Supporting Information

Inactivation Kinetics and Replication Cycle Inhibition of Adenovirus by Monochloramine

Aimee M. Gall,[1,3] Joanna L. Shisler,[2,3] Benito J. Mariñas[*1,3]

[1]Department of Civil and Environmental Engineering, [2]Department of Microbiology and College of Medicine, and [3]Safe Global Water Institute, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, United States

***Corresponding Author [**Phone: (217) 333-6961; E-mail: marinas@illinois.edu]

Supporting Information

Summary of Contents:

Text S1. Monochloramine Disinfection Experiments

Monochloramine disinfection experiments were performed in 100 mL batch reactors using reagent grade chemicals in 1 mM carbonate buffered solution (CBS), 1 mM phosphate buffer solution (PBS), or 10 mM borate buffer solution (BBS) (see Table S1). These buffers were used for long-term pH stability, and HAdV-2 inactivation kinetics were comparable to experiments performed with CBS. Monochloramine was preformed immediately before use by slow addition of 50 mL of sodium hypochlorite solution to 50 mL of ammonium chloride solution with vigorous stirring. Prior to virus addition, the initial monochloramine concentration (C_0) was measured using the N,N-diethyl-p-phenylenediamine colorimetric method,[1] and the absence of dichloramines and trichloramines was confirmed by UV-Vis spectroscopy. Batch reactors were continuously stirred by magnetic mixing in a temperature controlled water bath, and temperature and pH were measured before and after each experiment to confirm stability. Immediately after virus addition, a virus sample was taken to determine the initial untreated virus concentration (N_0). Monochloramine was quenched in virus samples taken from the reactor using 0.1% sodium thiosulfate, and the time of quenching was recorded as the sample time. A second reactor containing only buffered water and viruses was used to determine N_0 for rapid inactivation kinetics. The monochloramine concentration was continuously monitored throughout and no measureable decay was observed. The average monochloramine concentration (see Table S1) was used to calculate the *CT*. The role of initial virus concentration (N_0= 2.39×10^4 to 1.39×10^6 PFU/mL) was investigated at pH 8 and 15 °C. Initial monochloramine concentration ranged from 2.71-13.4 mg/L as Cl_2. All experiments to characterize the inactivation kinetics with monochloramine were performed with the same virus stock of HAdV-2. For the mechanistic study, a new stock of HAdV-2 was used after confirming that the kinetics of inactivation matched that of the previous stock. Previous research found that HAdV-2 kinetics were independent of the ammonia nitrogen-to-chlorine molar ratio as long as ammonia is in excess to form monochloramine, and the present study typically used a midrange NH_3-N/Cl_2 molar ratio compared to previous work.[2] Figure S2 shows the effect of pH on the inactivation rate constants (k_1 and k_2) in eq. 2 and 3 solved by simultaneous fitting of all data sets to eq. 1 compared to the inactivation rate constants (k) previously reported by Sirikanchana et al. 2008 solved independently for each pH.

Text S2. Analysis of HAdV-2 Replication Cycle

The analysis of HAdV-2 replication cycle has been described previously and key steps in the HAdV-2 replication cycle are shown in Figure S2.[3] First, virus samples were obtained by treating HAdV-2 from 0-99.99% inactivation by monochloramine at pH 8 and 15 °C. All replication cycle analyses were performed in triplicate. Genomic damage to the amplicon regions was determined by extracting DNA from untreated and treated viruses. Subsequent assays were performed by first synchronizing the HAdV-2 infection in A549 cells. Untreated and monochloramine treated HAdV-2 were inoculated on cell monolayers and allowed to attach at 4 °C for 80 min. Unbound viruses were removed and flasks were incubated at 37 °C to synchronize the entry process. Total viral and cellular DNA and RNA were extracted at 0, 4, 12, 24, and 36 h post infection (p.i.) to quantify HAdV-2 attachment, viral genomic DNA replication, and viral early and late mRNA transcription. DNA and RNA were extracted using the DNeasy Blood & Tissue Kit and the RNeasy Mini Kit, respectively (Qiagen, Valencia, CA) as described previously.[3] All RNA samples were digested with RNase-free DNase I (Qiagen, Valencia, CA) and purified to remove genomic DNA contamination.

