

Viability of Viruses in Suspended Aerosols and Stationary Droplets  
as a Function of Relative Humidity and Media Composition

Kaisen Lin

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Linsey C. Marr, Committee Chair  
Amy Pruden  
Peter J. Vikesland  
Gabriel Isaacman-VanWertz

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**ABSTRACT**

The transmission of some infectious diseases requires that pathogens can survive (i.e., remain infectious) in the environment, outside the host. The viability of pathogens that are immersed in aerosols and droplets is affected by factors such as relative humidity (RH) and the chemical composition of the liquid media, but the effects of these stressors on the viability of viruses have not been extensively studied. The overall objective of this work was to investigate the effects of RH and media composition on the viability of viruses in suspended aerosols and stationary droplets. We used a custom rotating drum to study the viability of airborne 2009 pandemic influenza A(H1N1) virus across a wide range of RHs. Viruses in culture medium supplemented with material from the apical surface of differentiated primary human airway epithelial cells remained equally infectious for 1 hour at all RH levels tested. We further investigated the viability of two model viruses, MS2 and  $\Phi 6$ , in suspended aerosols and stationary droplets consisting of culture media. Contrary to the results for influenza virus, we observed a U-shaped viability pattern against RH, where viruses retained their viability at low and extreme high RHs, but decayed significantly at intermediate to high RHs. By characterizing the droplet evaporation kinetics, we demonstrated that RH mediated the evaporation rate of droplets, induced changes in solute concentrations, and modulated the cumulative dose of solutes to which viruses were exposed as droplets evaporated. We proposed that the decay of viruses in droplets follows disinfection kinetics. Lastly, we manipulated the chemical composition of media to explore the stability of viruses as a function of pH and salt, protein, and surfactant concentrations. Results suggested that the effects of salt and surfactant were RH and strain-dependent. Acidic and basic media effectively inactivated enveloped virus. Protein had protective effect on both non-enveloped and enveloped viruses. Results from this work has advanced the understanding of virus viability in the environment and has significant implications for understanding infectious disease transmission.

Understanding the Viability of Viruses in Suspended Aerosols and Stationary Droplets  
as a Function of Relative Humidity and Media Composition

Kaisen Lin

**GENERAL AUDIENCES ABSTRACT**

Pathogenic organisms, including bacteria, viruses, fungi, protozoa, and helminths, cause infections that are responsible for substantial morbidity and/or mortality. For example, it is estimated that influenza has caused 9 million to 45 million illnesses and 12,000 to 61,000 deaths annually since 2010 in the United States. The spread of certain diseases relies on people touching the pathogenic organism on surfaces or inhaling it from the air. Successful transmission requires that the pathogen survive, or maintain its infectivity, while it is in the environment. The survival of pathogens can be affected by temperature, humidity, composition of the respiratory fluid carrying them, and other factors. However, there is limited research investigating the effects of these factors on the survival of viruses in the environment. In this work, we studied the effect of relative humidity (RH) on the survival of viruses, including influenza virus and two other types of viruses, in inhalable aerosols and larger droplets. We found that influenza viruses survive well in aerosols across a wide range of RH levels for at least 1 h. Conversely, the two model viruses survived best at both low and very high RHs, such as found indoors in the wintertime or in tropical regions, respectively, but had a pronounced decay at intermediate RHs. By measuring how fast droplets evaporated, we found that RH affected their chemistry and determined the total amount of stress that viruses were exposed to. This explained why a “U-shaped” survival pattern was observed against RH. We also investigated the survival of viruses in droplets containing different components. Results indicated that the effects of salt, surfactant, protein, and droplet pH depended on RH and the type of virus. The outcomes of this work are meaningful in predicting the survival of viruses in aerosols and droplets of various compositions in the environment and could provide insight on developing strategies to minimize the spread of infectious diseases.

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# 1. Introduction

## 1.1 Background

Pathogenic organisms, including bacteria, viruses, fungus, protozoa, and helminths, may cause infections that are responsible for substantial morbidity and/or mortality <sup>1</sup>. Some diseases rely on the spread of pathogenic organisms in the environment, from infected hosts to susceptible hosts via aerosol, direct contact, oral, and fomite transmission. Successful transmission requires that the pathogen survive, or maintain its infectivity, while it is in the environment. Studies on the persistence of pathogenic organisms in solutions, on material surfaces, and in the air have shown that the survival of pathogenic organisms varies by strain, composition of the surrounding media, and environmental factors <sup>2-11</sup>.

