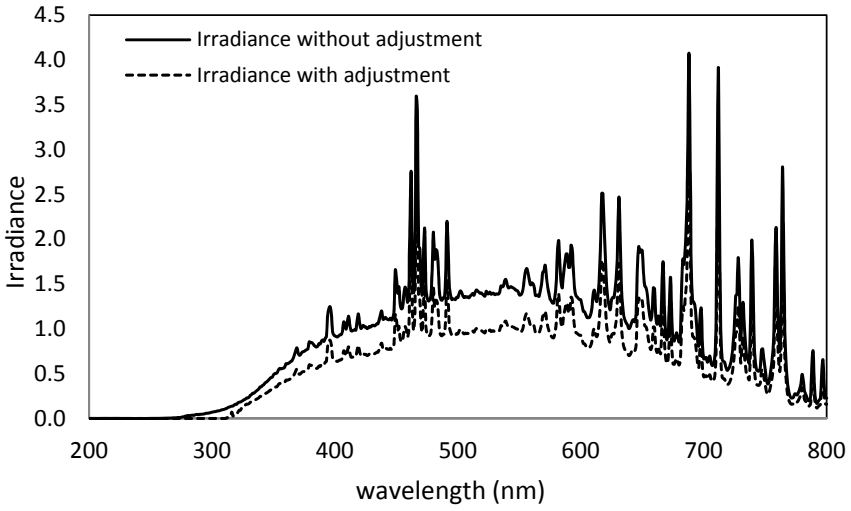


Figure 4.1 Irradiation spectrum from sunlight simulator

4.4 Results

4.4.1 Direct photolysis

Sunlight is an important factor that affects virus persistence in water. The distribution of wavelength and intensity of sunlight differs with depth in the water column. UVB, which is the major part of sunlight that causes virus inactivation, is usually quickly attenuated in water with increasing depth and light absorbing compounds. The remaining part of sunlight that penetrates the water column is largely UVA and visible light. Many studies have been performed to shown the virucidal effect of both UVB and natural sunlight (Fujioka and Yoneyama 2002; Malley 2004). In this study, we would like to examine the effects of UVA and visible light on virus inactivation. The direct photolysis experiment investigated phiX174 inactivation under UVA and visible light at 6 intensities (0, 175, 245, 385, 455, 525 W/m^2) over 4 hours.

The change of viable virus ratio (ln (N_t/N_o)) with time (t) is shown in Figure 4.2. The inactivation rate constant $k_d(h^{-1})$ which was derived from Chick-Watson Equation $dN/dt=-k_dN$ is shown in Figure 4.3 for different irradiation intensities.

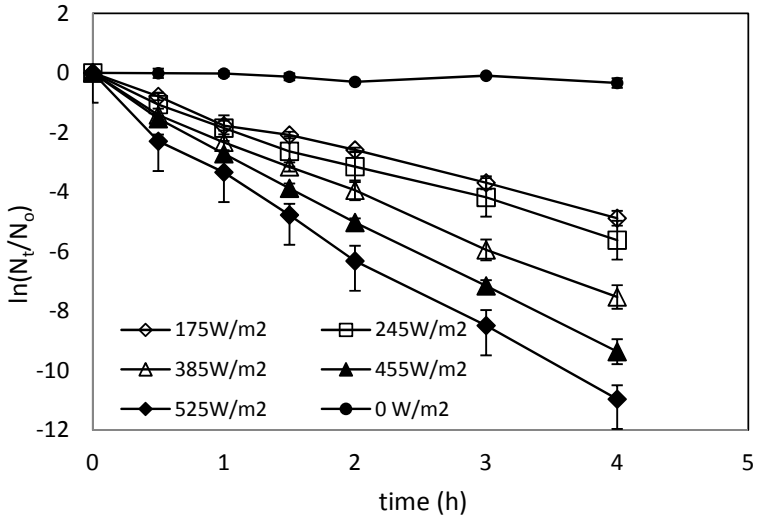


Figure 4.2 Change in phiX174 concentration with time at different irradiation intensities

Results from Figure 4.2 showed that compared with dark condition, phiX174 inactivation increased significantly under UVA and visible light over 4 hours, which proved the virucidal effects of the long wavelength spectrum of sunlight. As this experiment was carried out in buffered ultra pure water, the phiX174 inactivation could be due to direct damage of the virus structure. Under different irradiation intensities, the phiX174 inactivation rate increased with irradiation intensity from 175 W/m^2 to 525 W/m^2. This phenomenon suggests that the UVA and visible light induced phiX174 inactivation rate is dependent on irradiation intensity. It can be observed from Figure 4.2 that under UVA

and visible light, at different irradiation intensities, phiX174 followed a log linear inactivation. A log linear inactivation curve usually indicates uniform susceptibility among the virus population, which implies that UVA and visible light did not cause differential community change in the virus population.

Assuming the UVA-Visible light induced coliphage phiX174 inactivation followed the Chick-Watson Equation

$$\frac{dN}{dt} = -k_d N \qquad 4.1$$

where N = the concentration of virus (PFU/ml)

t = time (h)

k_d = Chick's law rate constant (h^{-1})

k_d was calculated for each inactivation curve at different irradiation intensities, as the slope of a linear regression curve of the ln transformed virus survival ratio (N_t/N_o) versus time. The change of k_d with irradiation intensity, I, is shown in Table 4.2 and Figure 4.3. The inactivation rate constant k_d (h^{-1}) was found to be linearly correlated with irradiation intensity.

Table 4.2 phiX174 inactivation rate constant k_d (h^{-1}) at different irradiation intensities (W/m^2) at 0 salinity in NOM free water

Intensity (W/m^2)	175	245	385	455	525
k_d (h^{-1})	1.26	1.47	1.96	2.41	2.88
SD	0.13	0.51	0.27	0.18	0.36

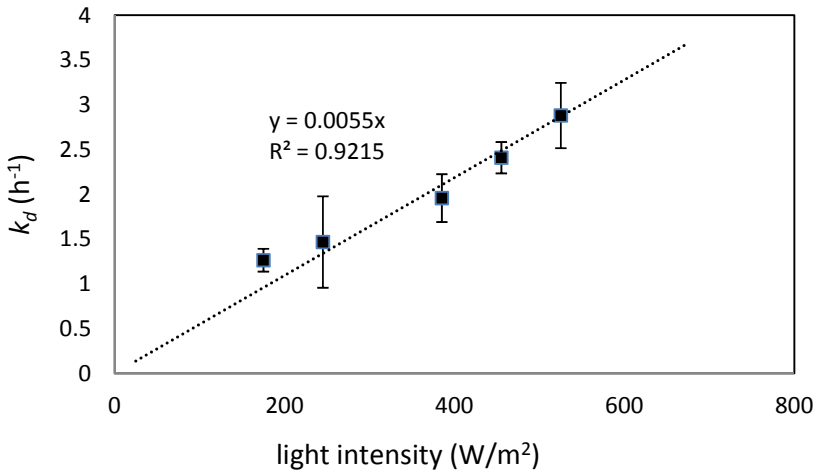


Figure 4.3 Correlation between phiX174 inactivation rate constant (h^{-1}) and irradiation intensity

4.4.2 Effect of Salinity

In order to evaluate the effect of salinity on virus inactivation, especially under UVA and visible light irradiation, inactivation experiments were performed at different salinities with constant light intensity and in dark. The irradiation intensity was fixed at 315 W/m^2 for light experiment, and six levels of salinity were set, ranging from 0 (fresh water) to 30 ppt (seawater). PhiX174 inactivation in dark was carried out as comparison, and four levels of salinity were set, ranging from 0 (freshwater) to 30 ppt (seawater). The inactivation of phiX174 at different salinities is shown in Figure 4.4.

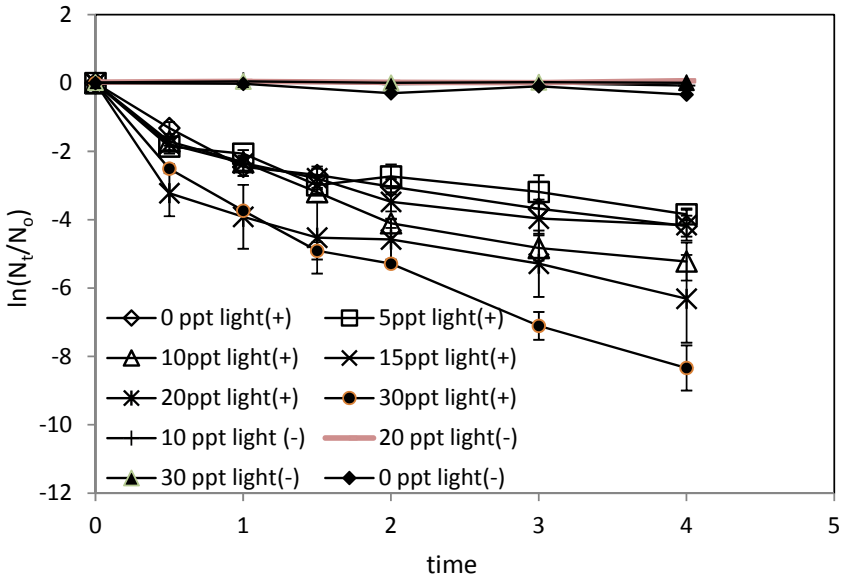


Figure 4.4 Change in phiX174 concentration with time at different salinities

Results from Figure 4.4 showed that salinity did not cause any significant difference in phiX174 inactivation in dark condition over 4 hours. However, when samples were irradiated, greater virus inactivation was observed for all salinities (0 to 30 ppt).

For the samples that were irradiated, phiX174 inactivation exhibited similar dependence on salinity as for UVA and visible light irradiation. As salinity increased from 0 to 30 ppt, phiX174 inactivation was also increased. Therefore, higher salinity was found to cause greater inactivation. Another effect of salinity on phiX174 inactivation was its influence on the shape of the inactivation curve. Unlike inactivation under UVA-Visible light at zero salinity, the inactivation of phiX174 at higher salinities followed a non log-linear inactivation curve. A non log linear inactivation curve usually indicates the existence of subgroups of different characteristics in the virus population,

with some subgroups being more resistant, as when experimental conditions remained unchanged, the heterogeneity was often found to be the major reason of the tailing effect observed in microbial survival curve (Cerf 1977). The ln based inactivation rate constant k(h^{-1}) for each inactivation curve was obtained from non linear regression and the results are shown in Table 4.2 and plotted against salinity in Figure 4.5.

Table 4.3 phiX174 inactivation rate constant k (h^{-1}) at different salinities (ppt) under constant UVA and visible light in NOM free water

Salinity (ppt)	0	5	10	15	20	30
k (h^{-1})	1.26	1.17	1.58	1.32	1.93	2.39
SD	0.24	0.20	0.24	0.31	0.85	0.28

Results from the inactivation experiments at different salinities showed that compared with the inactivation rate at 0 salinity, no significant increase in inactivation rates were observed at salinities of 5 and 15 ppt ($p=0.53$ and 0.11, respectively), while significant increases in inactivation rates was observed at 10, 20 and 30 ppt ($p= 0.03$, 0.002 and 0.008, respectively) if $p \leq 0.05$ is considered significant. However, if the difference is only considered significant when $p \leq 0.01$, there was no significant increase in phiX174 inactivation rates when salinity increased from 0 to 15 ppt. Significant increases in phiX174 inactivation rates were observed at 20 and 30 ppt. Therefore, the phiX174 inactivation rate constants at salinities ≤ 15 ppt were

not significantly affected by salinity. Nevertheless, when salinity continued to increase beyond 15 ppt, higher salinity led to significantly greater virus inactivation.

Results from Figure 4.5 also show an interactive effect between sunlight and salinity when salinity is higher than 15 ppt. As sunlight is constant, the increased salinity caused accelerated sunlight induced phiX174 inactivation. The reason can be that higher salinity may have resulted in aggregation of viruses or change in virus susceptibility, which then led to higher inactivation (Brennecke 2009). However, when salinity is low (≤ 15 ppt), no significant interactive effect was observed between sunlight and salinity. The increased salinity does not result in significantly increased sunlight induced phiX174 inactivation. The phiX174 inactivation at low salinities was determined by irradiation intensity.

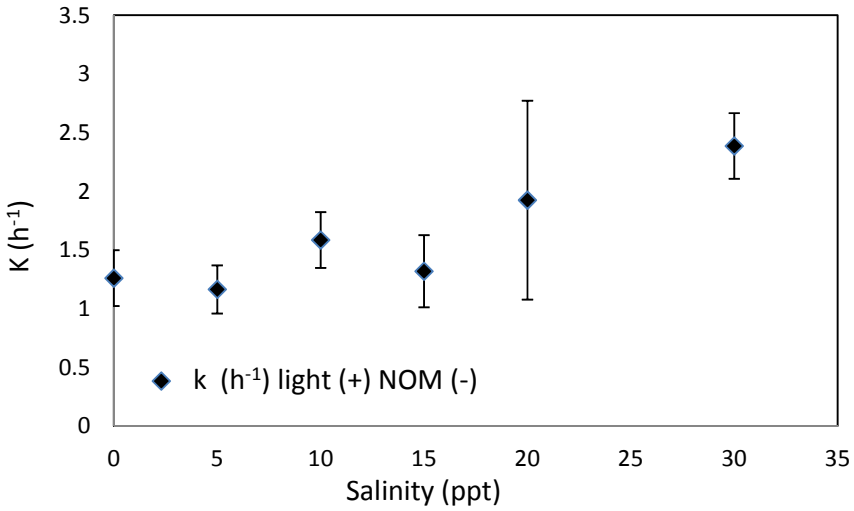


Figure 4.5 Correlation between inactivation rate constant k (h^{-1}) and salinity in NOM free water with constant sunlight intensity

4.4.3 Effect of salinity with light in NOM Rich Water

In order to evaluate the interactive effect of NOM and salinity on sunlight mediated phiX174 inactivation, experiments were carried out at different salinities with constant NOM concentration and light intensity. Sunlight intensity was fixed at 315W/m^2, and NOM concentration was set at 15 ppm.

Figure 4.6 shows the inactivation curve of coliphage phiX174 at different salinities under 315 W/m^2 irradiation and 15 ppm TOC. Over a period of 4 hours, greater phiX174 inactivation was observed at lower salinities, and less inactivation was observed at higher salinities. The inactivation curve at lower salinities was found to be linear with time. However, as salinity increases, deviation from linearity was observed in the virus inactivation curve. These results indicate that the presence of NOM does not cause formation of subgroups in the virus population, but the presence of high concentration of salts causes heterogeneity within the population. Compared with results from Figure 4.4 where higher salinity was found to cause greater virus inactivation at constant irradiation intensity, results from Figure 4.6 showed that in the presence of NOM, higher salinity led to lower virus inactivation. This change in the effects of salinity on virus inactivation at constant irradiation intensity is presumed to be caused by NOM, which indicates a strong interaction between the two parameters. The inactivation rate constants for each inactivation curve

in Figure 4.6 were obtained from non linear regression and are shown in Figure 4.7.