Text S3. Quantitative PCR (qPCR) and Two-Step Reverse Transcriptase qPCR (RT-qPCR)

Primer sets were previously designed for E1A and hexon genes in HAdV-2 as well as a cellular gene, β-actin, used as the housekeeping gene (Table S2).[3] Quantitative PCR was used to analyze the amount of E1A, hexon, and β-actin DNA or cDNA using SsoFast EvaGreen supermix (BioRad Laboratories, Inc., Hercules, CA) in methods we previously developed.[3] To analyze the quantity of mRNA present, first 0.5 µg of total RNA was converted by reverse transcriptase to cDNA, then analyzed by qPCR.[3]

Text S4. qPCR Data Analysis

Data obtained from qPCR and RT-qPCR assays was analyzed using the obtained quantification cycle, C_q, for each gene in a sample as previously described.[3] After running qPCR or RT-qPCR with each primer set for a given sample, the relative quantification and absolute quantification

were determined. The relative expression level, *R*, of DNA or mRNA was calculated using the $\Delta\Delta C_q$ equation:

$$R = 2^{-\Delta\Delta C_q} = 2^{-(\Delta C_{q,\text{sample}} - \Delta C_{q,\text{calibrator}})}$$

where the values ΔC_q are obtained as $\Delta C_{q,sample} = (C_{q,\text{sample}} - C_{q,\text{housekeeping\_gene}})$, and the as $\Delta C_{q,calibrator} = (C_{q,\text{N0}} - C_{q,\text{housekeeping\_gene for N0}})$. The housekeeping gene was β-actin, and the calibrator was the untreated virus sample (N_0). The relative expression level, *R*, is represented in the main text by the name of the gene for a particular sample divided by the untreated sample, $Gene/Gene_0$, for ease of comparison with the survival ratio, N/N_0. For example, the relative quantity of E1A DNA or mRNA at any time post infection for a monochloramine treated sample is represented by $E1A/E1A_0$, and for an untreated sample the value would be equal to one. Similarly, the C_q values obtained were also used to determine the absolute copy number of the gene in a sample. Standard curves were previously established and the resulting equations for each gene are shown in Table S2 with efficiencies of 93.6% for E1A, 87.0% for hexon, and 86.2% for β-actin.[3] The absolute quantification equations were used for solving the number of gene copies of DNA or mRNA in a sample. The housekeeping gene β-actin was used in all relative quantification analyses, and the absolute quantification of β-actin mRNA remained constant throughout all times post infection as shown in Figure S2.

Absolute quantification samples of genome replication were analyzed using a one-tailed *t* test to determine if genomic equivalents detected at 12, 24, and 36 h p.i. were statistically greater than the original baseline copies present at 4 h p.i. Resulting $P > 0.1$ were determined to be statically similar and are indicated by **ν** in the top plot of Figure 3. These samples had not replicated enough genomic DNA to be detected above the baseline and are therefore omitted in the relative expression analysis in Figure 2 (middle plot).

To determine correlations between relative quantification and the survival ratio, statistical analyses were performed using SigmaPlot V12 (Systat Software, Inc., San Jose, CA). The Pearson product moment correlation coefficient (*r*) was calculated between N/N_0 and each relative gene quantity at each time p.i. with $P < 0.05$ representing a significant correlation. A parallel line analysis then determined if the linear regression slopes were statistically similar ($P > 0.05$) between the slope of N/N_0 and the relative gene quantity.