Aerosol transmission and fomite transmission are important routes for the spread of many infectious diseases, such as influenza, tuberculosis, measles, and respiratory syndrome <sup>12</sup>, which cause millions of cases of illness every year. Pathogens can be released from a host in aerosols (particles small enough to remain airborne) and droplets (larger particles that quickly deposit onto surfaces). Aerosols and droplets are complex systems due to their varying size, high surface-to-volume ratio, and spatially heterogeneous composition. Pathogens that are immersed in droplets and aerosols experience a dynamic, highly variable microenvironment that affects their viability, as illustrated in **Figure 1.1**. Environmental factors and the surrounding media affect the microenvironment in which pathogens are immersed and thus modulate their viability in the environment.

### 1.1.1 Relative Humidity

Some infectious diseases exhibit a strong seasonal cycle <sup>13-16</sup>. For example, influenza epidemics tend to occur in the winter season in temperate regions and rainy seasons in tropical regions <sup>17-19</sup>. Other human respiratory pathogens, both bacterial and viral, also exhibit higher incidence in winter <sup>20,21</sup>. The seasonal pattern in infectious diseases can be attributed to many factors, such as seasonality in the survival of pathogens outside their hosts, host behavior, and host immune function, and abundance of non-human hosts, but the exact mechanism behind seasonal patterns in infectious diseases has not been identified.

Because environmental factors, especially humidity and temperature, also have seasonal cycles, there have been substantial efforts to investigate their roles in shaping the seasonality of certain diseases.

Studies with animal models (e.g., ferrets, mice, and guinea pigs) have revealed the importance of humidity and temperature on the transmission of influenza<sup>22-24</sup>. Recent studies have compared the impacts of relative humidity (RH), temperature, and absolute humidity on influenza virus survivability, transmission, and incidence<sup>25-28</sup>. Results from these studies indicate that RH is at least equally important as other environmental factors in influenza survival and transmission. At the same time, intervention studies have demonstrated that it is possible to reduce the incidence of respiratory infections by manipulating RH to a level that is less favorable for the survival and transmission of pathogens in built environments<sup>29-32</sup>.

The observed correlation between RH and disease transmission raises the question of how RH affects the survival of pathogens once they are expelled from infected individuals. Successful transmission requires that pathogens remain viable before infecting susceptible individuals. In order to better understand how RH affects disease transmission and incidence, it is essential to investigate the relationship between RH and the viability of pathogens carried in aerosols and droplets. In fact, there have been a number of studies exploring the survival of pathogens as a function of RH in suspended aerosols. A complex relationship between RH and viability of bacteria, with seemingly contradictory results, has been reported due to differences in bacterial species, aerosolization media, and experimental setup among studies<sup>33,34</sup>. For example, some studies have reported that Gram-negative bacteria had elevated death rates at either intermediate or high RH, while another study reported that the Gram-negative bacteria rod *Pasteurella* species had a lower decay rate at high RH levels<sup>35-37</sup>. Gram-positive bacteria, however, were generally found to have elevated death rates at intermediate RH<sup>38</sup>. Results on viral survival have been more consistent. Most viruses were found to survive best at both low (below 40%) and very high (over 90%) RH, while they survived worst at intermediate RH (between 40% and 90%)<sup>25,39,40</sup>. However, the underlying mechanism for the observed patterns remains unknown.

Aerosols and droplets containing pathogens evaporate after they are expelled into environments from infected individuals or emitted from natural environments to reach equilibrium with ambient RH. Evaporation causes shrinkage in size, which results in changes in solute concentrations, pH, and many other properties of the aerosol or droplet <sup>26</sup>. These changes alter the microenvironment surrounding microbes, and the magnitude of these changes determines the rate of microbial decay. However, evaporation kinetics and the resulting changes in chemical composition have been largely overlooked in explaining the observed patterns in the viability of microbes <sup>41</sup>. It is likely that the evaporation kinetics of aerosols and droplets varies with RH, and the resulting dynamics in solute concentrations should be considered in the inactivation of bacteria and viruses.

