ROLE OF TEMPERATURE AND SUWANNEE RIVER NATURAL ORGANIC MATTER ON INACTIVATION KINETICS OF ROTAVIRUS AND BACTERIOPHAGE MS2 BY SOLAR IRRADIATION

BY

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THESIS

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ABSTRACT

Indirect (UVA-visible spectrum) damage by exogenous sensitizers, indirect (UVB and UVA) damage by endogenous sensitizers, and direct UVB damage have been identified as three sunlight inactivation mechanisms for pathogens. This study investigated the role of temperature and Suwannee River natural organic matter (SRNOM) for each disinfection mechanism of rotavirus, an enteric virus, and MS2, a bacteriophage. For MS2 solutions irradiated at temperatures from 14-40°C, the inactivation rates followed the order: direct UVB damage > direct UVB damage in SRNOM solution > indirect UVA exogenous damage > indirect UVA endogenous damage. For rotavirus solutions irradiated at temperatures from 32-50°C, the inactivation rates (hr⁻¹) were: direct UVB damage > direct UVB damage in SRNOM solution > indirect exogenous damage > indirect endogenous damage. Below 25°C, the indirect exogenous and endogenous damage were experimentally similar for rotavirus. Above 50°C, rotavirus inactivation was primarily due to heat damage. To further assess the rotavirus exogenous damage, quencher tests conducted at 50°C in the presence of 20 mg/L TOC demonstrate that H₂O₂ is important for rotavirus inactivation at high temperatures. However, other tests conducted with commercial H₂O₂ suggest that it is the role of H₂O₂ via the photo Fenton or Fenton-like pathways that is important, and that ·OH radicals are primarily responsible for rotavirus inactivation. Efforts to quench or inhibit ·OH radical formation with Na-formate and EDTA, respectively, in the presence of 20 mg/L TOC resulted in instantaneous damage to rotavirus. No other data were collected on the direct effects of ·OH radicals. While indirect UVA exogenous damage was significant for MS2 over 14-40°C, this mechanism should be considered for rotavirus
over a higher range of temperature from 32 to 50°C. The observed difference in inactivation of MS2 and rotavirus suggested that it is crucial to investigate disinfection using enteric viruses.
To Mom, the Romero family, and to the loving memory of Dad: *J Guadalupe Romero*
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CHAPTER 1
INTRODUCTION

Wastewater can be appropriately treated for irrigation and thus help to reduce the increasing demand of potable water [1-3]. Although widely used worldwide [4], agricultural irrigation by wastewater in developing countries may not be properly treated at the same scale as in developed countries [5]. The waste stabilization ponds (WSP) method is a promising domestic wastewater treatment technology suitable for places where construction, operation and maintenance costs exceed land costs [6]. When properly designed, operated and maintained, WSP have been shown to provide high quality effluent able to meet the World Health Organization guidelines for safe use of wastewaters [7, 8]. Disinfection of pathogens in WSP by sunlight is capable of achieving significant inactivation of indicator organisms [9-11] and pathogens, including the recalcitrant Cryptosporidium parvum oocysts [12].

Despite the WSP well-established use, a clear understanding of WSP pathogen inactivation mechanisms is not yet available, especially for enteric viruses. Sedimentation of viruses adsorbed to particles was suggested as a primary removal mechanism in WSP [6]. However, a recent study showed evidence of norovirus adsorption to non-settleable particles, which may transport long distances [13]. Temperature, pH, dissolved oxygen, algae and organic matter (which affects light penetration) [14], appear to also play a significant role [10, 11]. Despite the different WSP conditions tested, consistently virus indicators display lower inactivation rates than bacteria indicators due to sunlight damage [9, 15]. Therefore, a better understanding of the sunlight disinfection mechanisms of viruses is needed.
Three sunlight disinfection mechanisms for pathogens have been identified [9]. The first mechanism is direct UVB damage to DNA, which forms pyrimidine dimers leading to the formation of photo-products that block DNA replication [16, 17]. The second mechanism is indirect endogenous damage by UVB and UVA, which initiates photo-oxidation leading to the formation of reactive oxygen species (ROS) that damage internal targets (i.e. nucleic acids, proteins) [18, 19]. The ROS formation is catalyzed by endogenous sensitizers (i.e. quinones, riboflavins) [18, 19]. The third mechanism is indirect exogenous damage by UV and the visible range, which causes photo-oxidative damage of the external structures by ROS formation catalyzed by the external (i.e. NOM, methylene blue, etc) sensitizers in solution [20].

Some of the most comprehensive sunlight inactivation studies of virus indicators reported varying dominant disinfection mechanisms. Studies have shown that RNA bacteriophages are effectively inactivated by the indirect exogenous sunlight contribution [9, 15, 21]. Still, other RNA, somatic phages, and F-DNA phages were shown to only be inactivated by direct UVB radiation in sunlight [9, 15]. A recent study reported that of the ROS produced by the irradiation of NOM, singlet oxygen was the most significant MS2 virus disinfectant [21]. Thus, inactivation of phage MS2 by the ubiquitous exogenous sensitizer NOM could serve as an important disinfection process of viruses in surface waters. Whether enteric viruses will be inactivated by sunlight following the same mechanism as for the MS2 bacteriophage is still unknown.