Table S1. Experimental conditions for monochloramine disinfection of HAdV-2

pH	Temp (°C)	Reactor	C_0 (mg/L as Cl_2)	$C_{average}$ (mg/L as Cl_2)	N_0 (PFU/mL)	NH_3-N/Cl_2 molar ratio
6	5	1 mM CBS	10.40	10.30	7.63E+05	3.0
6	5	1 mM PBS	9.37	9.05	9.50E+05	3.3
6	5	1 mM PBS	11.02	10.52	1.33E+06	2.8
6	5	1 mM PBS	10.40	10.13	2.31E+06	3.0
7	5	1 mM PBS	12.11	12.04	1.06E+06	2.5
7	5	1 mM PBS	12.21	11.79	1.04E+06	2.5
8	5	1 mM CBS	9.66	9.54	1.03E+06	3.2
8	5	1 mM CBS	13.35	13.12	9.13E+05	2.3
9	5	10 mM BBS	10.83	10.59	1.81E+06	2.8
9	5	10 mM BBS	12.61	12.30	1.48E+06	2.4
10	5	10 mM BBS	10.31	10.22	1.31E+06	3.0
10	5	10 mM BBS	11.90	12.23	1.59E+06	2.6
6	15	1 mM CBS	4.82	4.77	8.13E+05	3.2
6	15	1 mM PBS	9.16	8.93	5.13E+05	6.7
6	15	1 mM PBS	8.98	8.73	1.50E+06	3.4
6	15	1 mM PBS	10.12	9.83	2.81E+06	3.0
7	15	1 mM PBS	9.37	9.27	8.75E+05	3.3
7	15	1 mM PBS	9.90	9.69	2.31E+06	3.1
7	15	1 mM PBS	9.30	9.18	1.34E+06	3.3
8	15	1 mM CBS	3.14	3.08	5.50E+05	3.3
8	15	1 mM CBS	8.61	8.61	6.38E+05	3.6
8	15	1 mM CBS	10.40	10.13	1.06E+06	3.0
8	15	1 mM CBS	10.01	9.91	1.39E+06	3.1
8	15	1 mM CBS	11.78	11.80	1.70E+05	2.6
8	15	1 mM CBS	12.07	11.77	2.39E+04	2.6
8	15	1 mM CBS	5.11	5.00	1.38E+06	2.0
*8	15	1 mM CBS	10.74	10.49	1.03E+06	2.9
*8	15	1 mM CBS	10.55	10.40	9.88E+05	2.9
*8	15	1 mM CBS	10.36	10.21	7.63E+05	3.0
9	15	1 mM CBS	8.86	9.29	9.63E+05	3.5
9	15	1 mM CBS	10.33	10.13	1.18E+06	3.0
9	15	10 mM BBS	12.40	12.26	1.00E+06	2.5
10	15	1 mM CBS	9.87	9.87	7.88E+05	3.1
10	15	10 mM BBS	12.19	12.16	9.00E+05	2.5
10	15	10 mM BBS	12.83	12.52	1.31E+06	2.4
10	15	10 mM BBS	8.88	8.71	1.41E+06	3.5
6	30	1 mM CBS	2.71	2.55	5.69E+05	3.8
6	30	1 mM CBS	3.16	3.10	8.88E+05	3.3
6	30	1 mM CBS	9.83	9.77	1.13E+06	3.1
6	30	1 mM PBS	3.33	3.24	3.58E+06	3.1
6	30	1 mM PBS	3.09	2.97	1.81E+06	3.3
7	30	1 mM CBS	2.71	2.61	8.25E+05	3.8
7	30	1 mM CBS	9.87	9.52	1.63E+06	3.1
8	30	1 mM CBS	2.92	2.81	1.04E+06	3.5
8	30	1 mM CBS	3.06	2.97	7.75E+05	3.4
8	30	1 mM CBS	4.96	4.77	7.88E+05	3.1
8	30	1 mM CBS	9.41	9.14	1.18E+06	3.3
9	30	1 mM CBS	2.71	2.61	1.13E+06	3.8
9	30	1 mM CBS	4.77	4.56	9.88E+05	3.2
10	30	1 mM CBS	3.09	2.85	5.38E+05	3.3
10	30	1 mM CBS	5.23	4.95	1.01E+06	3.0