### **1.1.2 Media Composition**

The chemical composition of the fluid that constitutes aerosols and droplets affects the viability of pathogens, but the actions of specific compounds are largely unknown. Often, aerosols and droplets emitted from different sources have distinct chemical and physical properties. For example, fluids expelled by infected patients when they exhale, talk, cough, or sneeze contain high levels of proteins and are usually highly viscous <sup>42</sup>, while sea spray aerosols contain more salts and organic compounds <sup>43</sup>. Since each component might have a different effect on a pathogen, its survival in aerosols and droplets that are generated from different sources may vary <sup>36</sup>. In studies of the survival of *E. coli* in aerosols, Cox found that airborne bacteria survived better in raffinose solution than in distilled water at all RH levels <sup>44</sup>. Other studies have also observed different patterns in the viability of microorganisms in different media <sup>36</sup>. However, these studies have evaluated the viability of microorganisms mainly in culture media, which are often not environmentally or physiologically relevant. Employing more representative fluids, by collecting them from the field or artificially reproducing them, is more challenging. Data on how specific media components might inactivate pathogens are lacking.

Viruses are responsible for diseases such as influenza, hepatitis, SARS, and many cases of gastroenteritis. However, the influence of media composition on viral viability in aerosols and droplets has

not yet been studied extensively in a controlled manner. Seemingly contradictory results on viral viability have been attributed to the use of different media<sup>39</sup>. Influenza A virus decayed by  $\sim 4 \log_{10}$  units in PBS droplets at 55% RH, whereas it decayed by only  $\sim 1 \log_{10}$  unit when droplets were supplemented with protein. It is possible that proteins protect viruses, but the exact mechanism by which individual compounds interact with viruses remains unknown.

In addition to the direct effects on viruses caused by chemicals, there can also be indirect effects stemming from variability in the evaporation kinetics of aerosols and droplets as a function of their chemical composition<sup>45,46</sup>. Aerosols consisting of pure water fully evaporate on the timescale of seconds<sup>47</sup>, while it takes minutes to hours for aerosols with high viscosity, such as secondary organic aerosols, to evaporate half of their mass<sup>48</sup>. Organic aerosols tend to form a glassy layer at the air-liquid interface as they evaporate. This glassy layer prevents aerosols from further evaporation by inhibiting mass exchange between liquid and air, slowing the enrichment of solutes in evaporating aerosols and mitigating the potential for lethal effects on bacteria and viruses.

## 1.2 Objectives

The overall goal of this study is to gain a more complete understanding of the effect of RH and individual media components of aerosols and droplets, as well as the interaction effect between the two, on the inactivation of pathogenic organisms in aerosols and droplets. Addressing this question may also enable advances in understanding the mechanism of pathogen inactivation in the environment. Specific objectives include:

(1) Investigate the viability of three model bacteria, two model viruses, and influenza virus in suspended aerosols and stationary droplets at various RH.

(2) Explain the observed patterns of microorganisms' viability against RH by characterizing the evaporation kinetics of droplets and estimating the resulting changes in solute concentration in evaporating droplets.

(3) Manipulate the chemical composition of droplets containing viruses to identify the effects of individual media components on the viability of viruses in the environment.

### **1.3 Organization of the Dissertation**

Chapter 2, *Influenza Virus Infectivity Is Retained in Aerosols and Droplets Independent of Relative Humidity*, partially addresses Research Objective 1. In this chapter, we investigate the viability of 2009 pandemic influenza A(H1N1) in suspended aerosols and stationary droplets over a wide range of RH levels. Influenza virus remains equally infectious for 1 hour at all RHs tested in both aerosols and droplets. We further examine the effect of extracellular material from human bronchial epithelial cells on the viability of influenza virus and find that it provides a protective effect against RH-dependent decay of viral viability.

