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Inactivation Kinetics and Replication Cycle Inhibition of Adenovirus by Monochloramine

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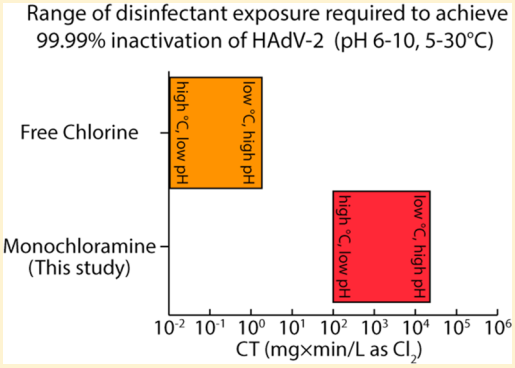
Supporting Information

ABSTRACT: Monochloramine is commonly used as a secondary disinfectant to maintain a residual in drinking water distribution systems in the United States. The mechanism by which waterborne viruses become inactivated by monochloramine remains widely unknown. A more fundamental understanding of how viruses become inactivated is necessary for better detection and control of viruses in drinking water. Human adenovirus (HAdV) is known to be the waterborne virus most resistant to monochloramine disinfection, and this study presents inactivation kinetics over a range of environmental conditions. Several steps in the HAdV replication cycle were investigated to determine which steps become inhibited by monochloramine disinfection. Interestingly, monochloramine-inactivated HAdV could bind to host cells, but genome replication and early and late mRNA transcription were inhibited. We conclude that monochloramine exposure inhibited a replication cycle event after binding but prior to early viral protein synthesis.



INTRODUCTION

Disinfection is an important drinking water treatment process for providing safe drinking water through inactivation of waterborne pathogens, including viruses. Free chlorine is currently the most widely used drinking water disinfectant throughout the world and in the United States. Although enteric viruses are adequately controlled with free chlorine disinfection, many utilities have changed to alternative disinfection schemes (e.g., monochloramine or low-pressure ultraviolet light disinfection) to meet recent national U.S. primary drinking water regulations targeting disinfection byproducts (DBPs).[1,2] Disinfection with monochloramine, formed from the reaction of ammonia with aqueous chlorine, provides a residual in distribution systems while reducing the level of formation of regulated DBPs.[3] Monochloramine is, however, a weaker disinfectant, requiring exposures higher than those of free chlorine to inactivate many types of waterborne pathogens, including enteric viruses.

Few studies have investigated the efficacy of monochloramine disinfection on enteric viruses, and within those studies, very few have examined multiple pH and temperature conditions.[4−15] Recent studies have focused on viruses listed on the U.S. Environmental Protection Agency (EPA) Contaminant Candidate List (CCL) that are known to be present in drinking water and may be regulated in the future.[16] Current regulations require 99.99% (4-log) inactivation or removal of enteric viruses.[17] Of the four viral pathogens listed on the CCL, including human adenovirus (HAdV), caliciviruses, enterovirus, and hepatitis A virus, HAdV is the virus most resistant to monochloramine disinfection and is also one of the least resistant to free chlorine.[10,11,16] The mechanism by which HAdV is so resistant to monochloramine inactivation remains unknown. Our previous study comparing untreated HAdV to monochloramine-treated viruses elucidated that viruses inactivated by less than 1-log were unable to synthesize the first early protein measured by immunoblotting at 12 h post infection (p.i.) in A549 cells, and treated viruses were unable to replicate their genomic DNA at 24 h p.i. measured by slot blotting to the same abundance as untreated viruses.[13] The aims of this research were to produce a highly comprehensive set of inactivation kinetics over the range of environmental pH and temperature conditions encountered in water treatment to achieve 4-log inactivation of HAdV-2 by monochloramine and quantitatively analyze what steps in the viral replication cycle become inhibited over the range of viral inactivation (i.e., 0- to 4-log) by monochloramine using quantitative polymerase chain reaction (qPCR) to analyze HAdV-2 attachment, genome replication, and mRNA transcription several times p.i. HAdV is listed on the CCL because it causes a variety of diseases, including gastroenteritis, respiratory disease, and conjunctivitis, and HAdV can be spread in contaminated water through ingestion, inhalation, or direct contact with eyes.[18−20] Viruses on the CCL could be regulated in the future if methods that can rapidly detect infectious viruses while differentiating them