*Indicates experiments used for qPCR based studies

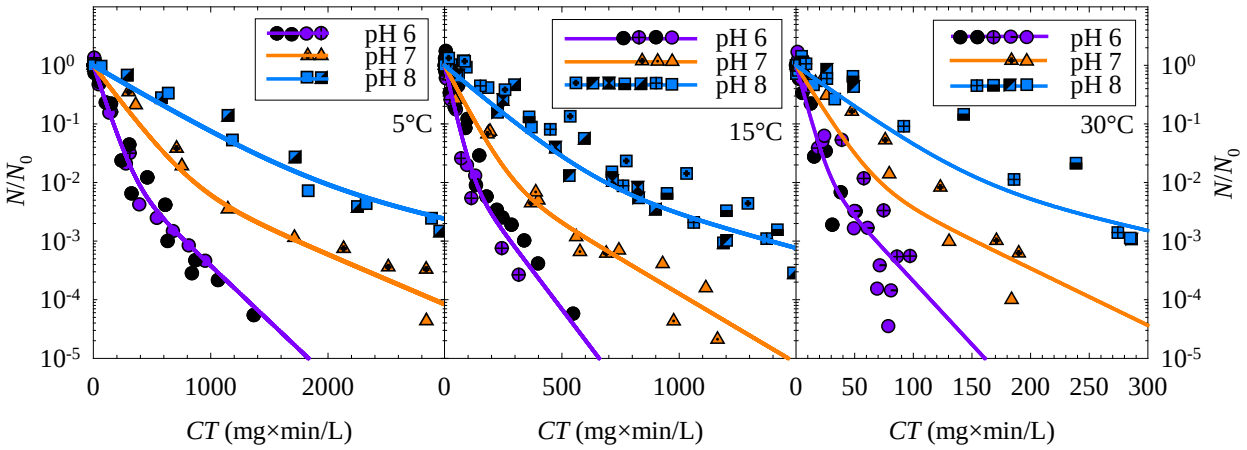

Figure S1. Effect of pH 6-10 on the inactivation kinetics of HAdV-2 by monochloramine at 5 °C, 15 °C, and 30 °C. Replicate experiments are indicated with different symbols. This figure is a reproduction of Figure 1 in the main manuscript with the horizontal axis expanded for ease of comparing model and data at pH 6-8.