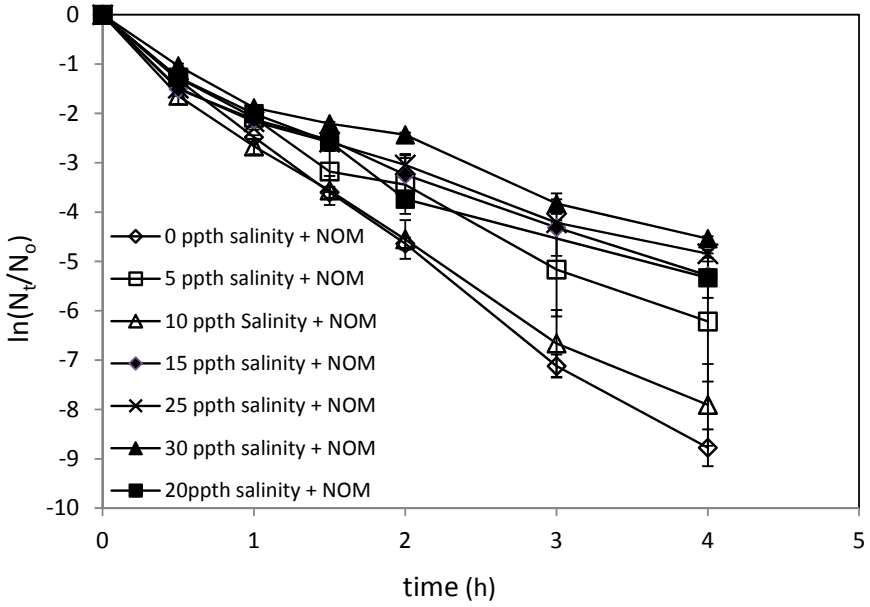


Figure 4.6 Change in phiX174 concentration in NOM (15ppm) rich water for different salinities at fixed irradiation (315W/m^2)

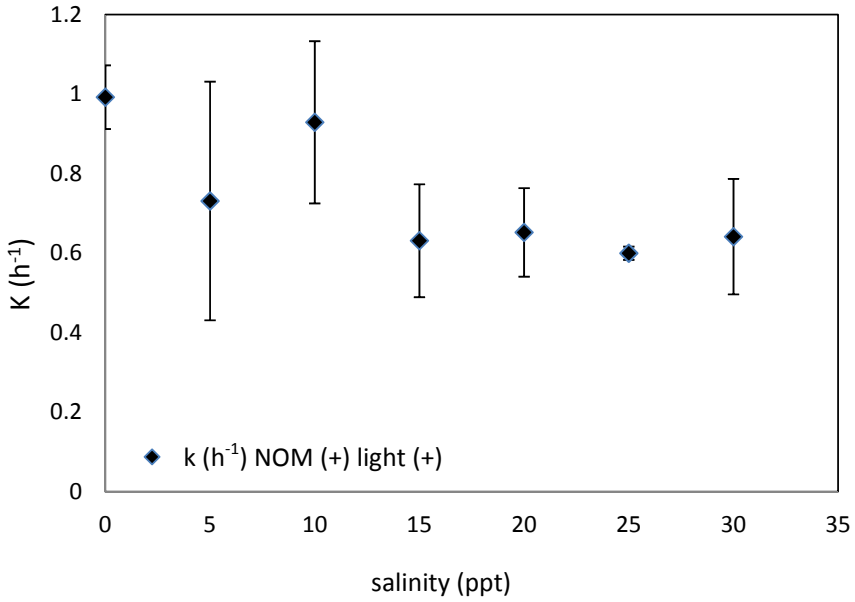


Figure 4.7 Correlation of phiX174 inactivation rate constant k (h^{-1}) and salinity in NOM (15ppm) rich water with irradiation ($315W/m^2$)

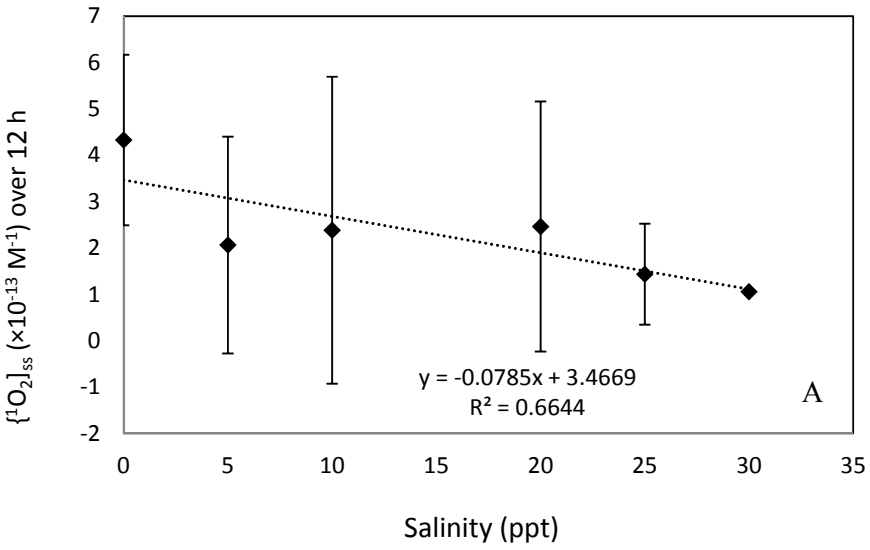
The mean values of phiX174 inactivation rate constants shown in Figure (4.7) exhibited a general trend of decrease with salinity, especially for salinity ≤ 15 ppt. However, a statistical test performed comparing the inactivation with every 5 ppt salinity increment showed that when salinity increased from 0 to 15ppt, there was a significant change in virus inactivation with salinity (p= 0.045, 0.015, and 0.026 for Salinity = 5, 10 and 15 ppt, respectively). However, when salinity continued to increase, no significant change was observed for phiX174 inactivation (p= 0.73 and 0.07 for salinity = 20 and 25 ppt, respectively). Inactivation at 30 ppt was found to be significantly changed as salinity increased (p= 0.003), which was probably due to the error bar. Therefore, the results indicate that virus inactivation at higher salinities (≥ 15ppt) in NOM rich water is not affected as much by salinity as at lower salinities (≤ 15 ppt).

4.4.4 ROS production in NOM rich waters with light at different salinities

As previous research found that the halides in water could increase both the production and quenching of ROS (Grebel, Pignatello et al. 2012), which was detrimental to viruses, the following experiment was performed to understand the effects of salinity on steady state concentrations of selected ROS, the change of which could lead to either increase or decrease in virus inactivation. As OH▪ and $^1O_2$ are two major ROS in the aquatic environments with known virucidal effects, their steady state concentrations at different salinities were measured in this experiment. The results are shown in Figures 4.8A and 4.8B.

The ANOVA test for results from Figures 4.8A and 4.8B showed that there was statistically significant difference for steady state concentrations of $^1O_2$ and OH▪ ($p=0.003$ and 0.001, respectively) at different salinities. With increased salinity, the steady state concentrations of $^1O_2$ and OH▪ were generally negatively correlated. However, the change in ROS concentration was rather small and to what extent this change affects virus inactivation under irradiation requires further investigation.

(A) [$^1O_2$]



(B) [OH▪]

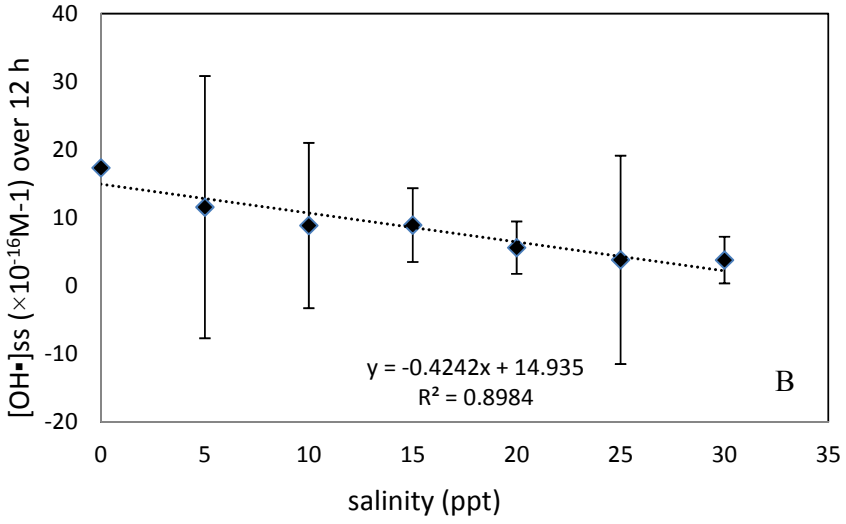


Figure 4.8 Steady state $^1O_2$ ([A]) and OH▪ ([B]) concentration at different salinity at constant irradiation intensity in NOM rich water

4.4.5 Aggregation

PhiX174 is one of the smallest viruses with an isoelectric point (IEP) of 6.6 (Mayer 2008). In our study where 1mM $NaHCO_3$ was used as buffer solution (pH ≈ 8.6), phiX174 was negatively charged. Virus aggregation at pH well above the IEP is usually considered negligible (Gerba 1984). However, aggregation of virus particles in liquid medium, which can be explained by the Derjaguin- Landau-Verwey-Overbeek theory (DLVO theory) as a result of dispersion forces and electrostatic repulsion, can be affected by salinity. Previous study found that increasing monovalent salt concentration (i.e. NaCl) in liquid medium led to the compression of the diffuse double layer and the reduction of stern potential of small solid particles (Elimelech, Jia et al. 1998). Therefore, a change in salinity may affect the stability of phiX174 suspension and result in aggregation. Similarly, the presence of NOM was found to significantly increase virus aggregation. The increase is usually due to the reduction in virus electrophoretic mobility and compression of virus diffuse layer in solution caused by the addition of NOM (Brady-Estévez, Nguyen et al. 2010). However, the study also observed that the continued increase in NOM concentration (0.5 mg/L to 5 mg/L) did not lead to a corresponding increase in virus aggregation, and the effects of salt (i.e. $CaCl_2$) on virus aggregation became insignificant in the presence of NOM. In order to understand the difference in phiX174 sunlight inactivation at different salinities in NOM free and NOM rich water, the effects of salinity and NOM on phiX174 aggregation were examined using a Malvern Nano ZetaSizer Analyzer. The samples from the microcosm were incubated at 4 ^{o}C for 24 h before subjected to particle size

analysis. The PDI (Polydispersity Index) values for the samples are shown in Table 4.4. A PDI value <0.1 usually indicates a monomodal dispersion, and PDI > 0.1 usually indicates a multimodal dispersion.

Table 4.4 PDI values for particle size measurements in different samples

Salinity (ppt)	0	5	10	15	20	25	30
NOM (+)	0.548	0.688	0.633	0.912	1	0.792	0.908
NOM (-)	0.317	0.352	0.257	0.331	0.385	0.195	0.185

The distribution of particles sizes is shown in Figure 4.9 and Figure 4.10.

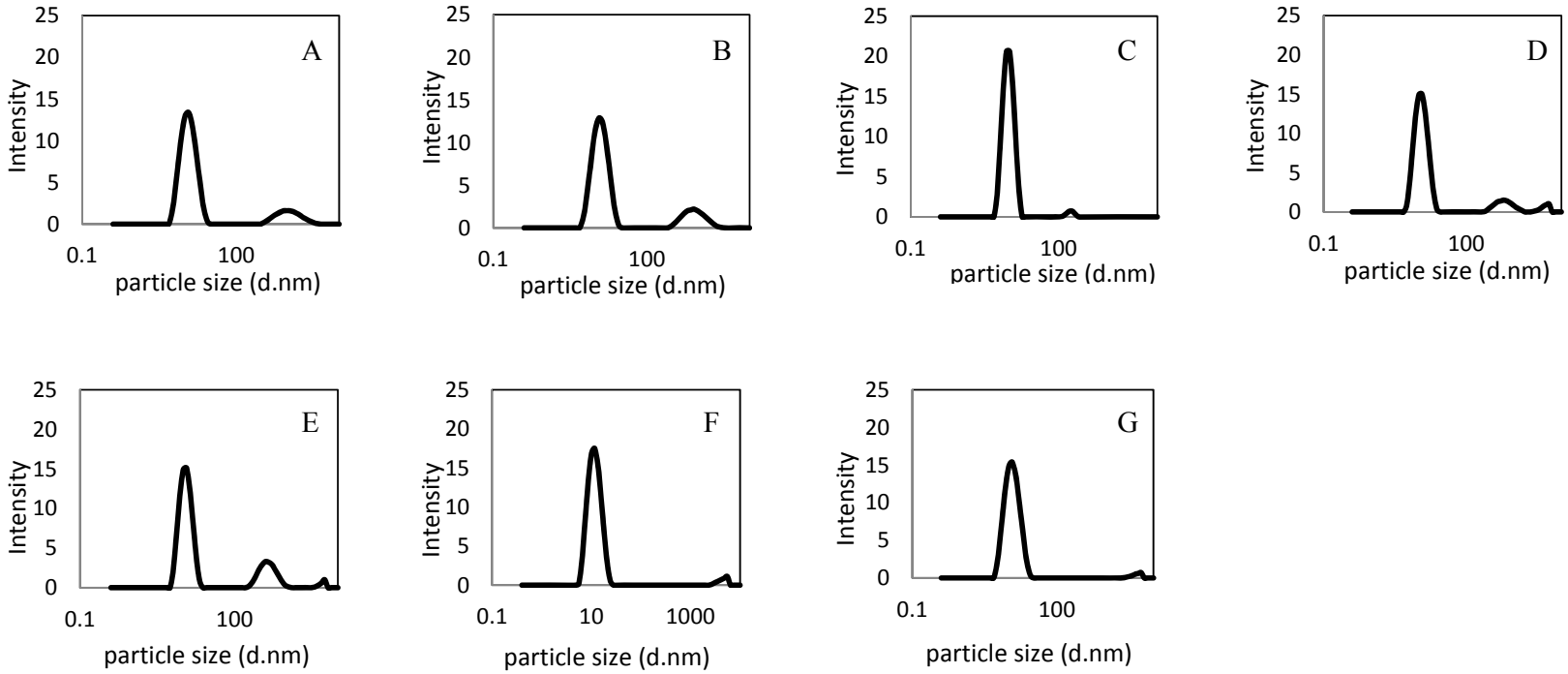


Figure 4.9 Distribution of phiX174 in NOM free water at different salinities (A, B, C, D, E, F, G = 0, 5, 10, 15, 20, 25, 30 ppt respectively)

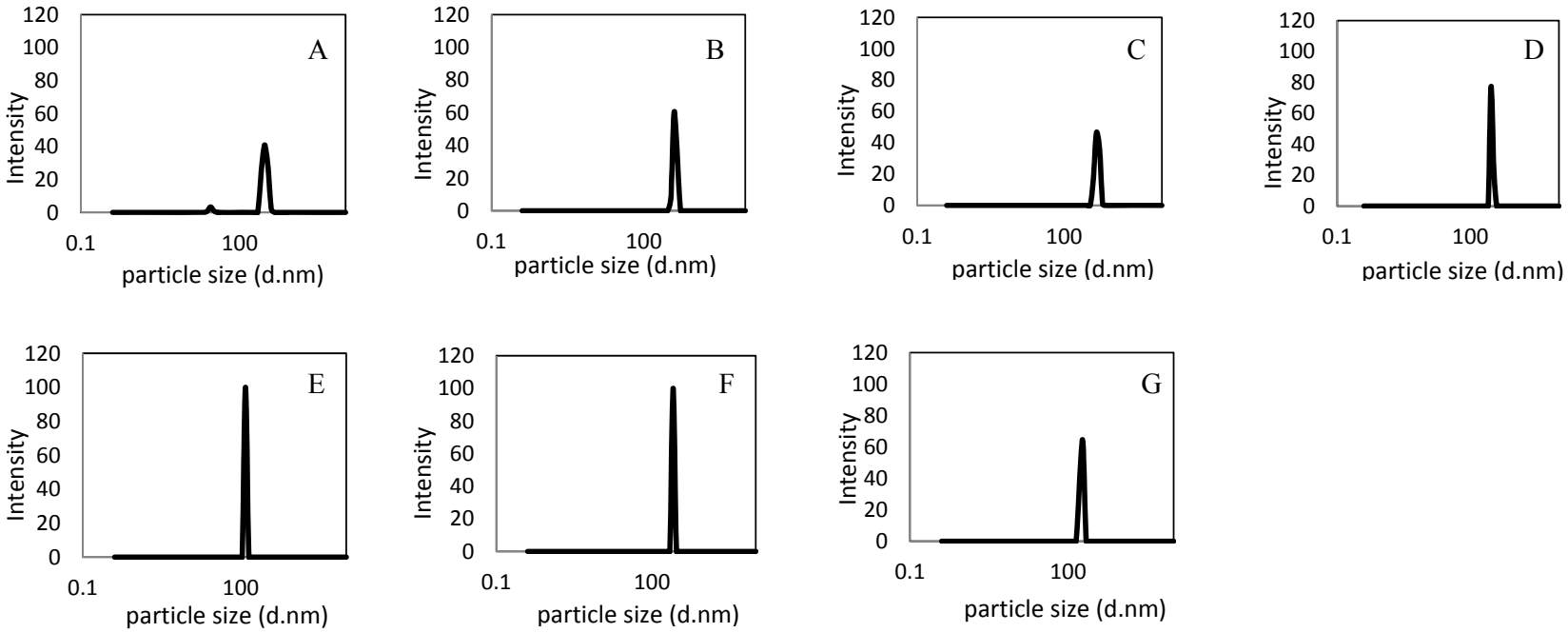


Figure 4.10 Distribution of phiX174 in NOM rich water at different salinities (A, B, C, D, E, F, G = 0, 5, 10, 15, 20, 25, 30 ppt respectively)

Results from Table 4.4 show that the PDI values obtained for all the samples were greater than 0.1. In particle size analysis, the best PDI is 0.0 (perfectly monodispersal solution) and the worst PDI is 1.0. This means that the phiX174 containing water samples in our experiment were multimodal rather than monomodal, Indicating the likelihood of the presence of virus aggregates. A PDI value greater than 0.7 usually indicates that the sample has a very broad size distribution and is not suitable for the DLS test. PDI values obtained in this experiment were all below 0.7 for NOM free water, and mostly above 0.7 for NOM rich water. These results indicate that phiX174 has a broader size distribution in the presence of NOM at all salinities. If the PDI values were compared among different salinities for NOM free water, no pattern was observed. The change in salinity did not cause any significant change in phiX174 size distribution. However, in NOM rich water, as salinity increased, the PDI values had an overall increasing trend. These results indicate that in the presence of NOM, an increase in salinity may lead to a broader size distribution of viral aggregates.

Figure 4.9 and Figure 4.10 show the size distribution of phiX174 at different salinities in NOM free and NOM rich waters respectively. In NOM free water samples (Figure 4.9), a major peak was observed at 17(±8) nm, which represented the dispersed phiX174 virus particles. This value is smaller than the commonly observed size for coliphage phiX174 (23-27 nm) (Mayer 2008). This could be due to the instrument differences. Apart from the major peak, another peak was observed at around 342 d.nm (diameter in nm) at 0 salinity, possibly the major size of aggregated viruses. This peak shifted slightly

towards larger sizes as salinity increased (458 d.nm at 30 ppt). indicating the possible formation of larger particles and change in virus aggregate size composition. As salinity increased from 15 ppt (Figure 4.9 D) to 30 ppt (Figure 4.9 G), a third peak appeared at around 1106 d.nm, and it replacing the second peak at salinities > 25ppt. These results show that the aggregation of viruses can be affected by salinity, with larger aggregates forming at higher salinities in NOM free water samples. Figure 4.10 shows the phiX174 size distribution in NOM rich water at different salinities. From the graph, it can be seen that at 0 salinity with 15 ppm NOM, only a small portion of the virus remained dispersed (as shown by the small peak). Most of the viruses formed aggregates with an average size ranging from 255 d.nm to-615 d.nm. As salinity increased, the sizes of viral aggregates were not significantly influenced. This indicates that the addition of NOM could cause significant aggregation of viruses in water, but in the presence of NOM, NaCl did not contribute to virus aggregation as significantly as in the absence of NOM, which is similar to the findings in a previuos study conducted with $CaCl_2$, where $CaCl_2$ was observed to significantly change virus aggregation in the absence but not in the presence of NOM (Brady-Estévez, Nguyen et al. 2010).