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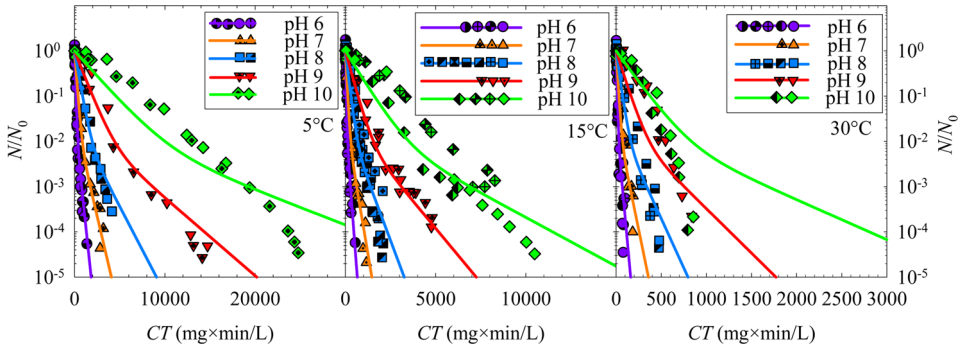

Figure 1. Effect of pH 6−10 on the inactivation kinetics of HAdV-2 by monochloramine at 5 °C (left), 15 °C (center), and 30 °C (right). Replicate experiments are indicated with different symbols. This figure is reproduced in Figure S1 of the Supporting Information with the horizontal axis expanded for ease of comparing the model and data at pH 6−8.

from inactivated viral particles, empty capsids, and free-floating nucleic acids are developed.[21] A more fundamental understanding of how viruses like HAdV become inactivated by disinfectants like monochloramine is needed for better detection and control of viruses in drinking water.

MATERIALS AND METHODS

HAdV-2 Viability and Monochloramine Disinfection. HAdV-2 (VR-846) was propagated using A549 human lung carcinoma cells (CCL-185) obtained from the American Type Culture Collection (Manassas, VA). HAdV-2 purification, A549 host cell culture growth, and viability using plaque assays have been described previously.[12,22] The methods used and range of conditions investigated for monochloramine disinfection experiments are described in the Supporting Information (see Text S1 and Table S1).

Analysis of the HAdV-2 Replication Cycle. The analysis of the HAdV-2 replication cycle has been described previously (see Text S2 and Figure S3).[22] HAdV-2 was untreated or treated with monochloramine (pH 8 and 15 °C) up to 99.99% (4-log) inactivation corresponding to a survival ratio (N/N_0) of 1 to 0.0001. Genomic damage to target amplicon regions (E1A and hexon genes) was determined by extracting DNA from untreated and monochloramine-treated viruses. Subsequent assays were performed by first synchronizing the HAdV-2 infection in A549 cells. Total viral and cellular DNA and RNA were extracted at 0, 4, 12, 24, and 36 h p.i. to quantify HAdV-2 attachment and viral genomic DNA (E1A and hexon genes) replication by qPCR, and viral early (E1A) and late (hexon) mRNA transcription by reverse transcriptase qPCR (RT-qPCR) (see Text S3).

RESULTS AND DISCUSSION

Inactivation Kinetics and Kinetic Model. The kinetics of HAdV-2 inactivation with monochloramine at pH 6−10 and 5−30 °C are shown in Figure 1. These results were generally consistent with previously reported data.[5,10−12] The inactivation data were represented with a two-population model similar in form to that proposed for the inactivation of HAdV-2 with free chlorine:[23]

$$\frac{N}{N_0} = \frac{N_1}{N_0}e^{-k_1 CT} + \frac{N_2}{N_0}e^{-k_2 CT} \quad (1)$$

where k_1 and k_2 are inactivation rate constants and N_1/N_0 and N_2/N_0 are the fractions of viruses in populations 1 and 2, respectively. Kinetic curves were characterized by an initial relatively rapid phase of inactivation, followed by a slower second phase of inactivation. N_2/N_0 was found to be independent of pH and temperature, and $N_1/N_0 = (1 - N_2/N_0)$. In contrast, k_1 and k_2 were found to be dependent on both pH and temperature according to the expressions