Rotavirus has been recognized as the most common cause of acute infectious gastroenteritis in children [22]; nonetheless all age groups are affected worldwide [23]. Rotavirus is highly infectious: it spreads via the fecal-oral route and it can retain their
infectivity for several days in water environments [22, 24]. Rotavirus is shed in high concentration \((10^{10}-10^{11} \text{ virions per gram of feces})\) for up to two weeks [22], the minimal infective dose is as little as once cell culture-infective unit [25], and it has been shown to be stable to temperatures up to 40°C and low pH (~3) for more > 24 hrs [26]. All of the documented rotavirus waterborne outbreaks have been associated with direct fecal contamination of a water supply or improper treatment [23]. The World Health Organization and the Australian National guidelines for safe use of wastewaters listed rotavirus as one of two enteric virus indicators in wastewaters as an attempt to correlate enteric viruses with fecal indicators (i.e. E. coli) [27].

Due to its resilient characteristics and current disease burden, particularly in the developing world [28], virus, especially rotavirus, transmission via water environments has been recognized as a potentially significant public health problem, and attempts are being made to develop viral standards for water quality [25]. In an effort to meet the Global Millennium Goals of 2015, WSP may be the primary option for wastewater treatment in developing countries [29]. For this reason, rotavirus is an excellent enteric virus model in the study of sunlight-mediated inactivation of viruses for wastewaters. The objective of this study is to investigate the roles of temperature and SRNOM on the three disinfection mechanisms for rotavirus and MS2 bacteriophage by simulated sunlight.
2.1 Model Viruses: MS2 and Rotavirus

MS2 phage was replicated and purified as described elsewhere [30]. An *Escherichia coli* (ATCC: 15577) stock was grown in tryptic soy broth solution and then inoculated with MS2. The replicated MS2 was purified by sequential centrifugation and microfiltration through 0.45-μm and 0.2-μm low-protein-binding polycarbonate track-etched membranes (Whatman Nucleopore, USA) to remove *E. coli* cell debris. To concentrate MS2 and to remove nutrients, microbial products, and other debris smaller than MS2 phages, a Millipore 100-kDa membrane (Koch Membranes, USA) and ultrafiltration unit (Whatman Nucleopore, USA) was used. The final MS2 stock, with a concentration of \( \sim 10^{11} \) plaque forming units (PFU) per mL, was re-filtered through a 0.2-μm membrane and stored at 4°C in sterilized 1 mM NaCl solution. The size distribution of the final MS2 stock was also measured by a Dynamic Light Scattering (DLS) instrument. MS2 enumeration was performed following the double agar layer procedure [31]. Following the latter procedure, the plates were incubated at 37°C for 16 hours for plaque formation. Only plaques in the range of 10 to 150 were considered for the MS2 enumeration.

Group A porcine rotavirus OSU strain was obtained from the American Type Culture Collection (catalog # VR892). Rotavirus was propagated in embryonic African green monkey kidney cells (MA-104 cells) and was extracted from the cell culture as described elsewhere [32]. Rotavirus was purified following the same protocol as for MS2 phages, except the microfiltration through the 0.2-μm membrane was not
performed. To prevent the denaturation of the outer capsid proteins [33], rotavirus was filtered in 1 mM NaCl and 0.5 mM CaCl₂ during the 100 kDa ultrafiltration. The final purified rotavirus stock (~10⁶ FFU/mL) was re-filtered through a 0.45-um membrane, measured by the DLS for size distribution, and stored in the dark at 4°C. Rotavirus infectivity assays, focus forming unit (FFU) tests, were carried out as described elsewhere [34].

2.2 Model Aquatic Natural Organic Matter

Suwannee River NOM (SRNOM) was obtained from the International Humic Substances Society (IHSS, St. Paul, MN). The procedure for NOM solution preparation was described elsewhere[35], and it consists of mixing 50 mg SRNOM in 50 mL of 1mM NaCl and 2 mM NaHCO₃ solution prepared with Nanopure (Millipore, Barnstead, USA) water of 17.3 MΩ cm resistivity. The SRNOM solution was stirred overnight with a final pH of 7.2. The next day, the SRNOM solution was filtered through a 0.22 μm acetate membrane and stored at 4°C in a 50-mL centrifuge tube covered in foil to protect it from the light. Total organic carbon (TOC) concentration of 230 mg/L for the final SRNOM solution was measured at the Illinois Sustainable Technology Center, at the University of Illinois at Urbana- Champaign.

2.3 Experimental Setup for Virus Inactivation

Solar disinfection experiments were conducted using a Atlas Suntest(r) XLS+ photosimulator (Chicago, IL) equipped with a xenon arc lamp. The solar simulator was also fitted with a window glass filter (Cat. # 56052372) that cuts off most of the
irradiance at 320 nm. Additionally, a second UVB filter (Newport 50.8 x 50.8 mm colored glass filter, FSQ-WG320) that completely covered each reactor, was used to filter out the UVB bleeding of the latter filter. A calibrated StellarNet Inc. spectrometer (S/N: 09110422) was used to measure the irradiance \((W \cdot m^{-2} \cdot nm^{-1})\) of the solar simulator from 280-700 nm. The solar simulator is not capable of irradiating wavelengths below the 280 nm range. The solar simulator intensity was set to 400 \(W \cdot m^{-2}\) for all experiments. The fraction of the UVB (280-320 nm) and UVA (320-400 nm) irradiance measured with and without the Newport filter was 4.71 and 6.24\%, respectively. The fraction of the UVB irradiance of natural sunlight measured at noon on March 5\(^{th}\), 2010 during a clear day in Urbana, IL was 5.86\%. The irradiance above the 320 nm range did not significantly differ between that measured with and without the Newport filter or that of natural sunlight.