Chapter 3, *Humidity-Dependent Decay of Viruses, but Not Bacteria, in Aerosols and Droplets Follows Disinfection Kinetics*, addresses Research Objectives 1 and 2. We determine the viability of three model bacteria and two model viruses in both suspended aerosols and stationary droplets at various RHs. Viability of bacteria generally decreases with decreasing RH. Viruses survive well at low and extremely high RHs, whereas their viability is reduced at intermediate RHs. We also characterize evaporation kinetics and estimate the change in solute concentrations as droplets equilibrate with ambient RH. The inactivation of bacteria appears to be influenced by osmotic pressure resulting from elevated concentration of salts as droplets evaporate. The inactivation of viruses follows disinfection kinetics and is governed by the cumulative dose of solutes.

Chapter 4, *Survival of Viruses in Droplets as a Function of Relative Humidity, pH, and Salt, Protein, and Surfactant Concentrations*, addresses Research Objective 3 and describes the interaction effect between RH and media composition on viral viability. We suspend viruses in solutions composed of different chemical compositions and investigate viral viability in droplets of each solution at various RH. Results suggest that the effects of sodium chloride and surfactant are RH and strain-dependent. Droplet pH does not affect the viability of non-enveloped viruses, but low and high pH effectively inactivate enveloped viruses. Protein has a protective effect on both non-enveloped and enveloped viruses.

Chapter 5 concludes this dissertation by summarizing the major outcomes of each chapter and making recommendations for future work.

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## Tables and Figures

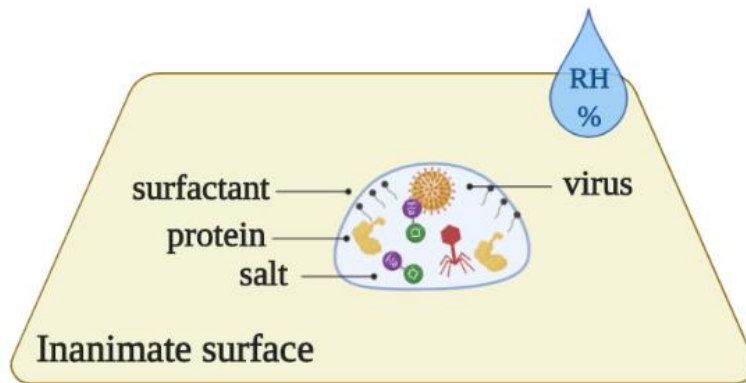


Figure 1.1 Virus-laden droplets containing chemical components on inanimate surface.

## **2. Influenza Virus Infectivity Is Retained in Aerosols and Droplets Independent of Relative Humidity**

Karen A. Kormuth<sup>a,1</sup>, Kaisen Lin<sup>b,1</sup>, Aaron J. Prussin II<sup>b</sup>, Eric P. Vejerano<sup>c</sup>, Andrea J. Tiwari<sup>b</sup>, Steve S. Cox<sup>b</sup>, Michael M. Myerburg<sup>d</sup>, Seema S. Lakdawala<sup>a,2</sup>, Linsey C. Marr<sup>b</sup>

- a. Department of Microbiology & Molecular Genetics, University of Pittsburgh School of Medicine, 450 Technology Drive, Pittsburgh, PA, 15219, USA
- b. Department of Civil and Environmental Engineering, Virginia Tech, Blacksburg, VA, 24061, USA
- c. Department of Environmental Health Sciences, University of South Carolina, Columbia, SC, 29208, USA
- d. Department of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh School of Medicine, 3469 Fifth Avenue, Pittsburgh, PA, 15213, USA

<sup>1</sup>K.A.K and K.L. contributed equally to this work.

<sup>2</sup>Correspondence: (412) 383-4062, lakdawala@pitt.edu

Karen A Kormuth, Kaisen Lin, Aaron J Prussin, Eric P Vejerano, Andrea J Tiwari, Steve S Cox, Michael M Myerburg, Seema S Lakdawala, and Linsey C Marr. “Influenza virus infectivity is retained in aerosols and droplets independent of relative humidity”, *The Journal of Infectious Diseases*, 2018, 218, 5, 739-747, by permission of Oxford University Press or the sponsoring society if the journal is a society journal.