$$k_1 = A_1 e^{-E_{a,1}/RT} e^{-B_1 \times pH} \quad (2)$$

and

$$k_2 = A_2 e^{-E_{a,2}/RT} e^{-B_2 \times pH} \quad (3)$$

where R is the ideal gas constant, T is absolute temperature, and A_1, A_2, B_1, B_2, $E_{a,1}$, and $E_{a,2}$ are fitting parameters. Simultaneous fitting of all data sets to eq 1 resulted in the following values: $N_2/N_0 = \exp(-3.505 \pm 0.393)$, $A_1 = \exp(31.13 \pm 2.56)$ L mg^{-1} min^{-1}, $E_{a,1} = 71130 \pm 6090$ J/mol, $B_1 = 0.834 \pm 0.057$, $A_2 = \exp(28.87 \pm 0.69)$ L mg^{-1} min^{-1}, $E_{a,2} = 68240 \pm 1690$ J/mol, and $B_2 = 0.799 \pm 0.015$. Notice that the model represented the data generally well (see Figure 1 and Figure S1) except for deviations observed for data sets at the two highest pH values investigated at 30 °C, which were excluded when the final fitting was performed. This model was similar to our previous kinetic model developed for HAdV-2 inactivation by monochloramine with an additional term for the secondary slower inactivation phase that was not previously observed with limited kinetic data.[12] Additionally, a lag phase was not observed in our current model potentially due to HAdV-2 stock preparations that resulted in fewer impurities and caused no measurable decay of monochloramine; our previous model solved fitting parameters for each pH condition separately, whereas our current model allowed for simultaneous fitting of all data sets.

Effect of Monochloramine on Genomic DNA Amplification. Viral DNA extracted from untreated and monochloramine-treated viruses was probed by qPCR, using primers specific for the E1A or hexon genes to determine if monochloramine damaged these selected regions of the HAdV-2 genome. The relative expression of each gene (E1A/E1A$_0$ and Hexon/Hexon$_0$) was determined for each virus sample subjected to increasing monochloramine exposure (CT > 0), and the untreated virus sample (CT = 0) was used as the calibrator. The relative copy numbers compared with the survival ratio (N/N_0) are represented in the top plot of Figure 2. As monochloramine exposure increased and survival ratio decreased, the relative quantity of both genes remained constant. Even as HAdV-2 approached 4-log inactivation, the targeted genes were amplified with the same abundance as the

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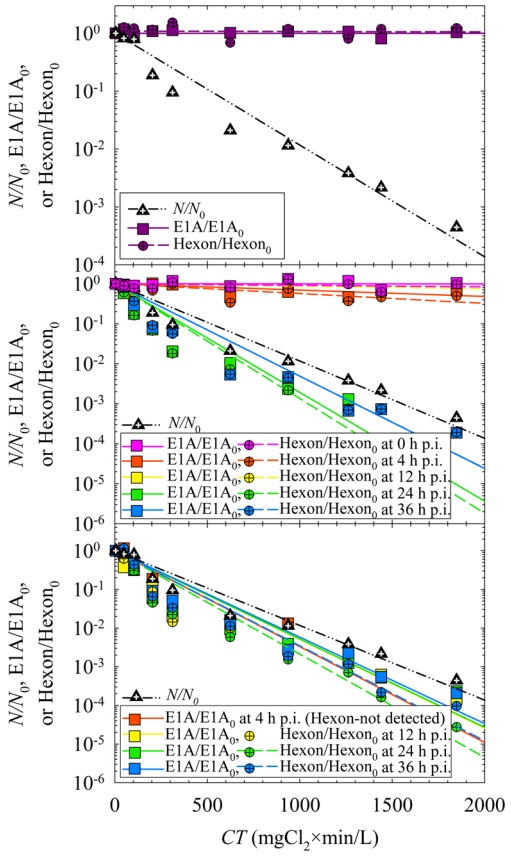