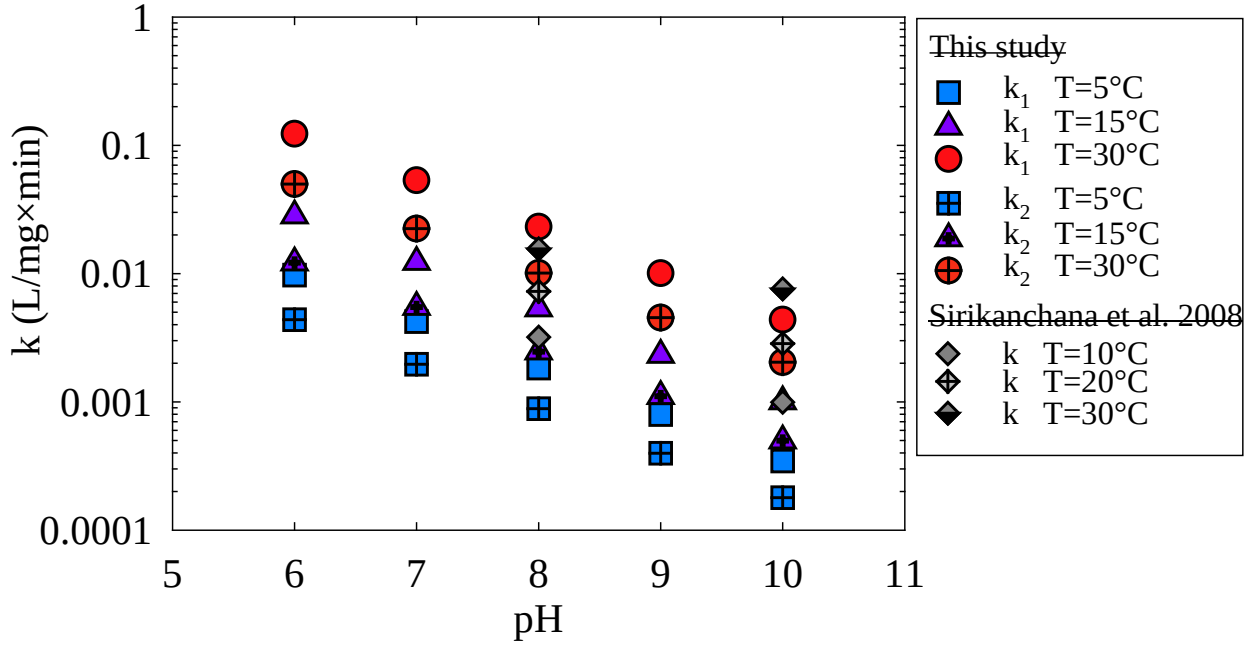

Figure S2. The effect of pH on the inactivation rate constants (k_1 and k_2) in eq. 2 and 3 compared to the inactivation rate constant (k) previously reported by Sirikanchana et al. 2008.

Table S2. Primer sequences and absolute quantification equations for each gene (after Gall et al. 2015).

	Gene	Primer Sequence	Genomic Position	Amplicon length (bp)	Absolute quantification	r^2
HAdV-2 (NCBI accession AC_000007.1)	E1A	Forward 5'-GAACCACCTACCCTTCACG-3'	673-778	106	C_q = -3.485 × $\log_{10}$(E1A copies) + 36.43	0.998
		Reverse 5'-CCGCCAACATTACAGACTCG-3'				
	Hexon	Forward 5'-AAAGCGGGCTACAAATAGG-3'	19400-19510	111	C_q = -3.678 × $\log_{10}$(Hexon copies) + 37.99	0.997
		Reverse 5'-CTTCGTTCCACTGAGATTCG-3'				
A549 host cells (NCBI accession NC_000007.14)	β-actin	Forward 5'-ATGAGGCTGGTGTAAAGCG-3'	5528803-5528905	103	C_q = -3.704 × $\log_{10}$(β-actin copies) + 38.38	0.999
		Reverse 5'-AGAACACGGCTAAGTGTGC-3'				