## **Abstract**

Pandemic and seasonal influenza viruses can be transmitted through aerosols and droplets, in which viruses must remain stable and infectious across a wide range of environmental conditions. Using humidity-controlled chambers, we studied the impact of relative humidity (RH) on the stability of the 2009 pandemic H1N1 (H1N1pdm) influenza virus in suspended aerosols and stationary droplets. Contrary to the prevailing paradigm that humidity modulates stability of respiratory viruses in aerosols, we found that viruses supplemented with material from the apical surface of differentiated primary human airway epithelial cells remained equally infectious for 1 hour at all RH conditions tested. This sustained infectivity was observed in both fine aerosols and stationary droplets. Our data suggest, for the first time, that influenza viruses remain highly stable and infectious in aerosols across a wide range of RH. These results have significant implications for understanding the mechanisms of transmission of influenza and its seasonality.

## **KEY WORDS**

Influenza virus, relative humidity, rotating drum, aerosol, mucus, respiratory airway cells, transmission

## 2.1 Background

Influenza viruses (IV) are highly successful pathogens that emerge every winter in temperate regions. Epidemiologically successful IV must replicate efficiently in humans and transmit via the airborne route <sup>1</sup>. Coughing, talking, and exhaling can release aerosols and droplets of varying sizes containing respiratory fluid and viral particles <sup>2-7</sup>. The spread of IV by either aerosol transmission (inhalation of infectious particles) or fomite transmission (self-inoculation from a contaminated surface) requires that IV remain infectious in a variety of environmental conditions <sup>8</sup>.

The risk of airborne disease transmission to a naïve host is linked to a combination of environmental and biological factors including ventilation in buildings, gravitational settling of respiratory droplets out of the air and onto surfaces, and biological inactivation of virus <sup>5,9,10</sup>. The link between the environment and IV transmission is evident in the seasonal cycles of influenza infections, particularly in temperate regions <sup>11,12</sup>, that coincide with seasonal variations in temperature and absolute humidity <sup>13-16</sup>. Relative humidity (RH) can affect airborne transmission of IV, as shown in the guinea pig model with deficient transmission at mid-range and very high RH <sup>9</sup>. Partial explanation for this observation may be biological inactivation of IV in aerosols at mid-range RH conditions, as suggested by the results of studies performed primarily in the 1960s <sup>17-22</sup>. We and others have previously shown that the presence of exogenous proteins in the virus solution can alter the pattern of viral decay in large stationary droplets in response to RH <sup>23</sup>, and can prolong the viability of viruses on surfaces <sup>24,25</sup>. However, these studies do not accurately represent the composition of droplets produced by the human respiratory system and fail to examine the impact of RH on the viability of circulating seasonal IV in aerosols.

Human respiratory droplets contain a variety of proteins including, but not limited to, mucins <sup>26,27</sup>, yet droplet composition is difficult to recapitulate in an experimental setting since the precise components and their concentrations are unknown. In some previous studies of IV viability <sup>17-23</sup>, proteins were present in the aerosolization media, but they were not mucins. A source of mucus that may help recapitulate a biologically relevant system to explore virus viability in a controlled experimental setting is extracellular

material (ECM) collected from the apical surface of human bronchial epithelial (HBE) cells cultures at an air-liquid interface.

In this work, we have studied the viability of the 2009 pandemic H1N1 (H1N1pdm) virus in suspended aerosols and stationary droplets over a wide range of RH. To mimic a physiologically relevant composition of the aerosols and droplets, we supplemented the suspension media with HBE ECM. Aerosol experiments took place within a custom rotating drum, which is designed to minimize loss of aerosols due to gravitational settling (**Figure 2.1**), as first described by Goldberg, Watkins et al.,<sup>28</sup>. Rotating drums have been widely used to study the viability of airborne bacteria and viruses under controlled environmental conditions<sup>17,22</sup>, though not since 1968 for IV<sup>29</sup>. We hypothesized detection of an RH-dependent decay of IV in aerosols, and were surprised to find sustained infectivity of IV at all RH conditions. To determine if this observation might extend to other viruses, we also investigated the survival of aerosolized  $\phi 6$ , a bacteriophage commonly used for studying pathogenic enveloped viruses in the environment<sup>30,31</sup>, with and without the HBE ECM. Our studies suggest that HBE ECM protects IV and  $\phi 6$  from RH-dependent decay.