Figure 2. Relative quantification (see Text S4) of E1A and hexon ($E1A/E1A_0$ and $Hexon/Hexon_0$) DNA or mRNA from virus samples subjected to increasing monochloramine exposure compared to the survival ratio (N/N_0) obtained via plaque assay. Values for each sample are relative to the untreated control at CT = 0. Linear regressions are shown for the sake of clarity only. (Top) Relative quantity of HAdV-2 genomic DNA amplicons extracted from virus samples to determine amplicon integrity. (Middle) Relative quantity of E1A and hexon genomic DNA to investigate the attachment to A549 monolayers 0 h p.i. and genomic DNA replication 4–36 h p.i. (Bottom) Relative quantity of early and late mRNA $E1A/E1A_0$ and $Hexon/Hexon_0$ transcription 4, 12, 24, and 36 h p.i.

untreated control, revealing that the E1A and hexon amplicon regions of the viral genomes were not damaged by monochloramine treatment. However, the regions amplified consisted of <2% of the entire HAdV-2 genome; therefore, we were unable to conclude if other regions of the viral genome were damaged by monochloramine. Because there was no loss of amplification of our target genes, our subsequent analyses were not affected by genomic damage of these regions due to monochloramine treatment.

Effect of Monochloramine on Virus Attachment. To assess if monochloramine exposure inhibited attachment to host cells, untreated and monochloramine-treated viruses were incubated on A549 cell monolayers at 4 °C, a temperature that allowed virus attachment but prevented entry. Unbound viruses were removed, and total DNA was extracted from cells and bound viruses and quantified by qPCR. The relative quantities of $E1A/E1A_0$ and $Hexon/Hexon_0$ at 0 h p.i. are shown in the middle plot of Figure 2 compared to the survival ratio of HAdV-2 (N/N_0). As the survival ratio decreased with increasing monochloramine exposure, the relative quantity of HAdV-2 genes remained constant. This revealed that monochloramine-treated HAdV-2 up to 99.95% inactivation was still able to bind to host cells.

Effect of Monochloramine on Genome Replication. The effect of monochloramine on HAdV-2 genome replication was assessed throughout the duration of one infectious cycle at 4, 12, 24, and 36 h post-synchronized infection. At 4 h p.i., the middle plot of Figure 2 shows that the same quantity of HAdV-2 DNA was present for treated and untreated viruses, consistent with the HAdV-2 genome replication not beginning until approximately 5–8 h p.i.[20] Genome replication is presented in terms of absolute copy numbers in the top plot of Figure 3. The untreated control ($N/N_0 = 1$) increased the amount of DNA copies by nearly 10^4-fold from 4 to 36 h p.i. Monochloramine-inactivated HAdV-2 produced genomic DNA copies but fewer than the untreated control. For example, HAdV-2 inactivated by nearly 2-log ($N/N_0 = 0.021–0.012$) by monochloramine produced nearly 2-log fewer copies than the untreated control at 36 h p.i. By 24 and 36 h p.i., the relative quantity of HAdV-2 DNA replicated correlated with the survival curve (N/N_0) of HAdV-2 (significant correlation; $r \geq 0.83$; $P < 0.05$) shown in the middle plot of Figure 2. This