4.5 Discussion

4.5.1 Effect of UVA and visible light

Our results from the direct photolysis experiment confirmed the virucidal effect of long wavelengths of sunlight (UVA and visible light). Compared with control samples in the dark, the samples irradiated at all intensities exhibited much greater inactivation over 4 hours. This result is consistent with

many previous studies which found sunlight being a major environmental factor controlling microbial survival (Kapuscinski and Mitchell 1983; Watts, Kong et al. 1995; Sinton, Finlay et al. 1999; Fujioka and Yoneyama 2002). However, most of the previous studies focused on the effect of UVA or visible light on bacteria or RNA viruses. Our result would provide an example for UVA inactivation of DNA virus.

In this study, as irradiation intensity increased from 175 W/m^2 to 525 W/m^2, the phiX174 inactivation increased accordingly. This observation indicated that the harmful effect of UVA and visible light on phiX174 is dependent on irradiation intensity. As discussed in Chapter 3, the damage on virus caused by UVA and visible light is probably on the genome through the generation of pyrimidine dimers and formation of single strand break (SSB) or double strand break (DSB) on DNA (Jiang, Rabbi et al. 2009). As irradiation intensity increases, it is believed that pyrimidine dimers, SSB and DBS are generated at a higher rate, which will lead to higher virus inactivation rate.

PhiX174 showed log linear inactivation at all irradiation intensities. Log linear inactivation curve usually indicates uniform virus 'resistance' to harmful treatment (Schaffner and Labuza 1997). The consistency in log linear inactivation curves showed that there was no change in phiX174 'resistance' under UVA and visible light irradiation at all intensities. The presence of UVA and visible light did not lead to heterogenization of the virus population which could result in differentiation of virus characteristics and lead to change in virus 'resistance'.

4.5.2 Effect of salinity and interaction with sunlight

The experimental results with varying salinity in NOM free water showed that salinity did not cause significant difference in phiX174 inactivation in dark conditions over 4 hours. However, the difference in inactivation might be observed at different salinities if the experiment was continued for a longer period of time. When the samples were irradiated by light (UVA and visible light), a significant effect of salinity on phiX174 inactivation pattern and rate was observed. As salinity increased from 0 ppt (freshwater) to 30 ppt (seawater), the phiX174 inactivation curve gradually showed a tailing effect, displaying a non log linear shape. The inactivation rate also increased with salinity, especially when salinity increased above 15 ppt. This result is consistent with some previous studies performed with avian influenza virus (Stallknecht, Kearney et al. 1990), 2009 Pandemic influenza A virus (Dublineau, Batejat et al. 2011) and murine norovirus(Lee, Zoh et al. 2008), where salinity was found to have a negative effect on virus survival.

Deviation from log linear microbial inactivation curve is usually because of the heterogeneity in virus 'sensitivity' and population (Cerf 1977; Peleg and Cole 1998; van Boekel 2002). Compared with phiX174 inactivation experiments at 0 salinity, the tailing effect observed at higher salinities in this study indicated that higher salt content could induce differentiation in virus population and formation of subpopulations, which appeared as differences in virus 'susceptibility' to sunlight irradiation. Salinity can affect virus 'susceptibility' by causing aggregation, change osmotic pressure, affect viral capsid stability, interfere with virus-host interaction, etc. Aggregation usually

occurs when the addition of ions compresses the virus particle diffuse layer and thus, changes the surface charge (Elimelech, Jia et al. 1998). However, virus aggregation at pH well above IEP is usually considered negligible (Gerba 1984). The results from the aggregation experiment showed that aggregation only happened for a small portion of the viruses, while the majority remained in dispersed form. This could be explained as phiX174 has an isoelectric point (IEP) of 6.6, and therefore under our experimental pH (8.6), aggregation was difficult to achieve due to the high charge density of both nucleic acid chains and protein chains. Change in osmotic pressure would lead to alteration in virus genome behavior (Cordova, Deserno et al. 2003), protein behavior (Perutz 1978), pressure exerted on virus capsid (Cordova, Deserno et al. 2003), permeability of viral capsid (Anderson, Rappaport et al. 1953), capsid conformation (Heggeness, Scheid et al. 1980), etc. The change in these properties would influence virus 'susceptibility' to harmful effects (sunlight irradiation) and affect inactivation rate. For many bacteriophages, the genome is strongly pressurized. The pressure provides the initial driving force for viral genome injection into host cells (Cordova, Deserno et al. 2003). The increased salinity could affect the viral genome injection force, interfering virus-host interaction and influencing virus quantification results using culture based methods such as the plaque assay. Therefore, the change in salinity can affect individual viral characteristics, virus population property and even the culture based quantification result, which would appear as changes in virus inactivation kinetics. This could also explain the synergistic effect observed in the study between sunlight and salinity which was observed in previous

studies using faecal coliforms and naturally occurring pathogens in urban sewage (Šolić and Krstulović 1992; Bordalo, Onrassami et al. 2002).

The observed results provide information on phiX174 inactivation under UVA and visible light with varying salinity and they could be used as a preliminary estimation of virus survival with similar properties under similar conditions.

4.5.3 Interaction of salinity, NOM and sunlight on virus inactivation

The phiX174 inactivation with varying salinity in NOM containing water under sunlight showed a different trend from those with varying salinity in NOM free water. Compared with NOM free water samples, less deviation from the log linear inactivation curve was observed in NOM containing water samples at all salinities. In contrast to the effect of salinity on phiX174 inactivation in NOM free water, the increase in salinity for NOM containing water led to a decrease in virus inactivation rate, which indicated that NOM acted as a protective agent for viruses at higher salinities.

As salinity increased from 0 ppt to 30 ppt in the experiment, the phiX174 inactivation gradually decreased with a more obvious tailing effect. The tailing effect observed in the inactivation curve could be explained similar to the discussion in section 4.5.2. However, the tailing effect for phiX174 inactivation observed at the same salinity in NOM rich water is less obvious than that in NOM free waters. This difference is presumed to be due to the presence of NOM. No clear aggregation was observed for phiX174 in NOM containing water for all salinities from Figure 4.10. However, the PDI values for all salinities were determined to be greater than 0.5 with most of them

close to 1, which indicate a wide particle size distribution. As salinity increased from 0 ppt to 30 ppt, the PDI value roughly followed an increasing trend. The increase in PDI value showed that the particle size distribution also became wider, which could be due to the increase in salinity and interactions between salt ions, NOM molecules and virus particles.

The $[OH\cdot]_{ss}$ and $[^1O_2]_{ss}$ were found to decrease at higher salinities in NOM rich water. The halides in the aquatic environments have been found to increase both the formation and quenching rates of ROS (Grebel, Pignatello et al. 2012), which might be the reason for the observed decrease in OH▪ and $^1O_2$ steady state concentrations. Large variation in OH▪ and $^1O_2$ steady state concentrations was observed in the experiment. This could be due to the experimental set up where loss of sample volume due to evaporation was possible. As NOM is a photosensitizer and radical quencher, the measurement method of OH▪ and $^1O_2$ steady state concentrations could also lead to the variation.

Higher salinity was found to increase virus-NOM binding affinity in a previous study (Templeton, Andrews et al. 2008). NOM could have provided a sheltering effect for associated viruses from sunlight irradiation and thus, reduced the virus inactivation rate at high salinity.

4.6 Conclusion

UVA-visible light, salinity and NOM can all contribute to virus inactivation in water environments and our results confirmed the interactive effects between these different environmental parameters. The combined effect of these factors on virus survival should not be treated as simple addition of the individual effects. Rather, the inactivation kinetics and mechanism should be determined by the intrinsic properties of the virus species, the characteristics of the virus population which could be affected by salinity and NOM, the 'dose' of 'lethal agent' (e.g. sunlight), aggregation of virus particles, and interaction between the different environmental parameters. Virus survival does not necessarily follow first order inactivation kinetics especially when multiple environmental factors are present. Instead, an sigmoidal survival curve was observed. This should be taken into consideration in modeling of water environments potentially contaminated by viral sources.

5 EFFECTS OF MICROCYSTIS ON PERSISTENCE OF PHIX174 IN AQUATIC ENVIRONMENT

5.1 Abstract

This study aimed to explore the potential impact of algal blooms on the survival and removal of waterborne viruses in aquatic environments by using microcystis and somatic coliphage phiX174 as model algae and model virus. For all the experiments, the growth of microcystis and removal of somatic coliphage phiX174 were closely monitored over time. The phiX174 concentration was determined by double agar layer plaque assay (USEPA 2001), and the microcystis density was determined by optical density (OD) measurement at 678nm. Individual samples were measured before and after filtration through a membrane with pore size of 0.22μm to count for the difference between unassociated viruses suspended in water column and the total viable viruses in the sample. Duplicate samples and duplicate measurements were used for all experiments. The first experiment was performed in the dark at 25 ^{o}C for 8 hours to evaluate the effects of microcystis on the persistence of phiX174 in the absence of algal cell growth. The result showed that over a period of 8 hours, no significant adsorption or inactivation of phiX174 was caused by microcystis. The second experiment was performed under optimum algae growth light condition at 25 ^{o}C for 24 hours. Two microcystis density levels were used for the experiment to

represent moderate and extreme algal bloom conditions. Results showed that over a period of 24 hours and under the experimental condition, no significant inactivation of somatic coliphage phiX174 was caused by microcystis activity. Experiments performed under strong light condition showed that algae could act like a source of NOM for phiX174 inactivation. The presence of algae could also reduce phiX174 inactivation due to light attenuation.

5.2 Introduction

An algal bloom refers to the rapid growth and accumulation of suspended algae or phytoplankton. It has been associated with many water quality deterioration problems such as foul odor and tastes generation, deoxygenation of bottom water, toxicity and food web alteration (Paerl, Fulton et al. 2001). Algal blooms are a natural phenomenon (Anderson, Glibert et al. 2002), but as human activity increases, excessive nutrients released into the environment increase the frequency and severity of these bloom. This phenomenon are believed to expand in a global scale (Anderson 1989; Hallegraeff 1993; Smayda 1997; Carstensen, Henriksen et al. 2007) .

Water borne enteric viruses (rotavirus, adenovirus, enterovirus, astrovirus, etc) are wide spread both in natural and man-made water systems (Haas, Rose et al. 1993; Ottoson and Stenström 2003; Bosch 2010) and pose health risks to humans through drinking water and recreational waters (Gerba, Rose et al. 1996; Crabtree, Gerba et al. 1997; Haas, Rose et al. 1999; Fong and Lipp 2005). To date, however, there have been no studies on the potential effects of algal blooms on the persistence of enteric viruses in aquatic environment.

Algae may affect the persistence of viruses in several different ways. First, the presence of algae may increase the removal of viruses from the water column through adsorption process, but it may also prolong the survival of virus in adsorbed form. Second, algal blooms can reduce sunlight penetration into the water column and thus, may reduce the direct photoinactivation of viruses and prolonging the survival of viruses. Third, algae are suspected to be photosensitizers, producing several different kind of reactive oxygen species (Zepp and Schlotzhauer 1983; Shimada, Akagi et al. 1991). The alteration in ambient reactive oxygen species concentration may thus, affect the exogenous oxidation decay of enteric viruses in aquatic environments.

Algae and enteric viruses have been detected in both reservoir and coastal waters in Singapore (Gin, Lin et al. 2000; Ng, Chan et al. 2005; Aw, Gin et al. 2009; Te and Gin 2011). In this study, we aim to provide insights into the potential effects of algal blooms on enteric virus survival.

Microcystis is a common blue-green algae detected in abundance in Singapore (Te and Gin 2011). It is small, has a spherical shape, and is usually suspended in the water column. Somatic coliphage phiX174 is a commonly used model virus to study the behavior of enteric viruses (Love, Silverman et al. 2010) and it has been detected frequently in Singapore waters (Aw and Gin 2010). The effects of microcystis on the removal of somatic coliphage phiX174 in aquatic environments will be examined in this study.

5.3 Methods

5.3.1 Virus and Host Bacteria Preparation

Somatic coliphage phiX174 (ATCC 13706-B1) and host bacteria *E.coli* CN-13 (ATCC 700609) were obtained from ATCC. Somatic coliphage phiX174 was propagated in the host bacteria, *E.coli,* using the double agar layer method. Briefly, 0.7% TSA mixed with log phase *E.coli* and somatic coliphage phiX174 was overlaid on to 1.5% TSA plates. The plates were then incubated overnight before the top layers from different plates were scraped off. The phiX174-*E.coli*-agar mixture was then centrifuged at 3000 rpm for 20 minutes, and then filtered through a membrane with pore size of 0.22μm. The filtrate was then purified.

PEG and NaCl were used to purify the somatic coliphage phiX174 stock. PEG 8000 (Sigma P5413) and NaCl (Sigma S3014) were added into phiX174 raw stock to form a final concentration of 8% and 0.5M respectively. The mixture was then kept at 4 ^{o}C for 12 hours. The mixture was centrifuged at 14,000 rpm for 60 minutes in 50ml centrifuge tubes (Falcon), and then the supernatant was discarded. The pellet was resuspended in sterile 1mM $NaHCO_3$ solution, and the titer was determined to be 3×10^9 PFU/ml. The purified stock was kept at 4 ^{o}C for experiments.

5.3.2 Algae preparation

The Microcystis culture was obtained from Dr. Te Shu Harn (NUS) and was originally isolated from a local reservoir and cultured in the laboratory. The culture was purified before use. Briefly, the culture was centrifuged at 8000 rpm for 25 minutes, and then resuspended in 1mM $NaHCO_3$ buffer solution.

This process was repeated twice to ensure no residue was left from the algae culture media. The culture was prepared on the day of the experiment.

5.3.3 Virus and Algae Enumeration

The concentration of phiX174 was determined by double agar layer plaque assay as described in USEPA method 1602 (USEPA 2001). The density of microcystis was determined by optical density (OD) at 678nm using a spectrophotometer (Hitachi U-2800).

Somatic coliphage phiX174 concentration was determined with duplicates measurements for duplicate samples. Optical density at 678 nm was measured in triplicate for each sample.

5.3.4 Dark Experiment

In order to evaluate the effect of microcystis cells on virus persistence in aquatic environments without light, microcystis cells were mixed with phiX174 and incubated in the dark for 8 h at room temperature before enumeration. Two sets of samples were prepared for the dark experiment. For each set, 130 ml sterile 1mM $NaHCO_3$ buffer solution was spiked with 100 µl purified somatic coliphage phiX174 stock in a sterile culture flask with a volume of 250ml. 2ml of microcystis stock was added into one flask. An equal amount of 1mM $NaHCO_3$ buffer solution was added to the other flask as control. The solutions were then gently shaken for 1 minute to achieve homogenous mixing. For each set, twelve T_{25} tissue culture flasks (Falcon) each were evenly distributed with 10 ml of the coliphage/microcystis mixture from the respective 250ml culture flask. For each set, every two T_{25} flasks

were grouped into pairs to work as duplicates, and 6 pairs of flasks were prepared for 6 time points.

Samples were placed under dark condition for 8 hours and the temperature was kept constant at 25 ^{o}C. Concentrations of somatic coliphage and algae were determined in duplicate at 6 time points corresponding to 0, 0.5h, 1h, 2h, 4, and 8 hr.

5.3.5 Optimum Algae Growth Light Experiment

Three sets of samples were prepared for the light experiment. For each set, 130 ml sterile 1mM $NaHCO_3$ buffer solution was spiked with 100 µl purified somatic coliphage phiX174 stock in 250ml culture flask. 1.5ml microcystis stock and 4.5 ml microcystis stock were added into two separate 250ml culture flasks respectively. An equal amount of $NaHCO_3$ buffer was added into the third flask which would function as a control for the light experiment. The mixtures were gently shaken for 1 minute to achieve homogenous mixing. For each set, twelve T_{25} tissue culture flasks (Falcon) each were evenly distributed with 10 ml of the respective coliphage/microcystis mixture from the respective 250ml culture flasks. For each set, every two T_{25} flasks were grouped into pairs to work as duplicates, and 6 pairs of flasks were prepared for 6 time points.

Samples were placed under light condition for 24 hours and set at the optimum light intensity for microcystis growth. The temperature was kept constant at 25 ^{o}C. Concentrations of phiX174 and density of microcystis were determined at 6 time points corresponding to 0, 2h, 4h, 6h, 10h, and 24hr.