## 2.2 Methods

### 2.2.1 Cells and viruses

Primary HBE cells were differentiated from human lung tissue following an approved Institutional Review Board protocol and maintained at an air-liquid interface<sup>32</sup>. HBE ECM was generated by pooling washes (150  $\mu$ L of PBS for 10 minutes at 37 °C) of the apical surface of uninfected HBE cells from multiple patients. Protein concentration of undiluted HBE ECM was found to be 100-500  $\mu$ g/mL (BCA assay, Thermo Fisher). MDCK cells (ATCC, CCL-34) were maintained in Eagle's Minimal Essential Medium (EMEM, Sigma) supplemented with 10% FBS, L-glutamine.

Influenza A Virus, A/California/07/2009 (H1N1)pdm09 Antiviral Resistance (AVR) Reference Virus M2: S31N NA: wild type (wt), FR-458 was obtained through the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA. Virus stocks were prepared in MDCK cells

until the onset of cytopathic effect, clarified by low-speed centrifugation, and concentrated by ultracentrifugation through a 30% sucrose cushion. Virus pellets were resuspended in 1:10 HBE ECM:L-15 media. Virus collected from HBE cells was prepared by infecting each Transwell with  $10^3$  TCID<sub>50</sub>/mL H1N1pdm virus as described in <sup>33</sup> and was collected in PBS at 48-72 hours post infection. IV samples were titered on MDCK cells as previously described <sup>34</sup>.

Bacteriophage  $\phi 6$  was propagated with its host *Pseudomonas syringae* according to established methods <sup>30</sup>. Virus was harvested in TSB media and stored at 4 °C.  $\phi 6$  stocks were concentrated and the pellets were resuspended in 1:10 HBE ECM:L-15 media in the same manner as IV. Samples collected from experiments were titered using plaque assays. Briefly, 50  $\mu$ L virus was mixed with 200  $\mu$ L *P. syringae* and 4.75 mL tryptic soy broth (TSB, Sigma) soft agar (0.75% w/v). The mixture was poured over a TSB plate and incubated at 25 °C for 24 hours prior to determination of viral titer.

### **2.2.2 Rotating drum experiment for aerosols**

A 27-L aluminum drum, modified from previous studies to fit inside a biosafety cabinet <sup>28</sup>, was built to investigate the stability of aerosolized viruses at various RH conditions. Three inlet ports introduced dry air, aerosols and saturated air generated by a Collison nebulizer (BGI Inc., MRE-3), and during sampling, make-up air (**Figure 2.1**). House air passed through a HEPA filter (Pall, 12144) and hydrocarbon trap (Agilent, BHT-4) to provide purified air to the nebulizer and dry air line. Three other ports were dedicated to an RH probe (Rotronic, HC2-C04), collection of samples, and exhaust flow passing through a HEPA filter to prevent the release of viruses. The drum rotated at a speed of 2.5 RPM, optimized to minimize aerosol losses. All IV aerosolization experiments were done inside a biosafety cabinet.

Seven RH levels, 23%, 33%, 43%, 55%, 75%, 85%, and 98%, were investigated, selected to span environmentally relevant conditions and correspond to levels readily achievable in the droplet experiments described below. The three lowest conditions (23%, 33%, and 43% RH) are representative of dry climates or heated indoor environments during winter. Two moderate conditions (55% and 75% RH) are typical of indoor environments during warmer seasons. The two highest RH conditions (85% and 98%) occur during

rainy periods everywhere and in tropical regions. RH in the drum was controlled by adjusting the flow rate of dry air (varied from 0 to 10 L/min depending on targeted RH), and the targeted RH was maintained within  $\pm 3\%$ . Temperature and RH were recorded prior to aerosolization and after collection of the aged sample. Temperature was maintained at  $25 \pm 1$  °C throughout all experiments. Representative RH data are provided in **Table A.1**.