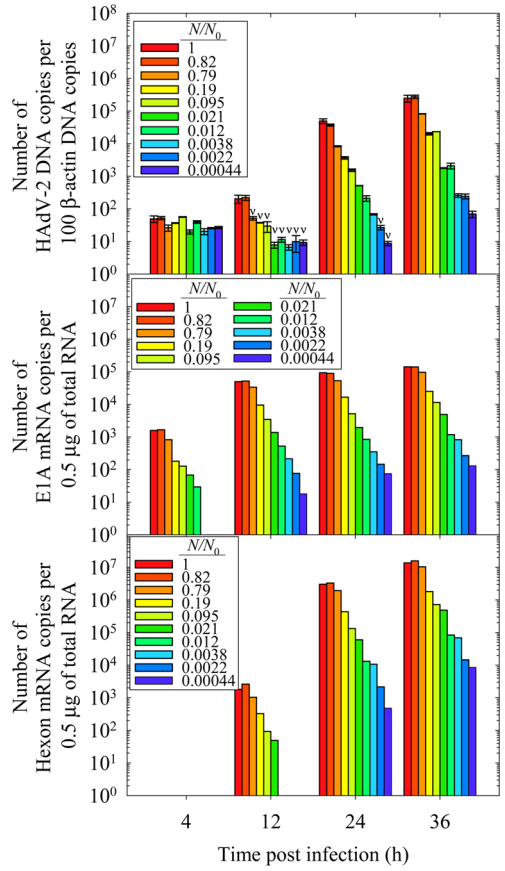

Figure 3. Absolute quantification (see Text S4) of HAdV-2 DNA or mRNA 4–36 h p.i. for untreated viruses ($N/N_0 = 1$) and viruses subjected to increasing monochloramine exposure ($N/N_0 = 0.82–0.00044$). (Top) HAdV-2 DNA copies per 100 β-actin DNA copies. Genome equivalents were measured by the average of E1A and hexon copies, and the error bars represent the standard deviation. Samples not statically greater than the baseline DNA copies at 4 h p.i. are indicated by ν ($P > 0.1$). (Middle) Absolute quantification of HAdV-2 E1A mRNA copies per 0.5 μg of total RNA. (Bottom) Absolute quantification of HAdV-2 hexon mRNA copies per 0.5 μg of total RNA.

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suggested that monochloramine inactivated HAdV-2 by inhibiting a replication cycle event at or before genome replication.

Effect of Monochloramine on mRNA Synthesis. Transcription of HAdV-2 early mRNAs (e.g., E1A) occurs before viral DNA replication, while late genes (e.g., hexon) are transcribed during genome replication. To determine if monochloramine treatment also inhibited late mRNA production, the levels of hexon mRNA in cells infected with treated or untreated viruses were quantified throughout one infection cycle. The absolute number of hexon mRNA copies per 0.5 μg of total RNA was quantified 4, 12, 24, and 36 h p.i. in Figure 3 (bottom plot) (see Text S3). Late mRNA transcription in HAdV-2 begins around 5−8 h p.i., and as expected, no detectable hexon mRNA was present 4 h p.i. By 12 h p.i., untreated and monochloramine-treated viruses to less than 1.7-log inactivation ($N/N_0 \geq 0.021$) produced detectable levels of hexon mRNA. The number of hexon mRNA copies increased gradually up to 10^7 copies at 36 h p.i. The number of hexon mRNA copies also increased from 12 to 36 h p.i. for monochloramine-treated HAdV-2 but overall produced fewer copies than the untreated control.

The transcription of an early gene (E1A) was also evaluated for HAdV-2 at the same times postinfection. E1A is the first viral gene transcribed after infection, detected by 1−2 h p.i.[20] Accordingly, as shown in Figure 3 (middle plot), E1A mRNAs were transcribed for the untreated control at 4 h p.i., and the number increased to 10^5 copies by 12 h p.i. The number of E1A mRNA copies for monochloramine-treated samples was smaller than the number for the untreated control, and the quantity of E1A mRNA decreased with an increasing level of inactivation at all times postinfection. By 24 and 36 h p.i., the amount of E1A mRNA had leveled off because the viral infection had undergone the transition to the late stage, and no additional E1A was needed. Housekeeping gene β-actin mRNA transcription remained at constant levels throughout the infection for untreated and treated HAdV-2 (see Figure S4), which verified β-actin as an effective normalizer gene.

The relative quantities of early and late mRNAs produced by HAdV-2 subjected to increasing monochloramine exposure that were calibrated to the untreated control (N_0) are shown in Figure 2 (middle and bottom plots). The relative quantity at 12, 24, and 36 h p.i. of early mRNA E1A (E1A/E1A$_0$) and late hexon mRNA (Hexon/Hexon$_0$) correlated to the survival ratio of HAdV-2 (N/N_0) measured by plaque assay (significant correlation; for E1A $r \geq 0.91$; for Hexon $r \geq 0.85$; $P < 0.05$). This indicated that monochloramine exposure resulted in blocking a step in the viral replication cycle at or before early mRNA transcription.