5.3.6 Strong Light Experiment

In order to examine the impact of microcystis on phiX174 survival under strong light conditions, microcystis and phiX174 culture were mixed and subjected to an irradiation of 450 W/m^2 inside a sunlight simulator. The first experiment intended to explore the relationship between phiX174 inactivation rate constant and microcystis density and concentration of ▪OH. Five microcystis densities were used and the OD for different samples were measured at 678 nm and determined to be 0.08, 0.135, 0.22, 0.32 and 0.45 respectively. The concentration of ▪OH was measured using HPLC with phenol as probe, as described by Kohn and Nelson in 2007 (Kohn and Nelson 2007).

The experiments were run for 2 hours with temperature maintained at 30 ^oC by a chiller. The $\log_{10}(N_t/N_o)$ values were plotted against time and the inactivation rate constant K was plotted against OD.

5.4 Results

5.4.1 Dark Experiments

In order to determine whether the presence of microcystis cells could remove phiX174 from the water column through adsorption or biological processes, we performed the experiments in the dark where purified microcystis was mixed with phiX174 and incubated for 8 hours. The change in phiX174 concentration (log10(Nt/No)) as total viable virus particles (filtration (-)) and non-associated virus particles (filtration (+)) in the presence of microcystis (microcystis (+)) and absence of microcystis (microcystis (-))are shown in

Table 5.1 and Figure 5.1. The microcystis density was monitored over the experiment and shown in Figure 5.2.

The relatively constant OD of microcystis shown in Figure 5.2 indicated that there was no significant change in microcystis density during the experiment. Results from Table 5.1 and Figure 5.1 showed the effects of microcystis on phiX174 survival in the dark. The experiment was carried out for 8 hours, which was sufficiently long to observe particle adsorption. The observations of total viable phiX174 (filtration (-)) in the absence of microcystis indicated that under dark conditions, no significant inactivation or aggregation of phiX174 occurred at room temperature. The $\log_{10}(N_t/N_o)$ of phiX74 in the presence of microcystis showed significant reduction from that in the absence of microcystis (Figure 5.1A) ($p=0.04$), which indicated that the addition of microcystis could have caused adsorption, inactivation or aggregation of phiX174 in the dark and thus, the total viable phiX174 measured became less abundant. This phenomenon could be due to the change in size distribution of phiX174 or the change of phiX174 surface charges. However, a comparison of $\log_{10}(N_t/N_o)$ values of non associated phiX174 (filtration (+))in the presence and in the absence of microcystis (Figure 5.1B) showed no significant difference ($p= 0.29$), which means the addition of microcystis did not cause any adsorption, inactivation or aggregation of non associated phiX174. Results from the dark experiment indicated that the addition of microcystis could not cause significant removal of phiX174 in the water column as non associated form, but it might affect the size distribution or fate of larger viral aggregates and thus, result in a lower total viable virus count.

Table 5.1 Change in phiX174 concentrations $\log_{10}(N_t/N_o)$ in dark conditions. (+/- indicate presence/absence of the factor)

Time (h)		0	0.5	1	2	4	8
Microcystis(-) filtration (-)	$\text{Log}_{10}(N_t/N_o)$	0	0.10	0.07	0.03	-0.18	-0.24
	SD	0.09	0.05	0.03	0.002	N.A.	0.33
Microcystis(+) filtration (-)	$\text{Log}_{10}(N_t/N_o)$	0	-0.02	-0.00	0.03	-0.22	-0.33
	SD	0.12	0.05	0.02	0.02	0.0	0.13
Microcystis(-) filtration (+)	$\text{Log}_{10}(N_t/N_o)$	0	-0.02	0.08	-0.02	-0.17	-0.15
	SD	0.03	0.02	0.01	0.05	0.01	N.A.
Microcystis(+) filtration (+)	$\text{Log}_{10}(N_t/N_o)$	0	0.08	-0.01	0.07	-0.05	-0.13
	SD	0.02	0.06	0.0	0.06	0.00	0.0

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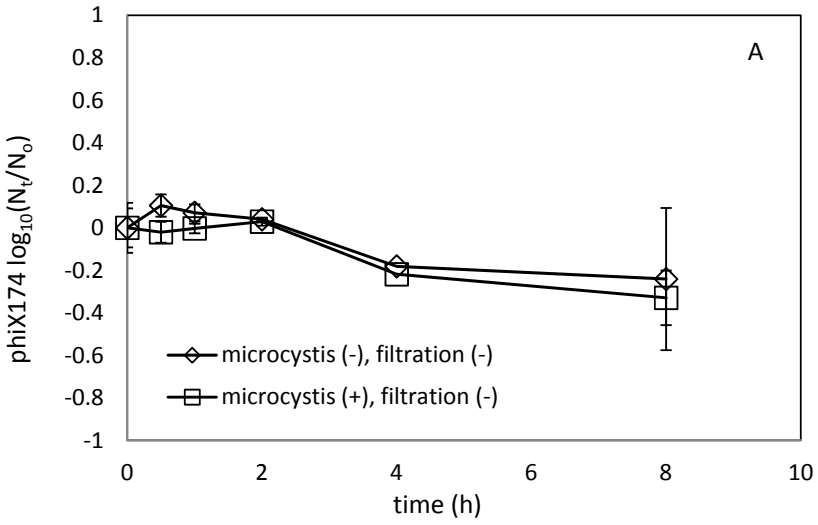


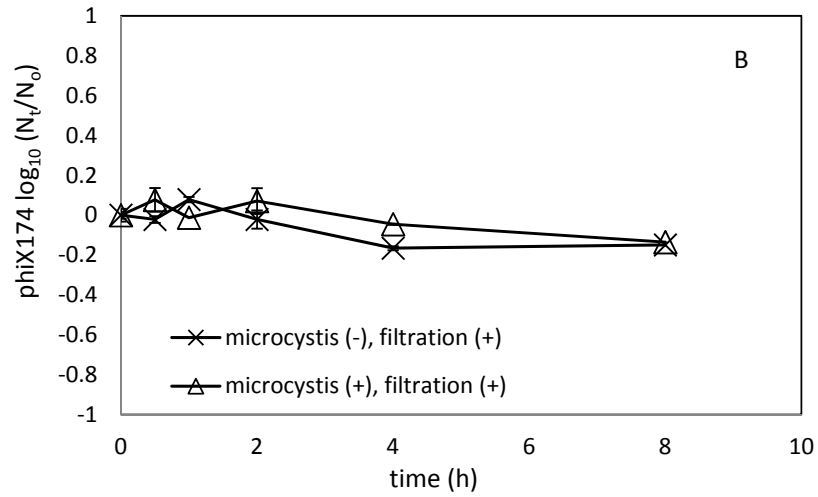


Figure 5.1 Effects of microcystis on persistence of phiX174

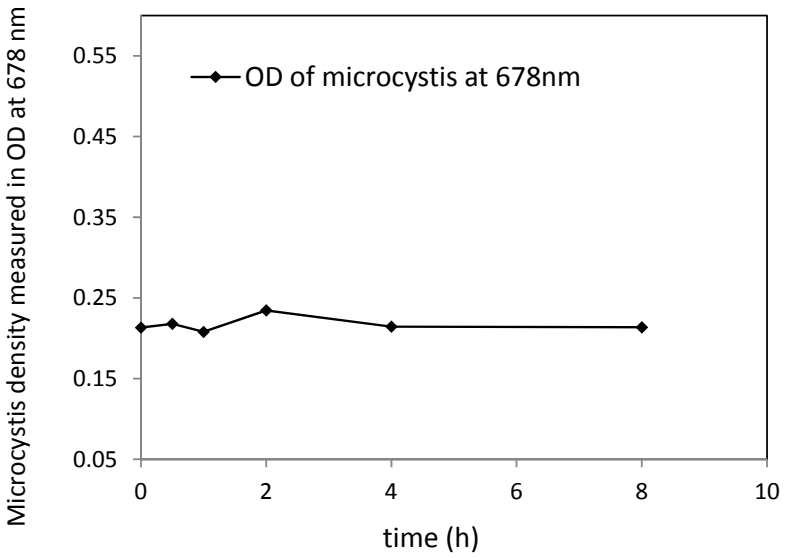


Figure 5.2 Microcystis density measured in OD at 678 nm

5.4.2 Optimum Algae Growth Light Experiment

In order to determine the effects of actively growing microcystis on phiX174 survival in the water column, we performed the experiments under optimum microcystis growth conditions in light where purified microcystis culture was mixed with phiX174 and incubated for 24 hours, which was considered long enough to observe the difference between two experimental conditions while the physicochemical parameters of the setup did not vary too much. The change in phiX174 concentrations ($\log_{10}(N_t/N_o)$) over time is shown in Table 5.2. The total viable phiX174 (filtration (-)) and non associated phiX174 (filtration (+)) survival under optimum growth light for microcystis is shown in Figure 5.3 A and Figure 5.3B.

Table 5.2 Change in phiX174 concentration ($\log_{10}(N_t/N_o)$) under optimum microcystis growth light intensity

Time (h)		0	2	4	6	10	24
Filtration (-) microcystis (-)	$\text{Log}_{10}(N_t/N_o)$	0	0.07	0.30	0.24	0.39	-0.14
	SD	0.06	0.02	0.08	0.04	0	0.12
Filtration (-) microcystis (+) OD 0.158	$\text{Log}_{10}(N_t/N_o)$	0	0.09	0.31	0.30	0.45	0.13
	SD	0.01	0.07	0.03	0.03	0.05	0.02
Filtration (-) microcystis (+) OD 0.45	$\text{Log}_{10}(N_t/N_o)$	0	0.17	0.27	0.32	0.45	0.25
	SD	0	0.05	0.04	0.01	0.11	0.11
Filtration (-) microcystis (-)	$\text{Log}_{10}(N_t/N_o)$	0	-0.05	-0.07	-0.36	-0.28	-0.41
	SD	0.09	0.04	0.02	0.18	0.18	0.07

Filtration (-) microcystis (+) OD 0.158	$Log_{10}(N_t/N_o)$	0	0.06	0.02	-0.22	-0.40	-0.20
	SD	0.02	0.07	0.09	0.45	0.03	0.04
Filtration (-) microcystis (+) OD 0.45	$Log_{10}(N_t/N_o)$	0	0.05	-0.17	-0.29	-0.22	-0.02
	SD	0.01	0	0.14	0.08	0.20	0.01

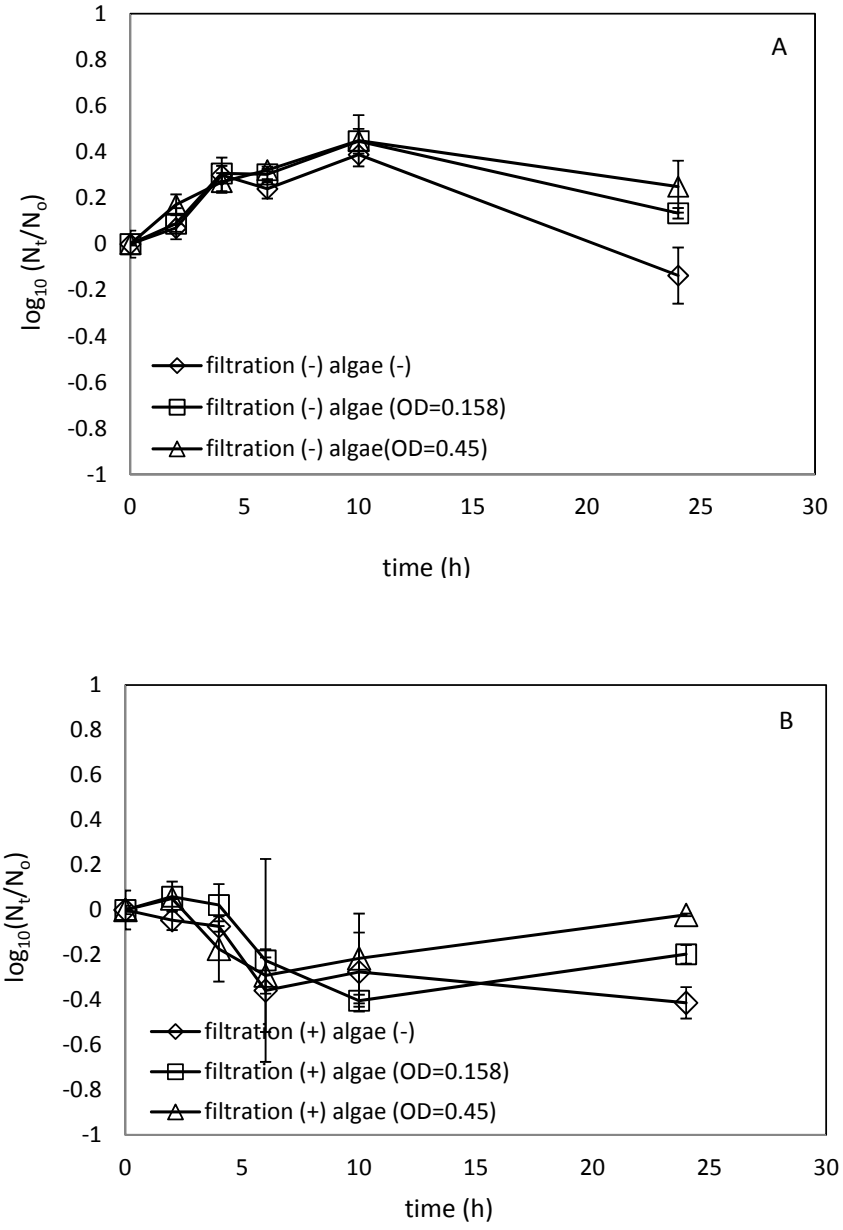


Figure 5.3 Effects of microcystis on persistence of phiX174 under optimum growth light for microcystis

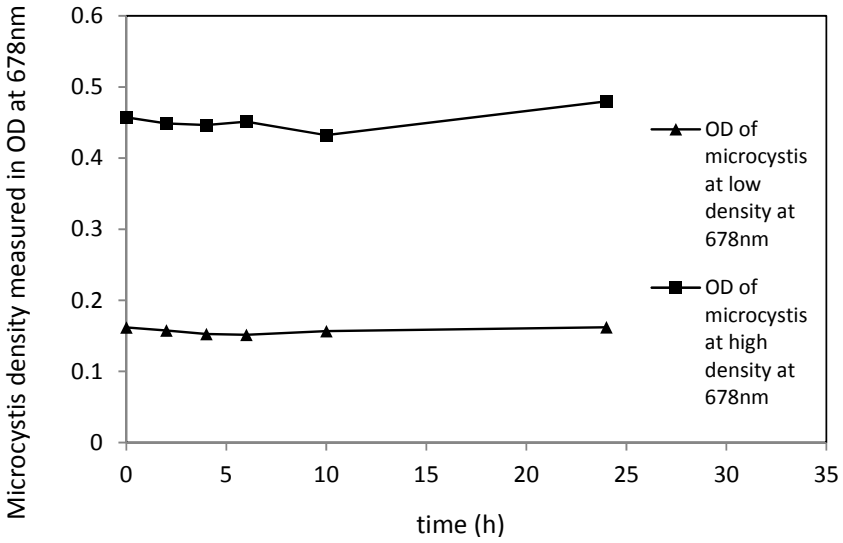


Figure 5.4 Microcystis density measured in OD at 678 nm under light

The ANOVA test showed that there was no significant difference for total viable phiX174 (filtration (-)) under optimum microcystis growth light (Figure 5.3A) (p=0.16) for a microcystis density of OD_{678}=0, 0.158 or 0.45. The observations for non associated phiX174 (filtration (+)) (Figure 5.3B) also showed no significant difference for microcystis at OD_{678}=0, 0.158 or 0.45 (p=0.26). The microcystis density during the experiment is shown in Figure 5.4 where it can be seen that the microcystis density did not experience any noticeable increase or decrease. Results from the optimum microcystis growth light experiment showed that the addition of microcystis at two different levels (OD_{678} =0.158 and 0.45, respectively) did not cause any significant change in phiX174 survival.

5.4.3 Strong Light Experiment

In order to examine the effect of microcystis on phiX174 survival under strong light conditions, we performed experiments with purified microcystis and phiX174 cultures under simulated sunlight at 450 W/m^2. The inactivation of phiX174 with time ($\log_{10}(N_t/N_o)$ at different microcystis densities is shown in Figure 5.5. If the inactivation is considered as first order kinetics, the corresponding inactivation rate constant k is shown in Figure 5.6.