Aerosolization experiments were conducted with IV in 1:10 HBE ECM:L-15 media,  $\phi 6$  in 1:10 HBE ECM:L-15 media, and  $\phi 6$  in TSB media. For each experiment, the corresponding virus-free media was nebulized to condition the drum to the desired RH. The aerosol flow rate was maintained at 3.9 L/min at all RHs, except 23% RH because nearly all inflow was distributed to dry air to achieve the desired RH. During conditioning, air at the targeted RH was collected into a separate polyethylene bag (Sigma, AtmosBag) to use as make-up air during sample collection to maintain constant RH. After conditioning, virus was aerosolized at the same flow rate for 20 minutes to fill the drum. While aerosolization proceeded, samples were collected onto 25 mm gelatin filters (SKC Inc., 225–9551) installed in stainless steel holders (Advantec, 304500) at a flow rate at 2 L/min for 15 minutes. These represented “unaged” samples. The drum was then sealed and viral aerosols were aged for 1 hour, which was chosen to represent typical air-exchange rates in residences and some office buildings<sup>35–39</sup>. 1 hour is longer than prescribed by ASHRAE, formerly the American Society of Heating, Refrigerating, and Air-Conditioning Engineers. Aged aerosol samples were collected in the same way as unaged samples, and the gelatin filters were dissolved in 3 mL of warm media, and stored at -80 °C prior to virus titration.

### **2.2.3 Correction for physical loss of aerosols**

A mass balance equation was applied to correct for loss of aerosols via gravitational settling and dilution during aging, assuming first-order decay for both processes. Instantaneous measurement of infectious virus titer was not possible, so this correction assumed that the measured virus titers represent time averages over the 15-minute sampling period. The particle physical loss coefficient ( $k_p$ , units of  $\text{min}^{-1}$ ) and dilution

coefficient ( $k_d$ , units of  $\text{min}^{-1}$ ) were determined as described in <sup>40</sup>. The equations for this correction are shown in the Supplemental Text.

#### **2.2.4 RH chamber experiment for stationary droplets**

Stationary droplets were incubated in a chamber with RH controlled using saturated salt solutions (**Figure A.3A and B**) <sup>23</sup>. Chambers were maintained within a biosafety cabinet at  $\sim 22$  °C. RH and temperature data were collected for each experiment (Onset, HOBO UX100-011; representative data in **Figure A.3C**). Following 1 hour equilibration to the desired RH, ten stationary 1  $\mu\text{L}$  virus droplets were incubated for 1 or 2 hours at each RH condition and then collected in 500  $\mu\text{L}$  L-15 media. 10  $\mu\text{L}$  enclosed virus samples were incubated outside the chamber during the experiment. Data are presented as *log* decay <sup>23</sup>. All data are available upon request.

### **2.3 Results**

#### **2.3.1 Aerosolized IV remains infectious at all RH conditions**

##### **Aerosolized IV remains infectious at all RH conditions**

To study the impact of RH on viability of airborne IV, we aerosolized 2009 H1N1pdm into a custom rotating drum (**Figure 2.1**), aged the aerosols for 1 hour, and then collected aerosols for analysis of infectivity by tissue culture infectious dose 50 (TCID<sub>50</sub>) on MDCK cells. The bulk virus solution was prepared in traditional tissue culture cells and supplemented with HBE ECM (**Figure 2.2A**) to simulate respiratory secretions that would be expelled from an infected person. We observed less than 0.5 log reduction in the amount of infectious H1N1pdm in the aged aerosols in the presence of HBE ECM at each RH tested, which is within the error of our assay (**Figure 2.2B**). There was no loss in virus viability in the bulk virus solution during the aerosolization process, which includes recirculation of larger aerosols that were trapped by the nebulizer, as virus titer before and after each experiment was unchanged (**Figure A.1**).

### **2.3.2 Addition of respiratory extracellular material protects $\phi 6$ viruses from decay at all RH conditions**

We found the 2009 H1N1pdm virus to be remarkably resistant to RH-induced decay at all RH conditions in our rotating drum. To further explore this finding, we conducted additional studies with an enveloped virus, bacteriophage  $\phi 6$ , in the presence and absence of HBE ECM. We aerosolized  $\phi 6$  in traditional laboratory growth media into the rotating drum and found that  $\phi 6$  decayed in aerosols in an RH-dependent manner after 1 hour (**Figure 2.3A**). In contrast,  $\phi 6$  in media supplemented with HBE ECM showed little to no decay at all RHs tested, as with H1N1pdm (**Figure 2.3B**). Taken together with the H1N1pdm data, we provide strong evidence that HBE ECM can protect enveloped viruses from RH-dependent decay in aerosols for at least 1 hour.