The reactors were immersed in an insulated water bath; an Isotemp 3013D refrigerated circulator maintained the temperature at the desired temperature (\(\pm 1^\circ C\)) inside the reactor by pumping thermostated water through stainless steel tubing in the water bath. All solutions were stirred by a Variomag electronic stirrer set to 130 rpm. All solutions for disinfection experiments, unless otherwise indicated, were conducted in 1 mM NaHCO\(_3\) to maintain a circumneutral pH.

For MS2 inactivation, each experiment consists of exposing a 50-mL pyrex beaker with 40-mL of the desired solution composition to the beam for 2-12 hrs. Each 50-mL reactor is covered in black cloth to diminish light reflection. The 40-mL sample consists of the following: 1 mM sodium bicarbonate buffer, SRNOM (0-100 mg/L SRNOM as TOC), and an initial MS2 concentration of \(10^8\) plaque forming units (PFU)
per mL (the stock concentrations are given in the chemical section). 100-μL of the MS2 working solution \(10^{10} \text{ PFU/mL}\) was added to each reactor, thus achieving an initial MS2 concentration of \(\sim 10^8 \text{ PFU/mL}\). For the aggregation experiments, 100-μL of the MS2 stock solution \(10^{12} \text{ PFU/mL}\) was added to each reactor, thus achieving an initial MS2 concentration of \(\sim 10^{10} \text{ PFU/mL}\). The DLS detection limit for MS2 is \(\sim 10^{10} \text{ PFU/mL}\).

For the reactors testing UV-A irradiation conditions, the colored-filter is placed on the reactor to completely cover the stirred solution. For those reactors testing UV-B conditions, the colored-filter was absent. 1-mL sample aliquots were collected in 1.7-mL centrifuge tubes at regular intervals and stored in the dark at 4°C until analyzed (normally within 2-12 hours after the sample collection).

A duplicate reactor, but without the virus spike, was included with each experiment to take temperature readings during each sample acquisition. The same sample volume is also collected from the duplicate reactor, so that all reactors have the same volume throughout the experiment.

For rotavirus inactivation experiments, a similar protocol was used. 0.5-1-mL of the rotavirus stock solution was added to each 10-mL pyrex beaker, thus achieving a \(10^4-10^5 \text{ FFU/mL}\) concentration in each reactor. The 10-mL beaker covered in black cloth is used for the 6-mL sample consisting of the compositions above. To overcome flooding of the 10-mL reactor in the water bath, the 10-mL beaker sits inside a 100-mL beaker with 35 mL of water. 200 μL sample aliquots were collected in 1.5-mL centrifuge tubes at regular intervals and stored in the dark at 4°C until analyzed (within the same day of the experiment).
2.4 Determination of Singlet Oxygen Concentration

Concentration of singlet oxygen produced by the irradiated SRNOM solution was measured by using furfuryl alcohol (FFA) as a quencher. Furfuryl alcohol (98%, Acros Organics) was dissolved in Nanopure water to a stock concentration of 50 mM. The final solution was filtered through a 0.22 µm membrane and stored in the dark at 4°C. 32-µL of the FFA stock solution at 50 mM was added to each 40 mL reactor, which contains 1 mM sodium bicarbonate buffer and SRNOM (0-40 mg/L SRNOM as TOC). Continuous stirring at 130 rpm by a magnetic stir bar allowed for a completely mixed reactor. 1.5-mL sample aliquots were collected in 1.7-mL centrifuge tubes at regular intervals and stored in the dark at 4°C until analyzed (normally within 1-2 days after the sample collection). A duplicate reactor is also included for the temperature readings.

Before the HPLC analysis, the 1.5-mL samples are centrifuged (Thermo: Sorval Legend Micro 17, 2007) at 13.3×Gx1000 min⁻¹ for 30 min. In a dark room, the samples are then transferred to 2-mL amber vials (Agilent Tech. Catalog # 5182-0558). The samples are analyzed by reverse-phase HPLC 1200 Series and an Eclipse XDB-C18 column (Agilent Tech. PN: 993967-902). The HPLC isocratic mobile phase contained 40% acetonitrile (HPLC grade, Acros Organic), 60% Nanopure water, and 0.1% acetic acid (~98% LC-MS grade, Fluka) was pumped at flow rate of 0.41 mL/min. The decay of FFA, singlet oxygen quencher with known quenching rates, was detected at 216 nm wavelength using a diode array detector (DAD). FFA had a retention time of 5.11 ± .05 minutes. The concentration of singlet oxygen was calculated as:

\[
[^{1}O_2]_{ss} = \frac{k_{exp}}{k_2}
\]  
(Equation 1)
where $[^1\text{O}_2]_{ss}$ is the steady state concentration of singlet oxygen (M) in the bulk, $k_{exp}$ is the experimentally determined pseudo-first order reaction rate constant (s$^{-1}$), $k_2$ is the second order reaction rate constant of the reaction between singlet oxygen and FFA (M×s$^{-1}$) [36-38]. Previous studies have reported activation energies of 0.5-8 kJ/mol for $[^1\text{O}_2]_{ss}$ oxidation reactions [38, 39]. Consequently, the temperature dependence of $k_{exp}$ is too low to significantly affect the $[^1\text{O}_2]_{ss}$ calculated by Equation 1. However, the quenching rate of FFA is temperature dependent (activation energy of 22 kJ/mol), and the reported $[^1\text{O}_2]_{ss}$ reflect this dependence [38].

2.5 Kinetic Data Analysis

The pseudo-first order inactivation rate, $k_{obs}$ (hr$^{-1}$), was obtained by finding the slope of the ln(PFU/mL) vs. time (hr) plots. The regression data analysis tool from Excel was used to calculate the $k_{obs}$ values and the corresponding 95% confidence interval of the duplicate samples. For the plots containing SRNOM, the $k_{obs}$ values were corrected for light screening as referenced elsewhere [40]. The spectrums of SRNOM containing solutions were measured using a UV-Vis spectrophotometer (UV 2450, Shimadzu) in 1 cm pathlength cuvettes (Plastibrand).

2.6 Quenching Experiments

To elucidate the disinfectant role of each species, selective quenchers that suppress the steady-state concentration of different ROS were tested. The quencher and respective concentrations used were 50 mM of sodium formate (MP Biomedicals) for OH-, 20 mM of L-histidine (Acros Organics) for $[^1\text{O}_2$, 200 U/mL of catalase (Aldrich) for
H₂O₂, and 5-20 mM of sorbic acid (Aldrich) for dissolved organic matter triplet state species. Additionally, commercially available H₂O₂ (30%, Fisher) and D₂O (99.8%, Acros) were used for direct inactivation of rotavirus.

2.7 Detection of Aggregation by Dynamic Light Scattering

A ZS90 Zetasizer instrument (Malvern, UK) was used to measure the size distribution of the MS2 and rotavirus samples in solution. With the known hydrodynamic diameters of MS2 and rotavirus [41], it was possible to determine the monodispersivity or aggregation of the desired solutions. The detection limit for MS2 and rotavirus in solution was ~10¹⁰ PFU/mL and ~10⁴ FFU/mL, respectively. A minimum of 2 measurements per sample were recorded.

2.8 Measuring Trace Metal Concentrations in SRNOM and Buffer Solutions

Trace metal concentrations were determined using inductively coupled plasma mass spectrometry with an ELAN Dynamic Reaction Cell instrument (PerkinElmer, Norwalk, USA) in the Microanalysis Laboratory at the University of Illinois at Urbana-Champaign. Samples were diluted to a total dissolved solid concentration of 0.25% and the light wavelength intensity from excited atom species was used to determine analyte concentrations.
CHAPTER 3
RESULTS AND DISCUSSION

3.1 Role of SRNOM on the Indirect (UVA) Inactivation of MS2 and Rotavirus at 23°C

3.1.1 Endogenous (UVA) Inactivation of MS2 and Rotavirus at 22-23°C

In the absence of SRNOM, we observed slight endogenous damage (~0.5-log units in at least 9 hrs, \( k_{\text{obs}} \approx 0.3 \text{ hr}^{-1} \)) for both viruses (Figure 1A). As previously reported for other RNA viruses [9, 21], MS2 and rotavirus are resistant to endogenous damage alone. No significant inactivation was observed in the dark in the presence or absence of SRNOM.

3.1.2 Role of SRNOM as an Exogenous Sensitizer (UVA+NOM) for MS2 at 22-23°C

Increasing SRNOM (reported as mg/L TOC) concentrations increased MS2 inactivation as observed for the 0, 10, and 20 mg/L as TOC plots (Figure 1A). The MS2 inactivation rates for the condition with 0, 10, and 20 mg/L TOC were 0.16 ± 0.04 (0.5-log units in 11 hrs), 0.53 ± 0.5 (~2.5-log unit in 11 hrs), and 0.91 ± 0.1 hr\(^{-1}\) (~4-log units in 11 hrs), respectively. No significant MS2 inactivation was observed in the dark for 0 and 20 mg/L TOC.

Previous work showed that the ROS formed by the irradiated NOM, singlet oxygen was the most important disinfectant for MS2 [21]. Therefore the positive increase in inactivation rates as SRNOM increases is attributed to the increase in singlet oxygen formation (Figure 2B). More recent studies showed that MS2 damage is largely linked to \(^1\text{O}_2\) oxidation of the viral capsid, more explicitly to the oxidation of the Cys101 amino acid [42, 43].

3.1.3 Role of SRNOM as an Exogenous Sensitizer for Rotavirus at 22-23°C
Unlike the MS2 virus, the rotavirus data show that there is experimentally no difference in rotavirus inactivation rates between the 0 and 20 mg/L TOC conditions (Figure 1A). It was inferred that rotavirus was resistant to the ROS produced, including [$\cdot^1$O$_2$], by the exogenous processes in the presence of SRNOM.