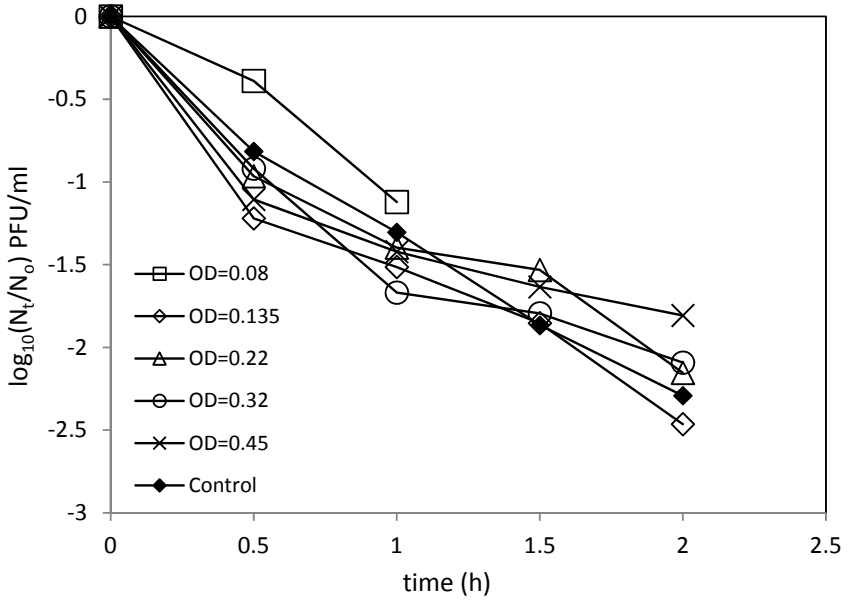


Figure 5.5 Inactivation of phiX174 with different microcystis concentrations at 450 W/m^2

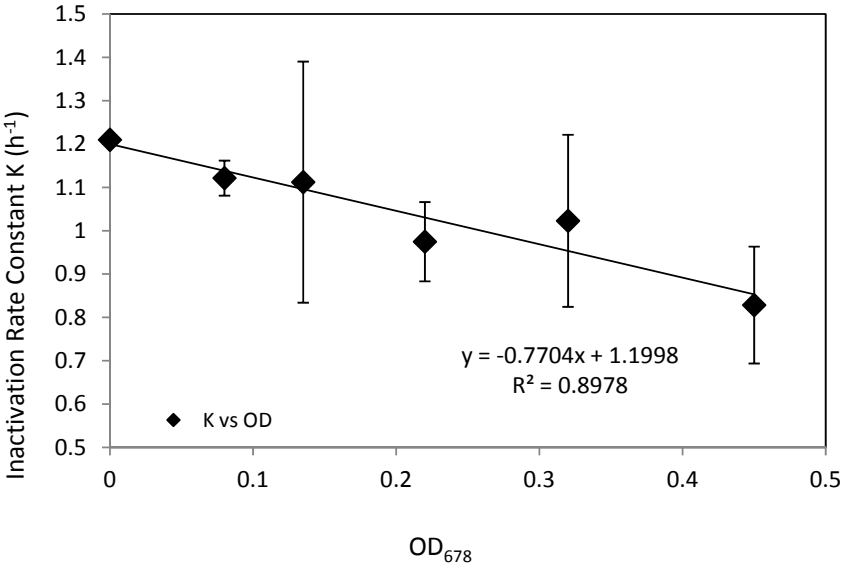


Figure 5.6 phiX174 inactivation rate constant with microcystis at different densities at 450 W/ m^2

Results from Figure 5.5 showed that phiX174 had similar inactivation at different microcystis densities. The inactivation rate constant from Figure 5.6 showed that phiX174 inactivation was slightly linearly related with microcystis density. At higher microcystis density, the phiX174 inactivation rate constant is generally lower. This is supposed to be due to the light scattering and absorbing caused by microcystis particles. The results from strong light experiment indicated that the abundance of microcystis could potentially shield virus from sunlight irradiation and provide a protecting effect.

5.5 Discussion

5.5.1 Adsorption

Results from the dark experiment showed that the addition of microcystis did not cause a significant removal of phiX174 from the water column through adsorption. Microcystis cells are usually negatively charged as their IEP is around 2.2 (Dittrich and Sibler 2005). PhiX174 has an IEP value of 6.6, and thus, is also negatively charged in our experiment water samples (pH=8.6). As both microcystis and phiX174 are negatively charged, adsorption is considered negligible.

5.5.2 Inactivation

Results from the optimum microcystis growth light experiment showed that the actively growing microcystis cells did not cause significant virus removal from the water column. This already was shown by the dark experiment where microcystis cells did not cause significant adsorption of phiX174.

The results from strong light irradiation showed that microcystis cells were lysed to form NOM. This resulted effect on virus inactivation was similar to the effect of NOM from other origins.

5.5.3 Environmental implications

The results from this study showed that the presence of microcystis did not pose any significant impact on the persistence of phiX174 under dark or weak light conditions. Under strong irradiation, microcystis cells lysed and acted as a source of natural organic matter, where its effect on virus removal was similar to the effect of natural organic matter. As the presence of microcystis

can affect light attenuation especially at high densities, it might work as protective mechanism for phiX174 in aquatic environments.

5.6 Conclusion

In this study, microcystis was studied as a model microalgae and its influence on phiX174 was investigated. The experimental results showed that microcystis cells could not remove phiX174 from the water column through either adsorption or inactivation in dark or under weak light conditions. Under strong light, microcystis could be lysed and affect phiX174 survival similar to NOM. Therefore, the presence of algae may potentially prolong virus survival and have a negative impact on microbial water quality.

6 MODELING OF PHIX174 INACTIVATION

6.1 Abstract

A mathematical model was formulated to predict the fate of viruses in environmental waters. Both the Chick-Watson model and Weibull model were used in the study. The experimental results showed that coliphage phiX174 inactivation induced by light at zero salinity and zero TOC followed a log linear relationship, which could be described by first order kinetics. However, the presence of salt or natural organic matter (NOM) influenced the phiX174 sensitivity to light, and resulting in an inactivation with a non log linear relationship. Under these conditions, the Weibull model was more appropriate. The validation experiments performed using environmental water samples showed that the mathematical model could not predict the phiX174 inactivation pattern and rate in environmental waters assuming the Chick-Watson model. However, the Weibull model was used, where sunlight was treated as a 'lethal agent', and salinity and NOM were treated as factors affecting phiX174 'sensitivity to the lethal agent'. Then, the phiX174 inactivation pattern and rate could be predicted satisfactorily.

6.2 Introduction

The presence of waterborne viruses, especially pathogens, can potentially cause human diseases (Leclerc, Schwartzbrod et al. 2002; Sinclair, Jones et al. 2009). In order to improve microbial water quality management, it is important to understand the survival pattern of respective pathogens in water environments. It would even be more beneficial to make predictions of

microbial contaminants concentration based on available microbial data and physicochemical properties of water environment. In this study, we proposed a new model to estimate the fate of viruses depending on the physicochemical environmental parameters.

Chick proposed first order inactivation kinetics in 1908 (Chick 1908) to describe microbial death as an analog to chemical degradation. Under this assumption, all microbial inactivation followed log linear curve which was described by the inactivation rate constant, K. The pseudo first order microbial inactivation kinetics was later developed into the Chick-Watson model which has been widely accepted and used for microbial survival estimation (Chick 1908; Watson 1908). However, since the late 1970s, especially the late 1990s, some researchers have found significant deviation of the experimental microbial survival curve from Chick-Watson model (Peleg and Cole 1998; van Boekel 2002). As a result, a number of different models have been proposed to describe non-log linear relationships such as the Weibull model (Peleg and Cole 1998), log-logistic (Cole, Davies et al. 1993) and Baranyi model (Baranyi and Roberts 1994). Peleg and Cole proposed the Weibull model to describe the more generalized microbial survival kinetics (Peleg and Cole 1998). The model can be expressed as

$$\ln(N_t/N_o)=-b(L)*t\text{^}n(L) \qquad 6.1$$

This model was based on the assumption that the virus 'sensitivity' towards a certain 'lethal agent' followed a Weibull distribution. The 'lethal agent' concentration independent exponent, n(L), defined the shape of the survival curve, and the rate constant, b(L), defined the slope. The overall inactivation

rate constant, b(L), followed a log logistic function and was dependent on its 'sensitivity' to the 'lethal agent' and dose of 'lethal agent' which could be described as k and L respectively. The rate constant b(L) could be expressed as

$$b(L) = \ln\{1+\exp(K(L-L_o))\} \quad 6.2$$

Exposure to sunlight is an effective way to reduce viral contamination in water environments (Fujioka and Yoneyama 2002; Love, Silverman et al. 2010). Other environmental parameters such as salinity, natural organic matter (NOM) and pH were also found to affect the virus survival pattern and rate (Stallknecht, Kearney et al. 1990; Kohn, Grandbois et al. 2007; Mylon, Rinciog et al. 2009; Silverman, Peterson et al. 2013). Sunlight can cause direct damage to the virus genome and thus can be treated as a 'lethal agent' for virus. Salinity and NOM, on the other hand, mainly affect sunlight mediated virus inactivation through alteration of virus 'resistance' to sunlight or indirect virus inactivation under sunlit conditions, even though they may also act as 'lethal agents' under other conditions. In this study, salinity and NOM were treated as 'non lethal agents'. The sunlight mediated virus inactivation in the presence of salinity and NOM was then analyzed by both the Chick-Watson model and the Weibull model. The accuracy and effectiveness of both the models were assessed and compared.

6.3 Methods

6.3.1 Chick-Watson Model

Overall, the phiX174 inactivation was treated as pseudo first order reaction following the Chick-Watson model. K_c was calculated from the slope of the regression line $\ln(N_t/N_o)$ against t (h). The model can be expressed as

$$\frac{dN}{dt} = -k_c N \qquad 6.3$$

or

$$ln\left(\frac{N_t}{N_o}\right) = -k_c t \qquad 6.4$$

The inactivation rate constant, k_c, was calculated as a function of sunlight intensity (W/m^2), salinity (ppt) and TOC (ppm) using multiple non linear regression from SPSS (IBM), which could be expressed as

$$k\_c = f(I, S, TOC)$$
$$= k\_I\ [I] + \mathrm{k}\_s\ [S] + k\_TOC\ [TOC] + k\_(I * S)\ [I][S]$$
$$+ k\_Ia\ [I]\ \exp(-j * [TOC]) + k\_(I * TOC)\ [I][TOC] + k\_(S$$
$$* TOC)\ [S][TOC] + k\_(I * S * TOC)\ [I][S][TOC] + M \qquad 6.5$$

where k_I, k_S, k_{TOC} are the inactivation rate constants due to light intensity, salinity and TOC, respectively. k_{Ia} is the indirect inactivation rate constant due to light intensity, j is the light attenuation coefficient due to TOC, k_{I*S}, k_{I*TOC}, k_{S*TOC}, $k_{I*S*TOC}$ are the inactivation rate constants due to the interaction of light intensity and salinity, light intensity and TOC, salinity and TOC, light intensity, salinity and TOC respectively. M is the inactivation rate constant caused by other environmental parameters.

Environmental water samples were then collected, and virus inactivation experiments were performed using the environmental water samples. The sunlight intensity, salinity and TOC values for these samples were input into equation (6.5). The k_c values obtained from experiments using the environmental water samples and model output were then compared.

The primary virus inactivation kinetics $\ln(N_t/N_o)= f(t)$ was also analyzed using the Chick-Watson model. The instantaneous virus inactivation equation can be expressed as

$$\ln\left(\frac{N_t}{N_o}\right) = -f(I,S,TOC) * t \qquad 6.6$$

6.3.2 Weibull Model

The phiX174 inactivation curve was treated as non log linear and fitted by a Weibull Model. The model can be expressed as

$$ln\left(\frac{N_t}{N_o}\right) = -bt^n \qquad 6.7$$

where

$$b = ln\{1 + exp[k_i(L - L_o)]\} \qquad 6.8$$

where

N = virus concentration (PFU/ml)

b = overall inactivation rate constant

n = overall inactivation exponent

k_i = fluence based virus inactivation rate constant by sunlight (cm^2/J)

In the equation, b (which follows a log logistic distribution) determines the slope of the survival curve, and n determines the overall shape of the survival curve. k_i is the coefficient that determines the dependency of b on the concentration of the 'virucidal factor' concentration, which is determined by the virus 'sensitivity' to the 'lethal agent'. L is the concentration of 'lethal agent', and L_o is the threshold value for the 'virucidal factor' to be effective.

When the Weibull model was used for analysis in this study, sunlight intensity (W/m^2), which can cause direct structural damage to virus, was treated as a 'lethal' agent. The light mediated virus inactivation can be expressed by equation (6.7). Salinity was treated as a factor that affected the virus 'sensitivity' to sunlight, which modified k_i. NOM was treated as a factor that affected the light attenuation in water, which modified L.

The experiment results from Chapter 4 showed that the effect of salinity on sunlight mediated virus 'sensitivity' could be approximately described by equation (6.8), with 'L_o' = 15 ppt.Therefore, the modification on k_i (from equation (6.8)) by salinity could be expressed as

$$m(S) = \ln\left(1 + \exp\left(k_s(S - S_o)\right)\right) \qquad 6.9$$

where

m(S) = modifier of K_i due to salinity (ppt)

k_s = coefficient for virus inactivation dependency on salinity (ppt^{-1})

S =salinity (ppt)

S_o = threshold value for salinity to be effective on virus inactivation

The modification on light intensity L (from equation (6.8)) by NOM (light attenuation) could be expressed as

$$m(TOC) = \exp(-j * [TOC]) \quad 6.10$$

where

m(TOC) = modifier of K_i due to TOC (ppm)

j = light attenuation coefficient due to TOC (ppm^{-1})

In addition to the modification of virus sensitivity and light attenuation, salinity and NOM were also found to affect the heterogeneity of the virus population, thus affecting the shape of the survival curve. The modification of exponent n by salinity and NOM can be described as

$$n(S) = (1 + k_1 * S) \quad 6.11$$

where

n(S) = modifier of n due to salinity (ppt)

k_1= coefficient of virus population heterogeneity dependency on salinity (ppt)

S = salinity (ppt)

And

$$n(TOC) = (1 + k_2 * [TOC]) \quad 6.12$$

where

n(TOC) =modifier of n due to TOC (ppm)

k_2 = coefficient of virus population heterogeneity dependency on TOC (ppm)

By substituting all the terms and including indirect virus inactivation into consideration, the sunlight mediated virus inactivation modified by salinity and NOM can be expressed as

$$\ln\left(\frac{N_t}{N_o}\right) = -\{\ln\langle 1 + \exp\{k_i * \ln[1 + \exp(k_s * (S - S_o))\} * I * \exp(-j * [TOC])\rangle + k_{id} * I * S * [TOC]\} * t^{(n*(1+k_1*S)*(1+k_2*[TOC])} \quad 6.13$$

where

I =sunlight intensity (W/m^2)

k_{id}= indirect virus inactivation rate constant

t = time (h)

All the coefficients were obtained from multiple non linear regressions using SPSS (IBM) based on lab experimental data.

Environmental water samples were collected, and an inactivation experiment was performed in the sunlight simulator. The measured virus inactivation $\ln(N_t/N_o)$ using double agar layer (DAL) method was then compared with the predicted value from equation (6.11).

6.3.3 Regression

Multiple non linear regressions (SPSS (IBM)) were used to estimate the coefficients. SPSS (IBM) was used in the analysis.

6.3.4 Water sample characteristics

The environmental water samples were collected from 9 different surface waters from Singapore. The physicochemical parameters such as TSS, TOC, TN, pH, salinity and total alkalinity were tested by Setsco Ltd. PhiX174 purified stock was spiked into the 9 environmental water samples and subjected to inactivation under sunlight. The inactivation over time was recorded and compared with predicted values from the empirical model. The characteristics of the water samples for the controlled experiment and environmental water samples are shown in Table 6.1 and Table 6.2 respectively.

Table 6.1 Characteristics of water samples for controlled experiment

Sample Name	light intensity (W/m^2)	TOC (ppm)	Salinity (ppt)
A	450	0	0
B	450	1	0
C	450	2	0
D	450	5	0
E	450	7	0
F	450	12	0
G	450	19	0
H	450	29	0
I	450	45	0
J	450	65	0
K	175	0	0
L	245	0	0
M	315	0	0
N	385	0	0
O	455	0	0
P	525	0	0
R	315	0	5

S	315	0	10
T	315	0	15
U	315	0	20
V	315	0	30
W	315	15	0
X	315	15	5
Y	315	15	10
Z	315	15	15
AA	315	15	20
AB	315	15	25
AC	315	15	30

Table 6.2 Characteristics of environmental water samples

Sample Name	light intensity (W/m^2)	TOC (ppm)	Salinity (ppt)	TSS(mg/L)	TN(mg/L)	pH	Total Alkalinity as $CaCO_3$
E1	315	1.2	27.09	12.8	0.39	7.8	101
E2	315	8.05	0.08	11.6	1.11	7.43	69.6
E3	315	1.52	27.81	25.6	0.37	7.94	1.3
E4	315	4.26	0.165	56	2.37	7.28	69.6
E5	315	4.72	0.11	28	1.65	7.75	57.7
E6	315	2.64	0.27	<10	0.91	7.87	58.7
E7	450	15.45	0.27	-	-	-	-
E8	450	9.93	0.106	-	-	-	-
E9	450	4.28	0.078	-	-	-	-

6.4 Results and Discussion

6.4.1 Estimation of overall inactivation rate constant K for pseudo first order kinetics

In order to estimate the virus inactivation rate or virus removal in waters with known physicochemical characteristics, the inactivation rate constant K was assumed to follow pseudo first order kinetics and determined using multiple non linear regression in SPSS as a function of sunlight intensity (I), TOC (ppm) and salinity (S) (ppt).In this analysis, the values of each of the unknown parameters in equation (6.5) were estimated from k under different I, S, TOC combinations using water samples in Table 6.1. The removal of virus over a certain period of time t (h) can be written as a function of k and t, where

k is given by equation (6.5),

$$k = f(I, Salinity, TOC)$$
$$= \mathrm{k}[I] + \mathrm{k}[S] + k_{TOC}[TOC] + k_{I*S}[I][S]$$
$$+ k_{Ia}[I]\exp(-j * [TOC]) + k_{I*TOC}[I][TOC]$$
$$+ k_{S*TOC}[S][TOC] + k_{I*S*TOC}[I][S][TOC] + M$$

The virus removal over a period of time, t (h), can be obtained from equation (6.4),

$$ln\left(\frac{N_t}{N_o}\right) = -k(I, S, TOC) \times t = -k * t$$

The regression results for k of the Chick-Watson equation and corresponding $\ln(N_t/N_o)$ values for water samples in Table 6.1 are shown in Table 6.5 and

Figure 6.1. The estimates of unknown parameters in equation (6.5) are shown in Table 6.3.