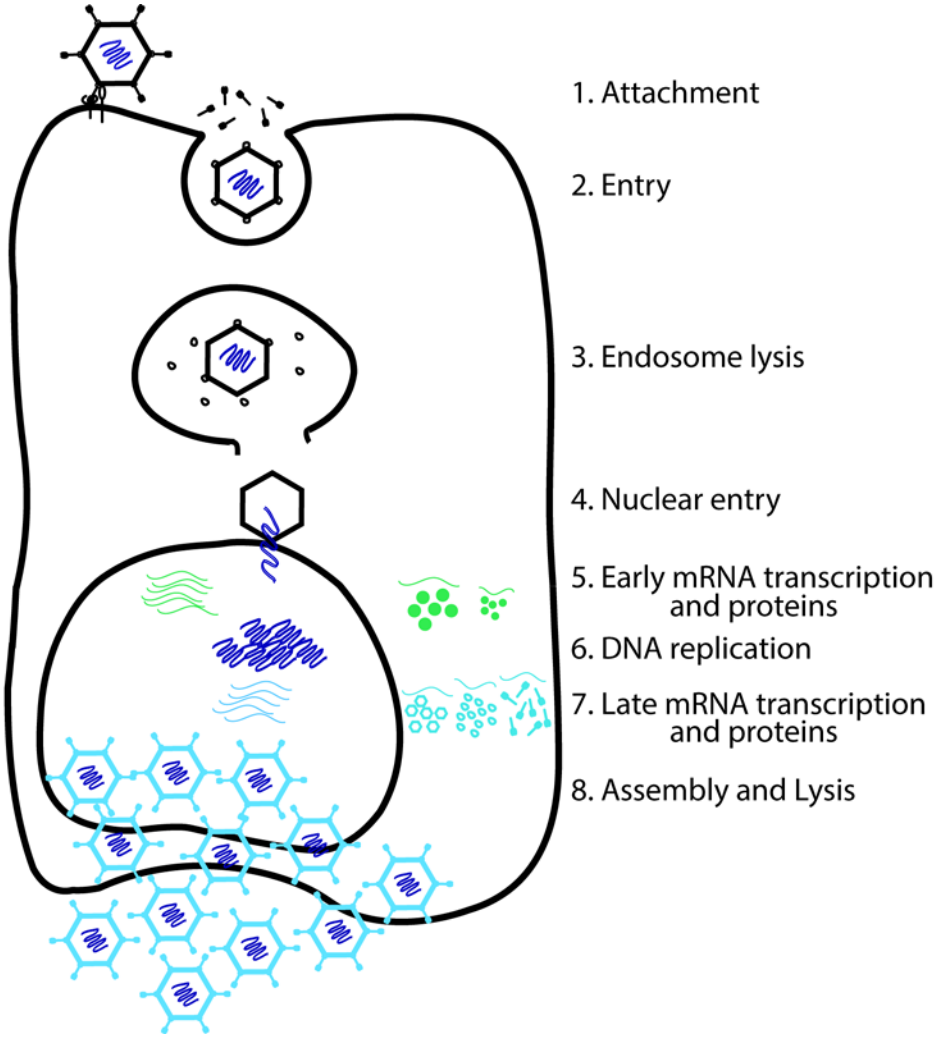

Figure S3. Key steps of the HAdV-2 replication cycle (after Gall et al. 2015).

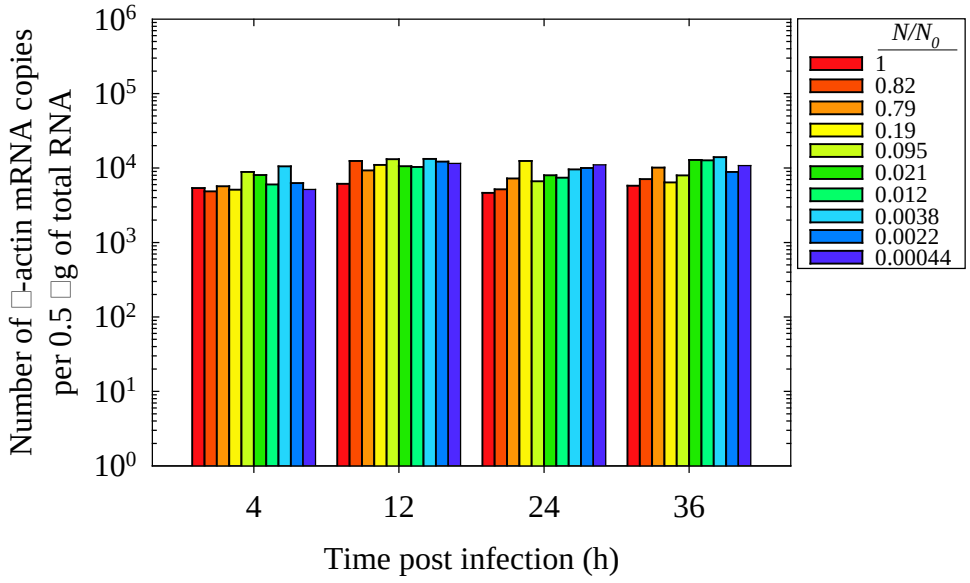

Figure S4. Absolute quantification of housekeeping genes β-actin mRNA copies per 0.5 µg of total RNA determined by a standard curve (see Text S4) for untreated viruses (N/N_0=1) and viruses subjected to increasing monochloramine exposure. Total mRNA was extracted for HAdV-2 infected A549 cells at 4, 12, 24, and 36 h p.i.

References

1. American Public Health Association (APHA); American Water Works Association (AWWA); Water Environmental Federation (WEF), *Standard Methods for the Examination of Water and Wastewater*. 21st ed.; APHA: Washington, D.C., 2005.
2. Sirikanchana, K.; Shisler, J. L.; Marinas, B. J., Inactivation Kinetics of Adenovirus Serotype 2 with Monochloramine. *Water Res.* **2008,** *42*, (6-7), 1467-1474.
3. Gall, A. M.; Shisler, J. L.; Marinas, B. J., Analysis of the Viral Replication Cycle of Adenovirus Serotype 2 After Inactivation by Free Chlorine. *Environ. Sci. Technol.* **2015,** *49*, (7), 4584-4590.