To elucidate the importance of ROS on rotavirus inactivation, the condition of 20 mg/L TOC in D$_2$O was tested at 22°C. D$_2$O has a lower [$\cdot^1$O$_2$] quencher rate ($k^O_{2O}$ = 1.6 x $10^4$ s$^{-1}$) than water ($k_d$ = 2.5 x $10^5$ s$^{-1}$) [44]. Therefore, when compared to water, the 20 mg/L TOC in D$_2$O increased the [$\cdot^1$O$_2$]$_{ss}$ in solution by a factor of ~10. As shown in figure 1B, the inactivation kinetics for MS2 and rotavirus at 23°C in D$_2$O greatly differ. The inactivation rate ratios between the exogenous (20 mg/L TOC) and endogenous (0 mg/L TOC) conditions were ~12 and ~2.5 for MS2 and rotavirus, respectively. The measured [$\cdot^1$O$_2$]$_{ss}$ at 20°C in the presence of 20 mg/L TOC in water and D$_2$O was 1.3 x $10^{-13}$ M and 1.2 x $10^{-12}$ M, respectively, which corresponds to a nearly 10 fold increase. No significant rotavirus inactivation was observed in the dark.

While MS2 inactivation is directly linked to $^1$O$_2$, rotavirus inactivation due to ROS is not relevant in aqueous conditions at ambient temperatures, but only noticeable at elevated $^1$O$_2$ concentrations (i.e. in D$_2$O). Increasing the TOC concentration does not significantly increase [$\cdot^1$O$_2$]$_{ss}$ in solution. The [$\cdot^1$O$_2$]$_{ss}$ in 20 and 40 mg/L TOC was 1.3 x $10^{-13}$ and 1.5 x $10^{-13}$ M, respectively (Figure 2B). Consequently, in aqueous solution and at ambient temperatures, $^1$O$_2$ formed by SRNOM is not expected to damage rotavirus.
Figure 1. (A) Exogenous and endogenous (UV-A) mediated inactivation and (B) Indirect exogenous and endogenous (UV-A) mediated inactivation kinetics of MS2 and RV at 22°C with varying SRNOM concentrations (0-20 mg/L TOC) dissolved in 1 mM sodium bicarbonate buffer. Sample aliquots were collected and temperature was measured at regular time intervals. The plots with SRNOM were corrected for light screening (UV: 280-400 nm) and the error bars correspond to the 95% confidence intervals.
Figure 2: Singlet oxygen concentration dependence on temperature (A) and (B) TOC. The apparent singlet oxygen concentration ($[^1\text{O}_2]_{\text{app}}$) (M) vs. temperature (°C) plot for 20 mg/L as TOC of SRNOM and 0.5 mg/L Rose Bengal by UVA and UVB irradiation. On the right, the $[^1\text{O}_2]_{\text{app}}$ vs. TOC (mg/L) at 22 and 30 °C by UVA irradiation. For each sample, the 20 mg/L TOC solution was prepared in 1 mM sodium bicarbonate buffer with an initial furfuryl alcohol (FFA) concentration of 40 μM. The temperature in each reactor was measured and sampled at regular time intervals. The data were corrected for light screening (280-400 nm) and the error bars denote 95% confidence intervals.
3.2 Role of Temperature on the Dark and Endogenous (UVA) Inactivation of MS2 and Rotavirus

Upon irradiation in the absence of SRNOM, no temperature dependence was observed for MS2 and rotavirus inactivation rates over the 14 to 43°C range (Figure 3A). Endogenous inactivation rates \((k_{obs} \pm 95\%\ CI)\) for rotavirus and MS2 from 14 to 43°C ranged from \(0.08 \pm 0.02\) to \(0.12 \pm 0.02\) and from \(0.16 \pm 0.04\) to \(0.33 \pm 0.04\ hr^{-1}\), respectively. For rotavirus, in the range of 50-60°C, inactivation dramatically increased with increasing temperature. At 50°C, the \(k_{obs}\) was \(0.23 \pm 0.18\ hr^{-1}\) (0.3-log units in 3 hrs), whereas at temperatures 60°C, heat damage was the dominant disinfection mechanism with \(k_{obs}\) values of \(> 30\ hr^{-1}\), corresponding to complete inactivation (3.5 log units) of all conditions tested within 6 min.