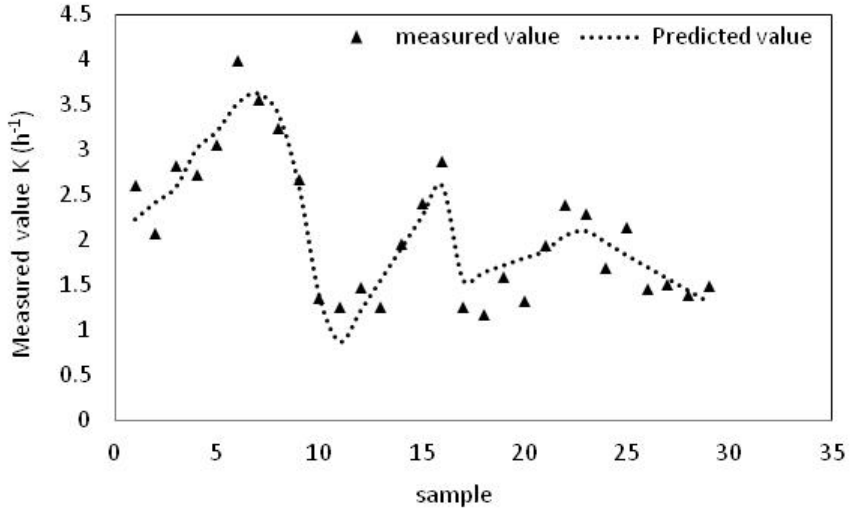


Figure 6.1 Multiple non linear regression for controlled experiment data

Figure 6.1 showed the results from multiple non linear regression in SPSS for water samples in Table 6.1. A total of 28 samples were analyzed in the study. The x-axis represented different water samples from Table 6.1. The first 10 water samples had fixed sunlight intensity and 0 salinity with varying TOC. The following 6 samples had varying sunlight intensity with constant salinity and 0 TOC. The next group of samples had 5 points, and they had varying salinity with constant sunlight intensity and 0 TOC concentration. The last 7 samples had varying salinity with constant light intensity and 15 ppm TOC. The dotted line represented the best fit (R^2=0.878) for the measured values as a function of sunlight intensity (I), salinity (S) and NOM (TOC) (equation (6.5)). The obtained values of k are shown in Table 6.5.

Table 6.3 Parameter estimates for the Chick-Watson Equation based on inactivation rate constant k

Parameter	k_I	k_S	k_{TOC}	k_{Ia}	k_{I*TOC}	k_{I*S}	k_{S*TOC}	$k_{I*S*TOC}$	j	M
Estimates	0.013	-32.318	-0.095	-0.008	6.532E-5	0.103	1.606	- 0.005	0.077	0.001
R^2					0.878					

Table 6.4 Parameter estimates for the Chick-Watson equation based on instantaneous phiX174 inactivation

Parameter	k_I	k_S	k_{TOC}	k_{Ia}	k_{I*TOC}	k_{I*S}	k_{S*TOC}	$k_{I*S*TOC}$	M	j
Estimates	0.012	-0.173	-0.095	-0.007	8.50E-05	0.001	-0.158	0	-0.077	0.089
R^2					0.872					

Table 6.3 shows the estimated values obtained for the unknown parameters in equation (6.5). k_I, k_{I*TOC}, k_{I*S}, k_{S*TOC}, j and M were found to have positive values, which indicated a positive or synergistic effect on virus inactivation. More specifically, the positive k_I indicated that virus inactivation would increase with sunlight intensity, which is consistent with our observations in the experiment. The positive values of k_{I*TOC}, k_{I*S}, k_{S*TOC} indicated that the presence of TOC and salinity would synergistically enhance sunlight mediated virus inactivation, which is also consistent with our experimental observations. Similarly, positive j indicated that the presence of NOM increases light attenuation. The positive M indicated a positive virus inactivation due to parameters that are not included in this study. However, the positive value of k_{S*TOC}, which indicated higher virus inactivation at higher salinity and higher TOC concentration, is contradictory to our experimental findings.

The parameters obtained from the regression in Table 6.3 were input into equation (6.5) and applied to environmental water samples in Table 6.2. The environmental water samples were then used for the phiX174 inactivation experiment. The predicted phiX174 inactivation rate constant k from equation (6.5) and the measured phiX174 inactivation rate constant k are shown in Table 6.6 and plotted in Figure 6. 2.

Results from Figure 6.2 showed the comparison between the predicted values of phiX174 inactivation rate constant k and the measured values. The predicted phiX174 inactivation rate constant k for all the environmental samples were higher than the measured values. The two seawater samples (E1 and E3) exhibited the most significant deviation. For these two samples, the

predicted inactivation rate constant k (h^{-1}) was more than 6 times higher than the measured value. The results for fresh water samples (E2, E4, E5, E6, E7, E8, E9) showed better prediction. The predicted inactivation rate constants for 6 of the 7 samples (E2, E4, E5, E6, E8, and E9) were about twice those of the measured values. The predicted value for sample E7 was found to be very close to the measured value. In general, the predicted values for fresh water samples showed relatively good linearity. This indicates that this method (equation (6.5)) could be applied for phiX174 inactivation estimation in fresh waters, but the final inactivation rate should be used with a factor of at least 2 to ensure a safe prediction.

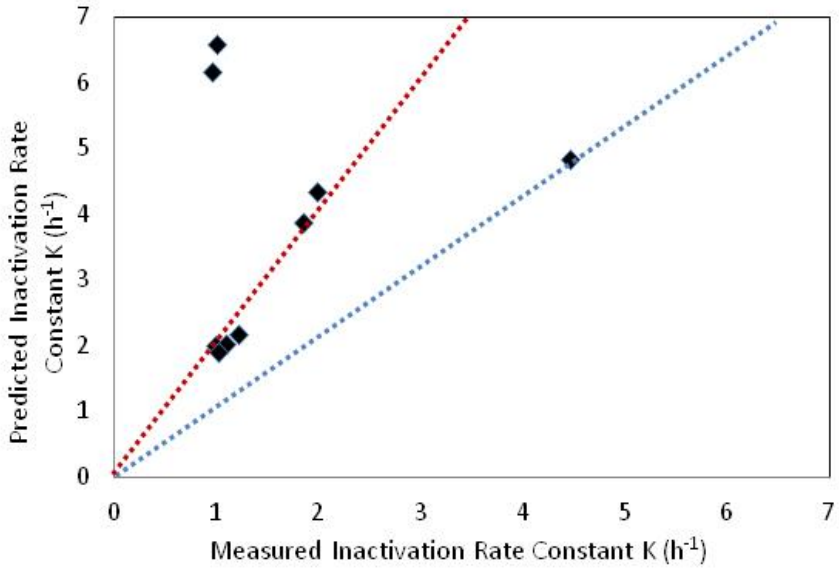


Figure 6.2 Comparison of the predicted and the measured phiX174 inactivation rate constant k

Table 6.5 Comparison of measured inactivation rate constant K, virus inactivation $\ln(N_t/N_o)$ with estimated k and $\ln(N_t/N_o)$ from both the Chick-Watson Equation and the Weibull equation for controlled experiment.

Sample Name	time (h)	measured K (h^{-1})	Chick-Watson Model estimated K (h^{-1})	meausred $\ln(N_t/N_o)$	Chick-Watson Model estimated $\ln(N_t/N_o)$	Weibull Model estimated $\ln(N_t/N_o)$
A	0	2.60	2.23	0	0	0
	0.5			-1.88	-1.14	-1.13
	1			-3.00	-2.28	-2.26
	1.5			-4.29	-3.41	-3.39
	2			-5.28	-4.55	-4.52
B	0	2.07	2.42	0	0	0
	0.5			-1.82	-1.24	-1.2
	1			-2.57	-2.48	-2.39

	1.5			-3.64	-3.72	-3.58
	2			-4.27	-4.96	-4.77
C	0	2.82	2.59	0	0	0
	0.5			-2.08	-1.33	-1.26
	1			-3.13	-2.67	-2.5
	1.5			-5.02	-4	-3.75
	2			-5.59	-5.33	-4.99
D	0	2.73	3.01	0	0	0
	0.5			-1.77	-1.55	-1.4
	1			-2.98	-3.1	-2.78
	1.5			-4.66	-4.65	-4.14
	2			-5.38	-6.2	-5.5
E	0	3.05	3.21	0	0	0
	0.5			-1.28	-1.66	-1.48
	1			-2.86	-3.31	-2.91
	1.5			-4.64	-4.97	-4.34
	2			-5.95	-6.62	-5.75
F	0	3.99	3.52	0	0	0
	0.5			-2.93	-1.81	-1.59
	1			-4.49	-3.62	-3.11
	1.5			-6.61	-5.43	-4.6
	2			-8.12	-7.25	-6.08
G	0	3.56	3.63	0	0	0
	0.5			-1.03	-1.86	-1.63
	1			-3.02	-3.72	-3.15
	1.5			-5.10	-5.58	-4.63
	2			-6.87	-7.44	-6.08
H	0	3.24	3.4	0	0	0
	0.5			-2.34	-1.74	-1.55
	1			-3.69	-3.49	-2.94
	1.5			-5.64	-5.23	-4.27
	2			-6.45	-6.97	-5.56
I	0	2.67	2.6	0	0	0
	0.5			-1.76	-1.38	-1.29
	1			-3.19	-2.76	-2.37

	1.5			-4.50	-4.13	-3.38
	2			-5.31	-5.51	-4.35
J	0	1.36	1.37	0	0	0
	0.5			-1.45	-0.83	-0.97
	1			-1.98	-1.66	-1.72
	1.5			-2.45	-2.5	-2.39
	2			-2.90	-3.33	-3.03
K	0	1.26	0.87	0	0	0
	0.5			-0.79	-0.42	-0.6
	1			-1.77	-0.84	-1.2
	1.5			-2.09	-1.26	-1.79
	2			-2.59	-1.68	-2.39
	3			-3.69	-2.51	-3.59
	4			-4.89	-3.35	-4.79
L	0	1.47	1.21	0	0	0
	0.5			-1.06	-0.6	-0.72
	1			-1.86	-1.2	-1.44
	1.5			-2.64	-1.81	-2.16
	2			-3.15	-2.41	-2.88
	3			-4.18	-3.61	-4.32
	4			-5.62	-4.82	-5.77
M	0	1.26	1.56	0	0	0
	0.5			-1.62	-0.78	-0.85
	1			-2.98	-1.57	-1.71
	1.5			-3.15	-2.35	-2.56
	2			-3.48	-3.14	-3.41
	3			-4.15	-4.71	-5.12
	4			-4.79	-6.28	-6.83
N	0	1.96	1.91	0	0	0
	0.5			-1.41	-0.97	-0.99
	1			-2.33	-1.94	-1.99
	1.5			-3.16	-2.9	-2.98
	2			-3.95	-3.87	-3.98
	3			-5.95	-5.81	-5.96
	4			-7.53	-7.74	-7.95

O	0	2.41	2.26	0	0	0
	0.5			-1.54	-1.15	-1.14
	1			-2.72	-2.3	-2.28
	1.5			-3.88	-3.45	-3.42
	2			-5.03	-4.6	-4.57
	3			-7.16	-6.9	-6.85
	4			-9.37	-9.21	-9.13
P	0	2.88	2.6	0	0	0
	0.5			-2.30	-1.33	-1.29
	1			-3.34	-2.67	-2.59
	1.5			-4.77	-4	-3.88
	2			-6.32	-5.33	-5.18
	3			-8.49	-8	-7.76
	4			-10.96	-10.67	-10.35
R	0	1.17	1.64	0	0	0
	0.5			-1.86	-0.82	-1.05
	1			-2.07	-1.65	-1.93
	1.5			-2.99	-2.47	-2.75
	2			-2.73	-3.3	-3.54
	3			-3.19	-4.95	-5.06
	4			-3.84	-6.59	-6.51
S	0	1.59	1.72	0	0	0
	0.5			-1.71	-0.86	-1.3
	1			-2.31	-1.73	-2.19
	1.5			-3.19	-2.59	-2.98
	2			-4.10	-3.45	-3.7
	3			-4.83	-5.18	-5.02
	4			-5.22	-6.91	-6.23
T	0	1.32	1.8	0	0	0
	0.5			-1.79	-0.9	-1.62
	1			-2.35	-1.81	-2.5
	1.5			-2.79	-2.71	-3.23
	2			-3.48	-3.61	-3.87
	3			-3.97	-5.42	-4.99
	4			-4.16	-7.23	-5.98

U	0	1.93	1.88	0	0	0
	0.5			-3.22	-0.94	-2.01
	1			-3.91	-1.89	-2.85
	1.5			-4.52	-2.83	-3.5
	2			-4.58	-3.77	-4.05
	3			-5.29	-5.66	-4.97
	4			-6.31	-7.54	-5.75
V	0	2.39	2.05	0	0	0
	0.5			-2.50	-1.02	-3.07
	1			-3.74	-2.04	-3.67
	1.5			-4.91	-3.06	-4.08
	2			-5.29	-4.09	-4.4
	3			-7.10	-6.13	-4.88
	4			-8.34	-8.17	-5.26
W	0	2.29	2.1	0	0	0
	0.5			-1.32	-1.07	-1.23
	1			-2.48	-2.14	-2.39
	1.5			-3.60	-3.21	-3.52
	2			-4.64	-4.28	-4.63
	3			-7.12	-6.42	-6.83
	4			-8.78	-8.56	-9
X	0	1.68	1.97	0	0	0
	0.5			-1.28	-0.99	-1.39
	1			-2.09	-1.98	-2.49
	1.5			-3.18	-2.98	-3.5
	2			-3.44	-3.97	-4.45
	3			-5.16	-5.95	-6.26
	4			-6.22	-7.93	-7.97
Y	0	2.14	1.83	0	0	0
	0.5			-1.64	-0.91	-1.58
	1			-2.67	-1.83	-2.61
	1.5			-3.56	-2.74	-3.49
	2			-4.55	-3.66	-4.3
	3			-6.66	-5.48	-5.76
	4			-7.91	-7.31	-7.09

Z	0	1.45	1.7	0	0	0
	0.5			-1.50	-0.84	-1.81
	1			-2.12	-1.67	-2.75
	1.5			-2.54	-2.51	-3.51
	2			-3.24	-3.34	-4.17
	3			-4.31	-5.01	-5.33
	4			-5.29	-6.69	-6.34
AA	0	1.50	1.57	0	0	0
	0.5			-1.27	-0.76	-2.08
	1			-2.00	-1.52	-2.91
	1.5			-2.58	-2.27	-3.54
	2			-3.74	-3.03	-4.07
	3			-5.33	-4.55	-4.96
	4			-4.84	-6.06	-5.7
AB	0	1.38	1.44	0	0	0
	0.5			-1.49	-0.68	-2.4
	1			-2.17	-1.36	-3.09
	1.5			-2.58	-2.04	-3.59
	2			-3.04	-2.72	-3.99
	3			-4.22	-4.08	-4.63
	4			-4.84	-5.44	-5.14
AC	0	1.48	1.31	0	0	0
	0.5			-1.03	-0.6	-2.78
	1			-1.88	-1.2	-3.3
	1.5			-2.21	-1.8	-3.65
	2			-2.43	-2.41	-3.92
	3			-3.83	-3.61	-4.34
	4			-4.54	-4.81	-4.66

6.4.2 Estimation of instantaneous phiX174 inactivation assuming pseudo first order kinetics

In order to predict phiX174 inactivation as a function of environmental parameters, initial concentration and time, a time dependent inactivation model was formulated. The overall inactivation was assumed empirically as a function of each environmental parameter and interaction of different parameters, and the influence of each environmental parameter on phiX174 inactivation was assumed to follow pseudo first order inactivation.

The viable phiX174 ratio after a period of time t (h) can be expressed as a modification of equation (6.14). The parameters in the equation were obtained using multiple non linear regression from SPSS (IBM) by inputting the phiX174 inactivation data into the equation.

$$ln\left(\frac{N_t}{N_o}\right) = -(k_I[I] + k_s[S] + k_{TOC}[TOC] + k_{Ia}[I]\exp(-j * [TOC]) + k_{I*TOC}[I][TOC] + k_{I*S}[I][S] + k_{S*TOC}[S][TOC] + k_{I*S*TOC}[I][S][TOC] + M) \times t \quad 6.14$$

The parameter estimates for equation (6.14) are shown in Table 6.4, and the regression results are shown in Table 6.5 and plotted in Figure 6.3.