Mechanistic Considerations of Monochloramine Disinfection. The results of this study revealed that HAdV-2 inactivated by monochloramine by >99.95% could bind to host cells. However, downstream replication cycle events were inhibited, including early gene transcription, genome replication, and late gene transcription. The results indicated that monochloramine inactivation of HAdV-2 inhibited a replication cycle event postbinding but at or before early mRNA synthesis. Our results of HAdV-2 inactivated up to 99.95% by monochloramine were consistent with our previous study of HAdV-2 inactivated up to 80% (less than 1-log) that found decreased levels of E1A protein synthesis measured by immunoblotting at 12 h p.i. in A549 cells and inhibited viral genome replication at 24 h p.i. measured by slot blotting.[13]

Our recent study of HAdV-2 inactivation by free chlorine showed similar results; free chlorine-treated HAdV-2 was able to bind to host cells, but viral gene transcription and genome replication were inhibited.[22] It was interesting to find that the HAdV-2 replication cycle is impeded postbinding but prior to early gene synthesis for both disinfectants despite the fact that there was a difference of more than 4 orders of magnitude in treatment efficiency between the two disinfectants. For example, a CT of approximately 0.5 mg×min/L was required to achieve 4-log inactivation of HAdV-2 by free chlorine at pH 10 and 5 °C;[23] in contrast, the CT required for the same level of inactivation with monochloramine at the same pH and temperature was approximately 25000 mg×min/L. One possible explanation for the differences in HAdV-2 inactivation rates by free chlorine compared to that with monochloramine is that the reaction rate constants for specific amino acids are up to 5 orders of magnitude higher for chlorination than for chloramination.[24] If the same key capsid proteins were modified by free chlorine and monochloramine disinfection leading to the same block in the HAdV-2 replication cycle, the reaction rate constants for the chlorination and chloramination of specific amino acids could explain the resistance of HAdV-2 to monochloramine disinfection. Additionally, chlorine transfer reaction rate constants from chloramine to amino acids have been shown to increase with a decrease in pH in the range of 6−10, further supporting the idea that monochloramine may cause protein modifications that lead to inactivation of HAdV-2, and as observed in Figure 1, inactivation occurred more rapidly under low-pH conditions.[25] Monochloramine and free chlorine most likely cause many modifications of the viral capsids and genome, and determining which modification results in inactivation will require further viral replication cycle studies. Studying if chlorine- and chloramine-treated HAdV-2 can successfully bind to its secondary receptor, escape from the endosome, traverse to the nucleus, and uncoat leading to nuclear entry will help pinpoint what region may be the target of inactivation.

Practical Implications. The results of this study have several practical implications for the control and detection of HAdV in drinking water. HAdV is known to be the most resistant CCL virus to monochloramine disinfection, and this study has characterized its inactivation over a range of environmental pH and temperature conditions. Monochloramine is usually applied as a secondary disinfectant for maintaining a residual in the distribution system. If infiltration or depressurization events occur in drinking water distribution systems leading to contamination, monochloramine would typically not be adequate to inactivate HAdV. Previous studies have also noted that the CT values recommended by the EPA for chloramines are insufficient for HAdV inactivation.[10,11] The CT values recommended for 4-log inactivation are 1988 mg×min/L at 5 °C and 994 mg×min/L at 15 °C for pH values of 6−9.[17,26] In contrast, our data showed that at pH 9 the exposures required are nearly 13000 mg×min/L at 5 °C and >5000 mg×min/L at 15 °C.

This study also contributes to the identification of potential molecular targets by demonstrating that the small amplicon regions of E1A and hexon genes are not the targets monochloramine disinfection, and that capsid protein or genomic DNA modifications inhibit a replication cycle event that occurs after binding but prior to viral mRNA transcription. Determining the specific protein or genome modification that leads to the inhibition of the viral replication cycle and thus the

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inactivation of HAdV would be very beneficial for the detection of infectious HAdV in drinking water. The modification that leads to inactivation may be unique for each disinfectant. An understanding of these modifications would allow the development of methods for distinguishing between infectious and noninfectious HAdV in drinking water by detecting these specific molecular modifications. The development of methods for rapidly detecting infectious viruses in drinking water would then allow CCL viruses like HAdV to become regulated in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.6b00079.

Monochloramine disinfection experiments, HAdV-2 replication cycle diagram, and qPCR data analysis (PDF)

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Notes

The authors declare no competing financial interest.

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