In the dark, the MS2 and rotavirus inactivation rates did not experimentally differ from those of endogenous effects in temperature range considered (14-60°C for rotavirus, 14-40°C for MS2). Dark inactivation rates with and without SRNOM had parallel trends. Therefore, SRNOM in the dark at high temperatures did not appear to have an effect on rotavirus. Consistent with previous temperature studies and reported rotavirus temperature resistance, the increased in temperature from 14 to 43°C did not appear to affect rotavirus over the time scale considered [26]. The study by Wood et al. [26] found rotavirus to remain stable at 30 and 40°C for at least 24 hr in viral growth media. The same study also reported a 1.5-log unit reduction at 50°C in 1 hr (estimated \(k_{obs}\) of \(~3.5\ hr^{-1}\)) and 3.25-log unit reduction at 60°C in 5 min (est. \(k_{obs}\) of \(~90\ hr^{-1}\)). Damage by heat was the predominant disinfectant for rotavirus in the 50 to 60°C range.
The decrease of infectious rotavirus at 50°C is attributed to inactivation and not to changes in the instability of the virus particles in solution, or aggregation. The size distribution of the two viruses at 25 and 50°C (42°C for MS2) was measured by DLS for 4 hrs. No aggregation for any virus was observed by in the time frame considered. Additionally, the dark controls with and without SRNOM at different temperatures for both viruses did not show significant viral decrease. Consequently, aggregation of viruses at different temperatures was insignificant.
Figure 3: (A) UVA and (B) UVB mediated inactivation of MS2 and Rotavirus as a function of time for 14-41 °C temperature range in 1 mM sodium bicarbonate buffer in 0 and 20 mg/L TOC. Each reactor was stirred at 130 rpm and irradiated with a solar simulator with an intensity of 400 Wm⁻². The plots have been corrected for light screening and the error bars correspond to 95% confidence intervals.
3.3 Role of Temperature on the Exogenous (UVA) Inactivation of MS2 and Rotavirus

3.3.1 Role of Temperature on the Exogenous (UVA+ NOM) Inactivation of MS2

In the presence of 20 mg/L TOC, a positive trend (slope of 0.03, $R^2 = 0.99$) between inactivation rates ($k_{obs}$) and temperature increase from 14 to 41°C was observed (Figure 3A). Singlet oxygen has been shown to be the most important disinfectant for MS2 [21]. However, as shown in figure 2A, the $[^1O_2]_{app}$ does not increase as temperature increases, but conformational protein capsid changes, diffusion and chemical reaction rates do [45-47]. The latter may enhance the overall MS2 sensitivity, thus increasing MS2 inactivation. Similarly, the increase in MS2 sensitivity may also enhance the contribution of other ROS on MS2 inactivation.

3.3.2 Role of Temperature on the Exogenous (UVA+NOM) Inactivation of Rotavirus

In the presence of 20 mg/L TOC, a positive trend (slope of 0.014, $R^2 = 0.96$) between inactivation rates ($k_{obs} \text{ hr}^{-1}$) and temperature increase from 14 to 43°C was observed (Figure 3A). The rotavirus slope of the $k_{obs}$ vs. temperature plot is low. Consequently, at temperatures below ~30°C, it was experimentally challenging to distinguish between different temperatures under the same condition. For temperatures higher than ~34°C and below ~50°C, the $k_{obs}$ values from 34 to 50°C increased from 0.3 ± 0.06 to 1.5 ± 0.3 hr$^{-1}$. In the same temperature range (34-50°C), the exogenous contribution exceed the endogenous damage by at least 1.8 times at 34°C and 3 times at 50°C.

Quenching tests were conducted at 50°C to identify which of the ROS was responsible for the inactivation of rotavirus by exogenous effects (see Figure 4). Singlet oxygen was the first suspect as it is responsible for MS2 inactivation. Although the tests
at 23°C in D₂O increased the rotavirus inactivation rate by a factor of 2.5 when compared to the aqueous solutions, the same comparison at 50°C did not show a difference. At 50°C, the leading disinfectant indirectly produced by SRNOM plays a dominant role in rotavirus inactivation. At 23°C the D₂O + NOM experiments were conducted for > 8 hrs to observe a 1-log unit decrease, whereas, at 50°C a ~2.5-log unit difference was observed after 3 hr irradiation in both buffer and D₂O. Additionally, \(^1\text{O}_2\) quencher tests were conducted with L-Histidine (20 mM). The inactivation rates for the conditions with NOM only, NOM in D₂O, and NOM + L-Histidine were experimentally the same, ~1.5 hr\(^{-1}\) (see Figure 4). Thus, the experimental results suggest that singlet oxygen was not primarily responsible for the rotavirus inactivation at high temperatures. Unlike MS2, Rotavirus resistance may be attributed to the inability of \(^1\text{O}_2\) to oxidize important and susceptible amino acids found on the outer-most exposed capsid proteins (VP4 and VP7) [42].

A 50 mM sodium formate was used to quench hydroxyl radicals. However, irradiation of sodium formate damaged rotavirus, as complete inactivation of rotavirus was observed after the first sample collection (36 min irradiation) in two separate experiments. No other information was gathered for this test. However, HO\(^-\) is produced and also efficiently consumed by NOM, which limits its steady-state concentration in waters illuminated by sunlight [48]. Due to the high NOM concentration in the system, HO\(^-\) formed directly by SRNOM was not expected to have a significant effect on rotavirus inactivation.