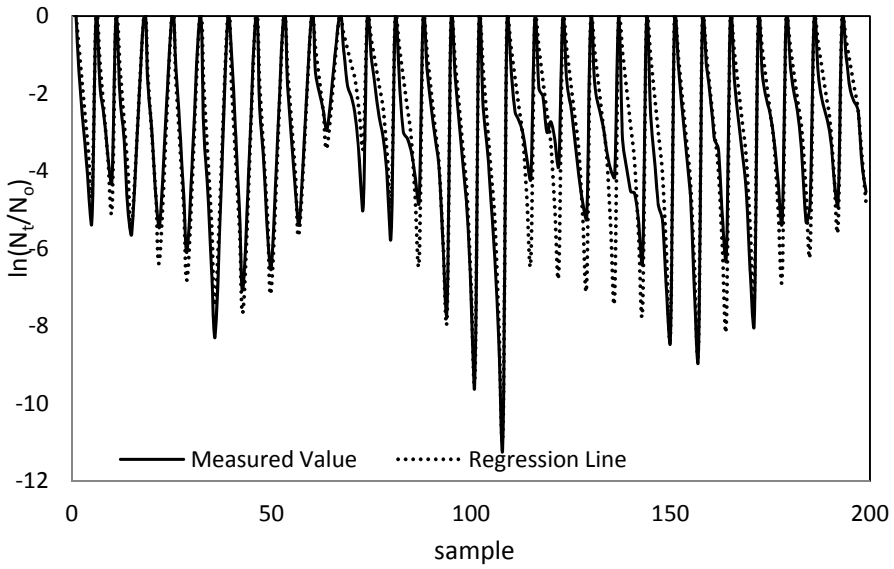


Figure 6.3 Multiple non linear regression of $\ln(N_t/N_o)$ for phiX174 based on the Chick-Watson Equation

Figure 6.3 shows the multiple non linear regression results of $\ln(N_t/N_o)$ with time for water samples in Table 6.1. The x-axis represents all the time points of all the water samples, and the y-axis represents the corresponding $\ln(N_t/N_o)$ value at that time point for that water sample. The solid line in Figure 6.3 represents the measured $\ln(N_t/N_o)$ values, and the dotted line represents the best fit for the experimental measurements assuming equation (6.14). The multiple non linear regression results showed an R^2 of 0.872 (Table 6.4), indicating a good approximation. Results from Table 6.5 show more detail than Figure 6.3. It can be seen that the regression results showed a more accurate prediction for samples with constant salinity. For samples with

varying salinity, the predicted values had a tendency of overestimating the total inactivation. The actual phiX174 inactivation curve differed significantly from the regression curve.

In order to examine the effectiveness of equation (6.14) with parameter estimates in Table 6.4, environmental water samples were tested for phiX174 inactivation. The results are shown in Table 6.6 and Figure 6.4.

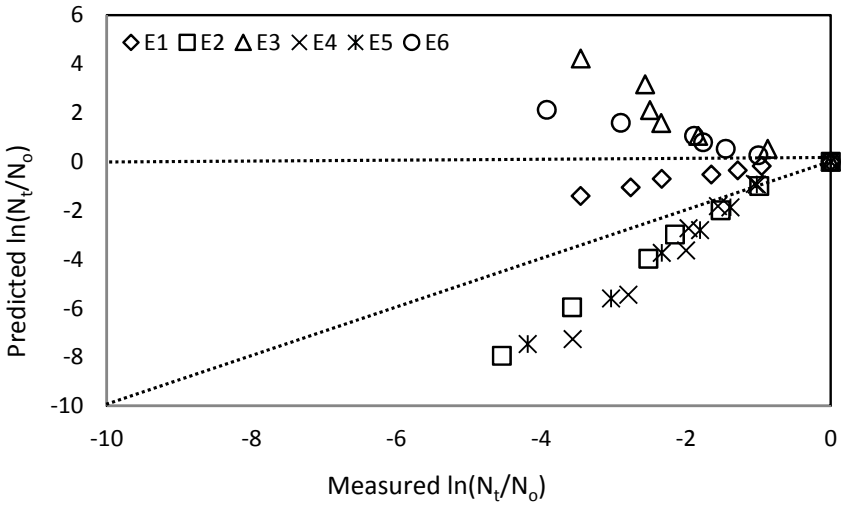


Figure 6.4 Comparison of predicted and measured $\ln(N_t/N_o)$ of environmental water samples based on empirical model from Chick-Watson equation

The predicted $\ln(N_t/N_o)$ values for 6 environmental water samples (E1, E2, E3, E4, E5 and E6) over 4 hours were plotted against the measured $\ln(N_t/N_o)$ values obtained from plate count in Figure 6.4. The results showed a totally random distribution of predicted values. No correlation between the predicted values and measured values was observed. Among these samples, $\ln(N_t/N_o)$ of phiX174 in E3 (seawater) and E6 (freshwater) are predicted positive values, indicating an increase in viable virus count. The predicted $\ln(N_t/N_o)$ values in

E1 (seawater) showed inactivation, but the rate was found to be lower than the measured values. The predicted $\ln(N_t/N_o)$ values for E2, E4 and E5 showed greater inactivation than those that were actually measured. The results indicated that the proposed empirical equation (equation (6.14)) could not predict the instantaneous phiX174 sunlight inactivation in water samples with different salinity and NOM concentrations.

Table 6.6 Comparison of measured inactivation rate constant k, virus inactivation $\ln(N_t/N_o)$ with estimated k and $\ln(N_t/N_o)$ from both Chick-Watson Equation and Weibull equation for environmental water samples

Sample Name	time (h)	measured k (h^{-1})	Chick-Watson Model estimated k (h^{-1})	Measured $\ln(N_t/N_o)$	Chick Watson Model estimated $\ln(N_t/N_o)$	Weibull Model estimated $\ln(N_t/N_o)$
E1	0	0.95	6.16	0	0	0
	0.5			-0.96	-0.175	-0.35
	1			-1.29	-0.35	-0.69
	1.5			-1.65	-0.525	-1.03
	2			-2.34	-0.7	-1.38
	3			-2.77	-1.05	-2.06
	4			-3.46	-1.4	-2.74
E2	0	1.21	2.17	0	0	0
	0.5			-0.99	-0.99	-0.35
	1			-1.52	-1.99	-0.69
	1.5			-2.15	-2.98	-1.03
	2			-2.52	-3.97	-1.37
	3			-3.58	-5.96	-2.05
	4			-4.54	-7.95	-2.73
E3	0	0.99	6.58	0	0	0
	0.5			-0.87	0.53	-0.35

	1			-1.83	1.06	-0.69
	1.5			-2.34	1.58	-1.03
	2			-2.50	2.11	-1.37
	3			-2.57	3.17	-2.06
	4			-3.45	4.22	-2.74
E4	0	0.97	2.01	0	0	0
	0.5			-1.05	-0.91	-0.35
	1			-1.56	-1.82	-0.69
	1.5			-1.97	-2.72	-1.03
	2			-2.00	-3.63	-1.38
	3			-2.80	-5.45	-2.06
	4			-3.57	-7.26	-2.74
E5	0	1.081	2.02	0	0	0
	0.5			-1.02	-0.93	-0.34
	1			-1.39	-1.87	-0.69
	1.5			-1.81	-2.80	-1.03
	2			-2.34	-3.73	-1.38
	3			-3.04	-5.60	-2.06
	4			-4.19	-7.46	-2.73
E6	0	1.008	1.90	0	0	0
	0.5			-1.00	0.27	-0.35
	1			-1.45	0.53	-0.69
	1.5			-1.76	0.80	-1.03
	2			-1.89	1.06	-1.38
	3			-2.90	1.59	-2.06
	4			-3.92	2.13	-2.74
E7	4	4.46	4.84			
E8	4	1.98	4.33			
E9	4	1.84	3.86			

6.4.3 Weibull Model

The Chick-Watson equation considers microbial inactivation as first order decay kinetics. However, we observed a non log linear inactivation curve in the experiments, especially with varying salinity. Therefore, the Weibull

model was used to take into consideration the shape of inactivation curve. The modified Weibull Model which takes into consideration the light intensity, salinity and NOM concentrations is shown as equation (6.13). The instantaneous phiX174 inactivation ($\ln(N_t/N_o)$) obtained in the experiments for water samples in Table 6.1 was input into equation (6.13) for multiple non linear regression analysis in SPSS. Samples K, L, M, N, O and P were used to determine the non modified Weibull parameters (equation (6.7)) for phiX174 sunlight (UVA and visible light) inactivation without the influence of salinity and NOM. The results are shown in Table 6.7.

Results from Table 6.7 showed that R^2=0.91, indicating a good fit for the experimental data. The regression results also showed that n(I), which is the shape factor for Weibull survival curve, was equal to 1 for phiX174 sunlight inactivation. When n(I) =1, the shape of the Weibull survival curve becomes linear and can be represented by first order kinetics (the Chick-Watson model). This finding suggests that the sunlight induced phiX174 inactivation follows first order kinetics and should have a log linear inactivation curve. The deviation from the log linearity observed in experiments is probably caused by the presence of other factors e.g. salinity or NOM. I_o was found to be 0, indicating no threshold value existed for sunlight (UVA and visible light) intensity in causing phiX174 inactivation. The value of k_i is the fluence based phiX174 inactivation rate constant, and it represents how rapidly phiX174 could be inactivated by UVA and visible light irradiation. This value can be directly used to compare phiX174 inactivation under the same irradiation

conditions with other viruses or the virucidal effects of UVA and visible light with other forms of irradiation.

The multiple non linear regression analysis of the instantaneous phiX174 inactivation using Weibull model are shown in Table 6.5 and plotted in Figure 6.5. The parameter estimates for equation (6.13) are shown in Table 6.8.

Table 6.8 showed that an R^2 =0.867 for the regression of instantaneous phiX174 inactivation ($\ln(N_t/N_o)$) was obtained using the Weibull model, indicating a good fit for the samples. The k_i value (k_i=0.011 cm^2/J at 30 ^oC) obtained was slightly smaller than the k_i value (k_i=0.0125 cm^2/J at 30 ^oC) obtained from the direct photolysis experiment in Table 6.7. Similar experiments were performed before, where k values were obtained for coliphage f2, EMCV and rotavirus inactivation (0.27, 0.09 and 0.71 cm^2/J at 40 ^oC respectively) by UVA (Wegelin, Canonica et al. 1994). The k_i value obtained in our experiment is comparable but smaller than the previous experiments. This could be because of the differences in viral species and experimental temperatures. The k_s value was found to be 0.041, representing the 'sensitivity' of phiX174 to changes in water salinity. $K_{id,}$ which represents the indirect phiX174 inactivation, had a value of zero, which means that indirect phiX174 inactivation was negligible compared with direct inactivation under the experimental conditions. K_1 and k_2 are the modifiers of the shape of phiX174 inactivation curve due to salinity and NOM, respectively. The k_1 and k_2 values were both found to be negative. The negative values indicate that the shape exponent n(L) for equation (6.13) is smaller than 1, thus, indicating an upward concave inactivation curve, which is consistent with experimental

observations. The value of j indicates the light attenuation due to NOM, consistent with our previous findings (Chapter 3).

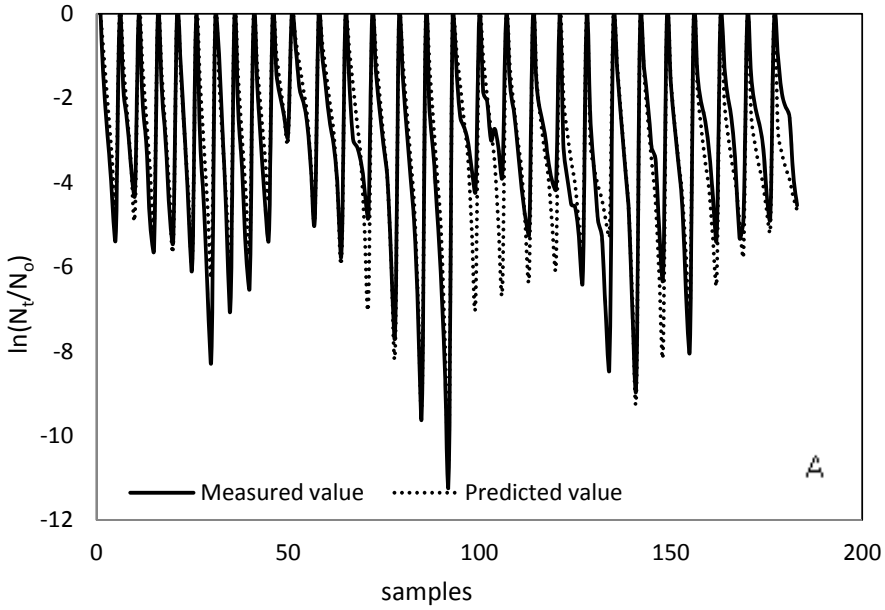
Table 6.7 Weibull model parameter estimates for direct photolysis

Factor	n(I)	I_o	k_i	R^2
sunlight	1	0	0.0125 cm^2/J	0.97

Table 6.8 Parameter estimates for Weibull model for sunlight mediated phiX174 inactivation in the presence of salinity and NOM

Parameter	k_i	k_s	k_1	k_2	j	k_{id}	S_o
Estimate	0.011 (cm^2/J)	0.041	-0.025	-0.003	0.041	0	15
95% CI	(0.01,0.012)	(0.033,0.049)	(-0.029, -0.020)	(-0.009, 0.003)	(0.031, 0.051)	0	-
R^2			0.867				

Figure 6.5A shows the multiple non linear regression of $\ln(N_t/N_o)$ for phiX174 using the Weibull Model for water samples in Table 6.1. The x-axis represents all the time points of all the water samples, and the y-axis represents the corresponding values of $\ln(N_t/N_o)$. The figure (Figure 6.5A) showed a good approximation for most of the samples, which confirms the regression results and the measured $\ln(N_t/N_o)$ values distributed along the 1:1 line (Figure 6.5B).



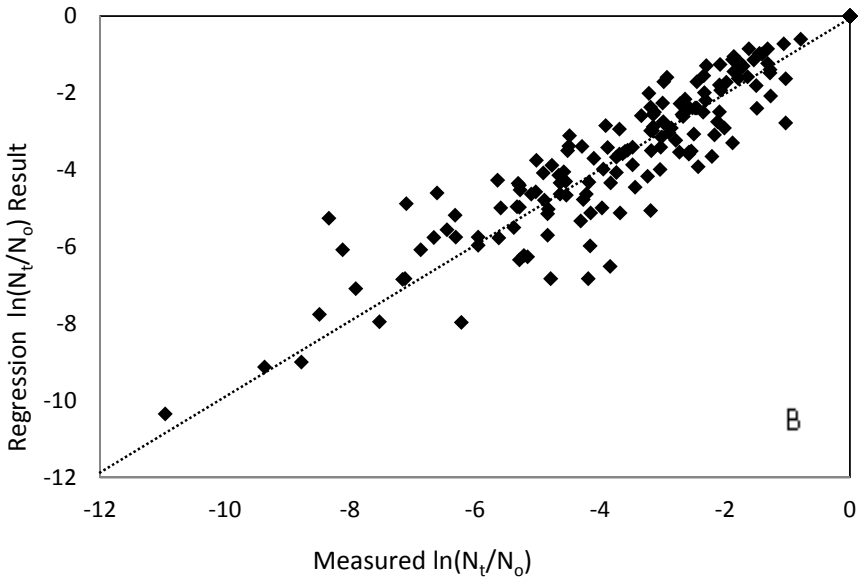


Figure 6.5 Figure 6.5 Multiple non linear regression of phiX174 inactivation using Weibull model (A) illustration of measured and regression values of $\ln(N_t/N_o)$ for all samples; (B) comparison between measured $\ln(N_t/N_o)$ and regression values

In order to examine the effectiveness of equation (6.13), 6 environmental water samples were tested (Table 6.2). PhiX174 inactivation in these environmental water samples determined by plate count was compared with the predicted inactivation using equation (6.13) with parameter estimates from Table 6.8. The results are shown in Table 6.6 and Figure 6.6.