Concentration of 5 and 20 mM of sorbic acid were also tested at 50°C to quench the dissolved organic matter triplet state (\(^3\text{DOM}^*\)) species [49]. \(^1\text{O}_2\), HO\(^-\), and \(^3\text{CDOM}^*\)
species have been identified as environmentally relevant for their contribution to organic pollutant degradation in sunlit aquifers [48]. Therefore, $^3$DOM could be responsible for rotavirus inactivation in the presence of 20 mg/L TOC at high temperatures. The experiments conducted with sorbic acid concentration of 5 and 20 mM in the presence of 20 mg/L TOC of SRNOM at 50°C were adjusted to a pH of 7.5 with NaOH. The irradiated solutions containing sorbic acid showed immediate inactivation of rotavirus. The solutions that also contained sorbic acid with or without SRNOM that were kept in the dark, showed inactivation rates that were experimentally similar to the dark control in buffer only ($k_{obs} \approx 0.5 \text{ hr}^{-1}$). These results suggest that irradiated sorbic acid damaged rotavirus. No other information was collected from the sorbic acid experiments.

$\text{H}_2\text{O}_2$ was quenched by 200 U/mL of catalase (see Figure 4). The catalase quenching experiment showed that rotavirus $k_{obs}$ decreased to experimentally equivalent $k_{obs}$ values of that of buffer alone ($0.5 \pm 0.3 \text{ hr}^{-1}$). The latter suggests that $\text{H}_2\text{O}_2$ is an important ROS for rotavirus inactivation at high temperatures. $\text{H}_2\text{O}_2$ is the least reactive of the investigated ROS, but is also a promoter of -OH radical formation in the presence of trace metals (via the photo Fenton and Fenton-like processes), which may indirectly influence rotavirus inactivation [50]. The trace metal source could be SRNOM or the sodium bicarbonate buffer. Trace metal concentrations (i.e. Fe$^{3+}$ and Cu$^{2+}$) of $< 1\mu\text{M}$ have shown to be significant in the photo Fenton and Fenton-like pathways [50]. At the time of writing this document, the results from the ICPMS analysis for trace metal quantification of the SRNOM and buffer solutions had not arrived.

To further assess the role of $\text{H}_2\text{O}_2$, experiments were conducted at 50°C with 10 $\mu$M EDTA in 20 mg/L TOC of SRNOM and $\text{H}_2\text{O}_2$ (50 $\mu$M) solutions, respectively. If the
Fenton and Fenton-like process is important, chelating any trace metals in solution should hinder the process. However, EDTA would also chelate Ca$^{2+}$, which is needed to stabilize the structure of infectious particles. Decreasing the concentration of free Ca$^{2+}$ induces solubilization of the outermost capsid proteins [51] responsible for attachment and entry into the cell, thus rendering rotaviruses non-infective. The experiments conducted with 10 μM EDTA showed instantaneous inactivation of rotavirus and no concrete data were gathered.

Additionally, inactivation tests were conducted in the dark at 50°C with commercially available H$_2$O$_2$ at concentrations of 0, 10, 100, and 1000 μM. The tests conducted at 0, 10, and 100 μM H$_2$O$_2$ did not experimentally differ; all showed a similar $k_{obs}$ value of ~ 0.45 hr$^{-1}$ (or 0.5-log units in 3 hr). The condition with 1000 μM H$_2$O$_2$ showed significant rotavirus inactivation, with a $k_{obs}$ value of 5.5± 3.3 hr$^{-1}$ (~2-log units in 1 hr). However, the H$_2$O$_2$ concentration is not expected to greatly exceed 100 μM in 20 mg/L TOC at 50°C. The photochemical accumulation rate of H$_2$O$_2$ in sunlight has been measured for several surface waters and groundwaters and was found to be 2.7 to 48 X 10$^{-7}$ mol L$^{-1}$ h$^{-1}$, in waters ranging from 0.53 to 18 mg L$^{-1}$ TOC, respectively [52]. The H$_2$O$_2$ concentrations have been measured as high as 32.5 μM in sewage ponds.

Nevertheless, the H$_2$O$_2$ accumulation rates over temperature are needed to fully evaluate the effects of H$_2$O$_2$. At the time this document is being written, the reagents needed to conduct the H$_2$O$_2$ accumulation rates vs. temperature have not arrived. Although rotavirus inactivation by the exogenous mechanism could be from secondary reactions, the presence of H$_2$O$_2$ is required.
Figure 4. Quencher tests were conducted under UVA irradiation at 50°C in buffered solutions to identify the ROS responsible for rotavirus inactivation due to exogenous effects. To quench $^1$O$_2$ and H$_2$O$_2$, 50 mM L-Histidine and 200 U/L catalase, respectively, were used. Catalase decreased the rotavirus inactivation rates (hr$^{-1}$) to the those of dark and 0 mg/L TOC conditions, suggesting that H$_2$O$_2$ is an important ROS for rotavirus inactivation. The test conducted in D$_2$O did not increase the inactivation rate when compared to the aqueous condition, suggesting that $^1$O$_2$ is not an important ROS for rotavirus inactivation.
3.4 Role of SRNOM on the Direct (UVB) Inactivation of MS2 and Rotavirus

Direct UVB damage was indisputably more important than UVA inactivation (Figure 3B). The ratio of the SRNOM-free $k_{obs}$ values at 14-43°C between UVB and UVA ranged between 9.7 to 13 for MS2 and 96 to 110 for rotavirus. For all temperatures considered and for both viruses under direct UVB irradiation, the SRNOM containing solutions had lower inactivation rates (0.6-0.8 times) than that of the SRNOM-free solutions, even after accounting for the light screening correction factor (280-320 nm).

UVB has been reported to be the most lethal portion of the sunlight spectrum for most indicator of waterborne pathogens [15, 53]. Although TOC has been the most successful predictor of UV attenuation in aquatic system [54], correcting for light screening does not effectively match the condition of SRNOM-free in solution. Our data suggest that SRNOM in solution may provide some shielding (as a coat) effects for direct UVB inactivation.

Inactivation by UV is largely based on the damage caused to the nucleic acids (DNA/RNA) of the cell or virus [17, 55]. The UV damage follows from the formation of pyrimidine dimers and photo-products of nucleic acids and nucleic acid lesions that hinder replication and transcription, which prevent the virus from multiplying. The UV absorbance of both DNA and RNA peaks at 260 nm [55], however, there is significant absorption at 280 nm. The ratio between the absorbance at 260 and 280 nm is used to assess nucleic acid purity [56]. The absorption of the shorter wavelengths of natural sunlight by the RNA genome is potentially responsible for the rotavirus and MS2 damage.
3.5 Role of Temperature on the Direct (UVB) Inactivation of MS2 and Rotavirus

As temperature increases from 14 to 43°C, the inactivation rates (k\text{obs}) for both viruses also increase (Figure 3B). Rotavirus exhibited a positive trend in the presence (slope of 0.1; R²=0.96) and absence of SRNOM in solution (slope of 0.14; R²= 0.89). Similarly, MS2 showed a linear trend for the solutions with (slope of 0.026; R²=0.96) and without SRNOM (slope of 0.042; R²= 0.96). For rotavirus, the ratio between the k\text{obs} at 43 and 14°C was 1.54 (without SRNOM) and 1.49 (with SRNOM). For MS2, the ratio between the k\text{obs} at 43 and 14°C was 1.2 (without SRNOM) and 1.6 (with SRNOM). The positive correlation between k\text{obs} values and temperature for both viruses is linked to the increase sensitivity of the RNA genomes and viral proteins (i.e. due to protein conformational changes, etc.) to direct UVB irradiation.
CHAPTER 4
CONCLUSIONS

4.1 ENVIRONMENTAL RELEVANCE

The data reported here shows that solar irradiation is important for viral
disinfection via the direct (UVB) or indirect (UVA) mechanisms. For the two viruses
investigated here, direct UVB damage with or without SRNOM was more important than
the UVA via the endogenous or exogenous mechanisms. The ROS formed during the
exogenous mechanism proved to be important for the disinfection of MS2 and rotavirus.
Yet, the exogenous mechanisms for MS2 and rotavirus differed in their dependent on
temperature. For rotavirus the exogenous damage became important at high temperatures
> 32°C, whereas for MS2, the damage was visible at low temperatures (14°C).
Additionally, the most damaging species formed through the exogenous mechanism
varied for each virus: $^1\text{O}_2$ and $\text{H}_2\text{O}_2$ were the most significant of the ROS for MS2 and
rotavirus, respectively. However, whether $\text{H}_2\text{O}_2$ damaged rotavirus directly or indirectly
has not been fully assessed. Work with enteric viruses has the unfortunate disadvantages
of being difficult to cultivate and extremely sensitive to reagents (i.e. EDTA, Na-formate,
sorbic acid), which conventional techniques for chemicals often lack. Based on the data
presented here, we conclude that damage by indirect mechanisms is organism dependent;
therefore it is crucial to study the solar disinfection of individual waterborne pathogens.

4.2 APPLICATION TO WSP

Our data suggest that sunlight irradiation is an efficient rotavirus disinfectant,
even in turbid waters (i.e. maturation ponds, the final pond of WSP configuration).
However, UV radiation is highly dependent on TOC, as it has been identified the most successful predictor of UV radiation attenuation in aquatic systems [57]. Because UVB is more rapidly attenuated by high TOC systems than UVA is [57], the effects of UVB, being the most damaging region of sunlight, would be important in the top region of the pond. Compared to UVA, the effectiveness of UVB on MS2 and rotavirus inactivation was calculated to be up to 10 and 100 times more efficient, respectively. Therefore, a shorter irradiation time (few hrs of full sunlight) would be needed at the very top of pond to achieve the WHO recommendation of 4-log unit pathogen reduction. The effects by UVA would dominate when UVB irradiation becomes attenuated in the water bed. With inactivation rates of $\sim 1$ (4-log unit of infectious particle reduction in 9 hrs) and $\sim 0.3$ hr$^{-1}$ ($\sim 1$-log unit reduction in 9 hrs) for MS2 and rotavirus, respectively, triggered by the exogenous effects at 23°C, 1 to 4 days of full sunlight would be needed to achieve the 4-log unit pathogen reduction. The latter suggest that slow mixing of these ponds to allow the viruses to be exposed to UVB irradiation and/or long retention times would effectively inactivate viruses.

4.3 FUTURE WORK

The next phase of the project will focus on elucidating the rotavirus damaged site by direct UVB and by the indirect exogenous mechanism. Understanding the sunlight disinfection mechanism of rotavirus is crucial in the design of other treatment technologies, including WSP that may exploit the weaknesses of these viruses. TEM, gel electrophoresis, and other similar methods will be used.
REFERENCES