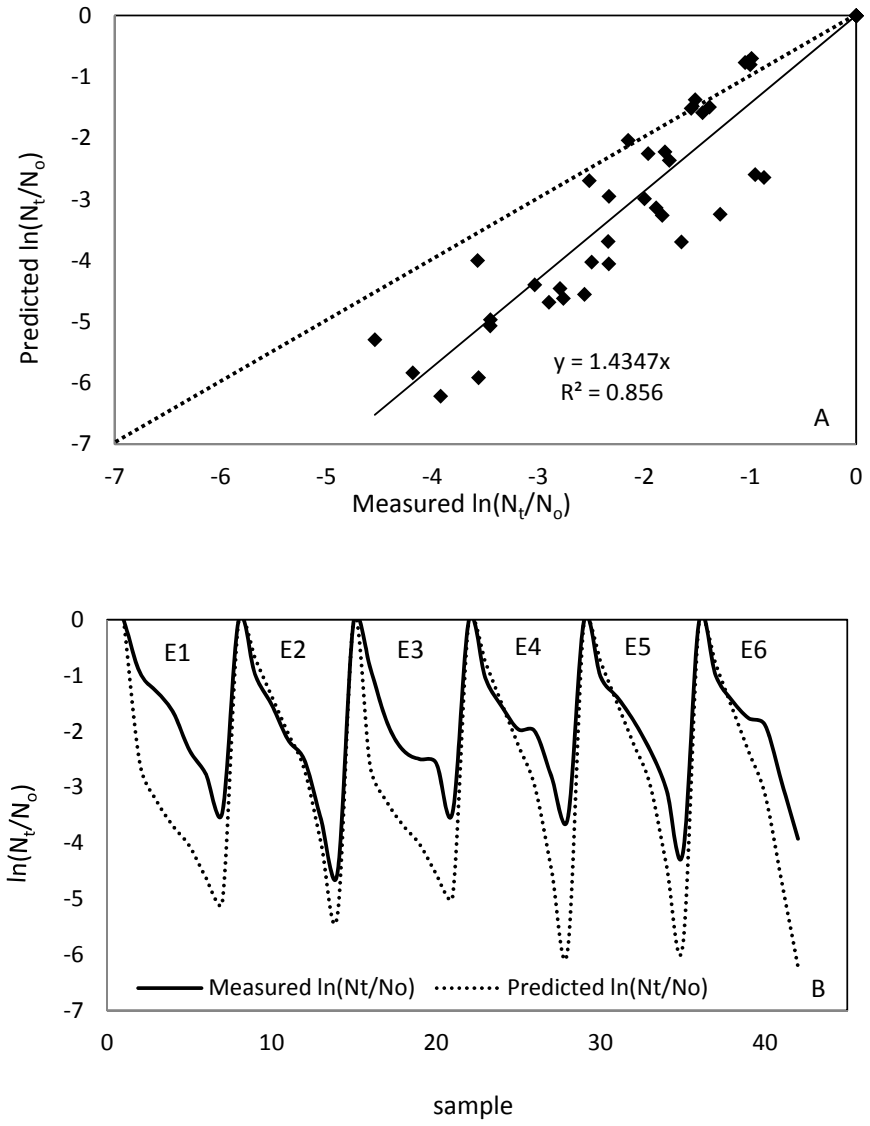


Figure 6.6 Measured $\ln(N_t/N_o)$ and predicted $\ln(N_t/N_o)$ using Weibull model for environmental water samples (A) comparison between measured $\ln(N_t/N_o)$ and predicted values; (B) illustration of measured and predicted values of $\ln(N_t/N_o)$ for all samples

The predicted values of phiX174 inactivation in the environmental water samples were compared with measured values and plotted in Figure 6.6A, with the measured values being the x-axis and predicted values using equation

(6.13) as y-axis. A few points fall on the 1:1 line, indicating a perfect approximation. However, most of the points fall below the 1:1 line, even though they show good linearity with a slope greater than 1 (k= 1.4347). The results indicated that equation (6.13) which was derived from the Weibull Model could predict the virus inactivation pattern well, but it tended to overestimate the inactivation rate. This deviation could be due to the presence of other environmental factors in the water samples that were not included in the controlled experiment. These factors may affect light attenuation (e.g. total suspended solids) or virus 'sensitivity' (i.e. pH and total alkalinity) and thus, result in a lower actual inactivation. Figure 6.6B shows the comparison between predicted inactivation and measured inactivation at each time point for all 6 samples (E1, E2, E3, E4, E5 and E6). The solid line represents the measured $\ln(N_t/N_o)$ values, and the dotted line represents the predicted $\ln(N_t/N_o)$ values using equation (6.13). The shape of the inactivation curve for most fresh water samples (E2, E4, E5 and E6) were predicted well, but a tendency to overestimate was observed. For sample E2, both the phiX174 inactivation rate and shape were predicted very well by equation (6.13). For seawater samples (E1 and E3), the model could predict the inactivation curve well, but overestimated the inactivation rate more significantly than for freshwater samples. These results indicate that the Weibull Model may be a good tool to estimate virus inactivation in aquatic environments. However, more parameters need to be included in the model construction to achieve a better fit for all situations.

In order to improve the Weibull Model fit, we included another environmental factor in the model, i.e. total suspended solids. Total suspended solids (TSS) can be treated as simple light scattering substance and its effect on virus inactivation can be simplified as a modification of equation (3.7)

$$I = I_o \times e^{-(\mu(NOM)\times[NOM])}$$

Taking total suspended solids into consideration, the above equation can be written as follows,

$$I = I_o \times e^{-(\mu(NOM)\times[NOM]+\mu(TSS)\times m(TSS))} \qquad 6.15$$

Substituting values for total suspended solids from Table 6.2 into equation 6.15, and adjusting equation (6.13) by equation (6.15), the following corrected prediction is obtained as shown in Figure 6.7.

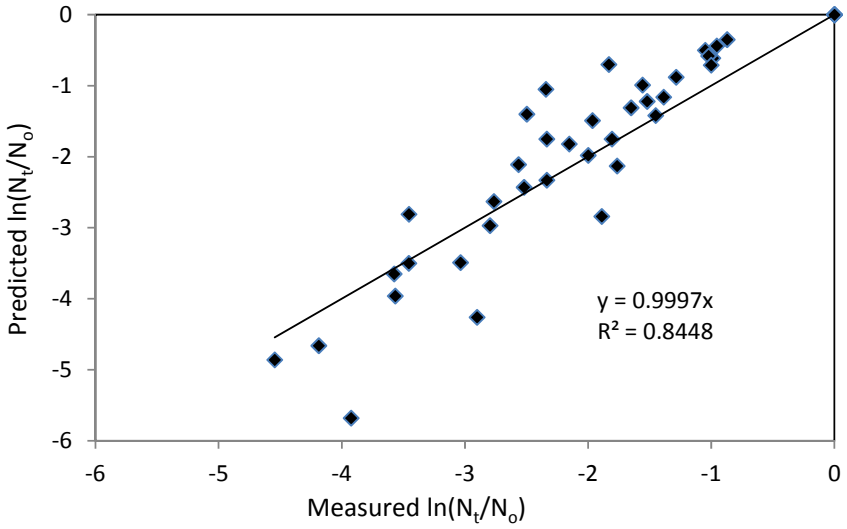


Figure 6.7 Measured $\ln(N_t/N_o)$ and predicted $\ln(N_t/N_o)$ using Weibull Model for environmental water samples taking TSS into consideration

It can be seen from Figure 6.7 that after taking into consideration total suspended solids, the ratio between the predicted values and measured values

was very close to 1. These model results prove that our proposed model equation (6.13) could be used effectively for phiX174 inactivation estimation for environmental waters.

6.4.4 Weibull Model sensitivity analysis

A preliminary sensitivity test was performed to show the change of model output when a certain factor changes. The results are shown in Table 6.9 (A, B, C) and Figure 6.8 (A, B, C).

It can be seen that the impact of sunlight intensity (Figure 6.9 C) on model output was rather constant with time (approximately 5.5 % variation with 20% change in input value). The output value is not as sensitive as input value. The impact of NOM and salinity on model output, however, is dependent on time. The model output is more sensitive with a certain degree of variation in the input value with longer experimental time. The model output is more sensitive to NOM than salinity when exposure time is short (2hr), but it is more sensitive to salinity than NOM when exposure time is long (8hr). This test result is similar with our experimental observations where the tailing effect was mainly caused by salinity. Overall, the model output values are not as sensitive as input values (~6% vs 20% and ~10% vs 20%).

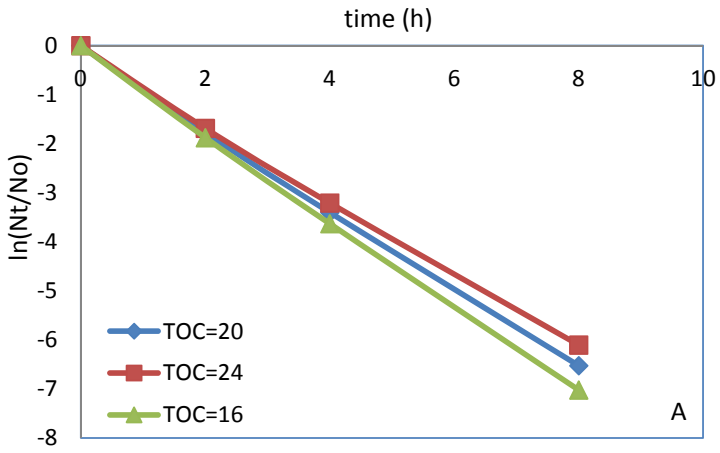
Table 6.9 Change of model output values when input values increase/decrease by 20%, A: NOM; B: Salinity; C: sunlight

Vary TOC	+/- by 20%					A	
Input	N_o	t(h)	S (ppt)	I(W/m^2)	TOC (ppm)	Output ($ln(N_t/N_o)$)	percentage variation

	1000	0	0	200	20	0	
	1000	2	0	200	20	-1.7733	
	1000	4	0	200	20	-3.40212	
	1000	8	0	200	20	-6.52706	
	1000	0	0	200	24	0	0
	1000	2	0	200	24	-1.68683	-4.876%
	1000	4	0	200	24	-3.20942	-5.664%
	1000	8	0	200	24	-6.10636	-6.445%
	1000	0	0	200	16	0	0
	1000	2	0	200	16	-1.87648	5.8187%
	1000	4	0	200	16	-3.63015	6.7026%
	1000	8	0	200	16	-7.02271	7.5938%

Vary Salinity	+/- by 20%					B	
Input	N_o	T (h)	S(ppt)	I(W/m^2)	TOC (ppm)	Output ($\ln(N_t/N_o)$)	percentage variation
	1000	0	15	200	20	0	
	1000	2	15	200	20	-1.62934	
	1000	4	15	200	20	-2.44831	
	1000	8	15	200	20	-3.67893	
	1000	0	18	200	20	0	0
	1000	2	18	200	20	-1.6104	-1.162%
	1000	4	18	200	20	-2.30445	-5.876%
	1000	8	18	200	20	-3.29761	-10.365%
	1000	0	12	200	20	0	0
	1000	2	12	200	20	-1.65121	1.3422%
	1000	4	12	200	20	-2.60543	6.4175%
	1000	8	12	200	20	-4.11109	11.747%

Vary intensity		+/- by 20%				C	
Input	N_o	T (h)	S(pt)	I(W/m^2)	TOC (ppm)	Output (ln(N_t/N_o)	percentage variation
	1000	0	0	200	20	0	
	1000	2	0	200	20	-1.7733	
	1000	4	0	200	20	-3.40212	
	1000	8	0	200	20	-6.52706	
	1000	0	0	240	20	0	0
	1000	2	0	240	20	-1.87182	5.5561%
	1000	4	0	240	20	-3.59114	5.5561%
	1000	8	0	240	20	-6.88971	5.5561%
	1000	0	0	160	20	0	0
	1000	2	0	160	20	-1.67799	5.375%
	1000	4	0	160	20	-3.21927	5.375%
	1000	8	0	160	20	-6.17626	-5.375%



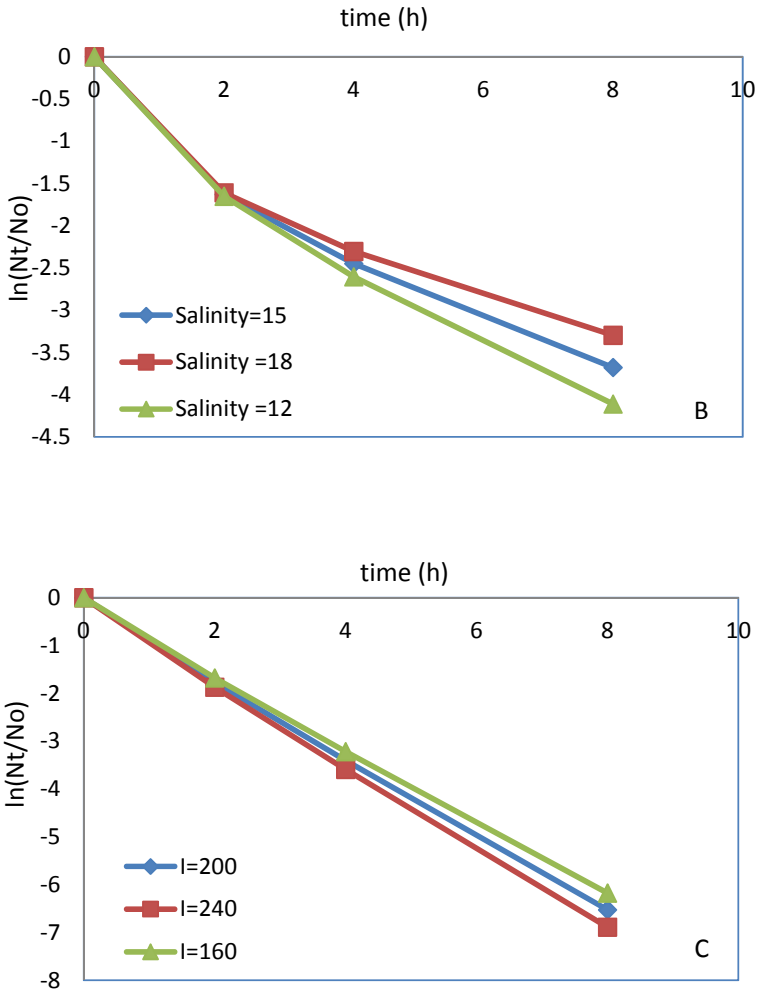


Figure 6.8 Change of model output values when input values increase/decrease by 20%, A: NOM; B: Salinity; C: sunlight

6.5 Conclusion

Comparing the two models, it can be seen that the Weibull model, which took into consideration the heterogeneity of virus populations, the change in virus population sensitivity and the non linear relationship between inactivation and environmental parameter concentration in virus inactivation processes, was a more appropriate model to estimate virus survival based on environmental

factors. When applying this approach, it was important to differentiate the 'lethal agent' from 'non-lethal agent' and categorize the environmental factors accordingly.

7 Conclusion

We investigated the inactivation kinetics and mechanisms of phiX174, a common surrogate virus for enteric viruses, induced by long wavelength sunlight (UVA and visible light) in the presence of natural organic matter (NOM) and salinity. In addition to providing information on the effects of each of the individual parameters on phiX174 inactivation, we also provided insights to the interactive effects of different parameters, revealing a complex virus inactivation mechanism.

The results from this study showed that in addition to the findings from previous studies where UVB was found to be the virucidal wavelengths from sunlight, UVA and visible light could also cause direct inactivation of phiX174, a single stranded DNA virus. Our results provide evidence for direct DNA damage caused by UVA and visible light (which was discovered in 2009).

The presence of NOM was found to influence virus inactivation through affecting light attenuation, generation of reactive intermediates and virus-NOM association. At low NOM concentrations (<11 ppm in this study), the increase in NOM concentration was found to increase virus inactivation. As NOM concentration continued to increase, virus inactivation started to decrease as light attenuation became the dominant process. Our results found that the dependence of virus sunlight inactivation on NOM followed a sigmoidal curve instead of a linear relationship which most previous studies assumed.

Unlike previous studies on salinity where salinity was treated as an equivalent environmental factor as sunlight and had debatable effects on virus inactivation, the results from this study showed that salinity should be treated differently from sunlight. Salinity affects the virus 'sensitivity' to harmful environmental factors such as sunlight, but it does not directly cause virus inactivation. The overall effect of salinity on virus inactivation is due to interactive effects with other parameters. For example, in this study, salinity was found to increase virus inactivation at high concentrations (>15ppt) in NOM free waters. However, the effect of salinity on virus inactivation in NOM rich water is different. In NOM rich waters, the initial increase in salinity from 0 to 15 ppt caused a rapid decrease in virus inactivation, but further increases in salinity did not have any significant impact.

This study has also determined the values of several virus inactivation coefficients, namely k_i (phiX174 inactivation coefficient by UVA and visible light), j (light attenuation coefficient by NOM), k_s (phiX174 sensitivity due to salinity), which can be used for other kinetics and modeling studies.

The presence of microcystis was not found to affect virus survival either through adsorption or inactivation. However, after microcystis cells were lysed, they could affect virus inactivation as NOM.

Based on the controlled experiment involving UVA/visible ight, NOM and salinity, we proposed an empirical model to predict virus survival, which can be written as follows,

$$\ln\left(\frac{N_t}{N_o}\right) = -\{\ln\langle 1 + \exp\{\mathrm{K_i} * \ln\,[1 + \exp(\mathrm{K_s} * (\mathrm{S} - \mathrm{S_o}))\} * \mathrm{I}$$

$$* \exp(-\mathrm{j} * [\mathrm{TOC}])\rangle + K_{id} * I * S * [TOC]\}$$

$$* \mathrm{t}^{(\mathrm{n}*(1+\mathrm{K_1}*\mathrm{S})*(1+\mathrm{K_2}*[\mathrm{TOC}])}$$

Validation with environmental samples showed that the predicted inactivation rates were consistently higher than measured values and this was believed to be caused by the presence of other parameters in the environmental water samples. The corrected model taking into consideration total suspended solids was found to predict virus inactivation rates close to measured values (predicted values/measured values=0.9997, R^2= 0.8448), which can be applied directly and written as follows,

$$\ln\left(\frac{N_t}{N_o}\right) = -\{\ln\langle 1 + \exp\{\mathrm{K_i} * \ln\,[1 + \exp(\mathrm{K_s} * (\mathrm{S} - \mathrm{S_o}))\} * \mathrm{I}$$

$$* \exp(-\mathrm{j} * [\mathrm{TOC}] - \mu(\mathrm{TSS}) * \mathrm{m}(\mathrm{TSS}))\rangle + K_{id} * I * S * [TOC]\}$$

$$* \mathrm{t}^{(\mathrm{n}*(1+\mathrm{K_1}*\mathrm{S})*(1+\mathrm{K_2}*[\mathrm{TOC}])}$$

This model can be used to predict survival of other viruses in the environment with proper adjustment of the parameters. Ultimately, such models could be applied in quantitative microbial risk assessment of recreational waters and thus, serve to protect public health.

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