### **2.3.3 Adjustment for physical loss of aerosols within a rotating drum**

The rotating drum reduced, but did not eliminate, physical losses of aerosols due to gravitational settling. Furthermore, the sample of aged aerosols collected at the end of the 1 hour aging period was subject to dilution by RH-conditioned make-up air. Therefore, a mass balance equation specific to each aerosolization medium was applied to account for these processes, assuming first-order decay for both gravitational settling and dilution (**Figure A.2**). As expected, physical loss of aerosols due to settling was greater at higher RH because they evaporated less and were larger. Application of this equation to the data allows measurement of the change in viral infectivity unbiased by physical loss of aerosols within the drum. Corrected  $\log_{10}$  decay values confirmed the lack of decay of H1N1pdm (**Figure 2.4A**) and  $\phi 6$  (**Figure 2.4B**) in the presence of HBE ECM.  $\phi 6$  experienced up to 2  $\log_{10}$  decay at 75% and 85% RH without HBE ECM (TSB in **Figure 2.4B**), confirming our observation that it protects aerosolized viruses from RH-dependent decay.

### **2.3.4 IV within stationary droplets remain infectious at all RH conditions**

To test whether HBE ECM also protected IV from decay in stationary droplets, we performed a series of studies with 2009 H1N1pdm in stationary, 1  $\mu$ l droplets exposed to the same RH conditions used in the rotating drum. RH was maintained using saturated aqueous salt solutions within an enclosed chamber (**Figure A.3A**) and, along with temperature, was continuously monitored during each experiment to ensure stability of the specified RH (**Figure A.3B and C**). Consistent with our analysis of  $\phi$ 6 and 2009 H1N1pdm viability in aerosols, we observed that the H1N1pdm virus experienced very little decay in infectivity in stationary droplets containing HBE ECM at each RH tested (**Figure 2.5A**). In contrast, there was an RH-dependent decay of virus infectivity in droplets lacking exogenous HBE ECM, with peak loss at 55% RH. Decay in virus infectious titer exceeded 1  $\log_{10}$  across all RH levels, and the concentration decayed over 2  $\log_{10}$  at 55% RH. Further, we found this protective effect at 55% RH to be dependent upon the concentration of exogenous ECM. We supplemented H1N1pdm with 1:5, 1:10, 1:50, and 1:100 dilutions of HBE ECM, finding that the 1:50 and 1:100 samples decayed similarly to (-) HBE ECM control virus after 1 hour at 55% RH (**Figure 2.5B**). Similar experiments in droplets comparing viability of virus collected from either infected transformed tissue culture or primary differentiated HBE cells also indicated a lack of RH-dependent decay in the HBE propagated viruses (**Figure A.4**). This result indicates that the protection observed with HBE ECM collected from uninfected cells behaves similarly to ECM from infected cells that would be present in expelled aerosols in nature.

### **2.3.5 IV viability in stationary droplets is not dependent upon virus concentration**

The  $\phi$ 6 and 2009 H1N1pdm bulk virus solutions used for our rotating drum experiments had titers ranging from  $10^8$ - $10^9$  plaque forming units/mL or TCID<sub>50</sub>/mL. Individuals infected with H1N1pdm shed approximately  $10^5$  RNA copies/mL, as determined by quantitation of virus from nasopharyngeal specimens<sup>41,42</sup>. To assess whether the sustained infectivity of IV over a range of RH conditions was dependent upon virus concentration, we determined the viability of 10-fold serial dilutions of H1N1pdm ( $10^9$ - $10^4$

TCID<sub>50</sub>/mL), in stationary droplets (**Figure A.5**). We observed very little decay of infectivity for all virus concentrations and all RH tested.

## 2.4 Discussions

Here we provide new insights into the interplay of humidity and respiratory virus viability in expelled aerosols and droplets that has long been proposed to impact IV transmission. We have previously demonstrated that release of submicron aerosols containing IV correlated with efficient transmission of 2009 H1N1pdm in the ferret model <sup>43</sup>. In both humans and animal models, aerosol transmission of human IV has been suggested to be equally or more efficient than fomite transmission <sup>44-46</sup>.

RH has previously been shown to impact the viability of IV in aerosols and stationary droplets <sup>5,17-22</sup>. Contrary to prevailing wisdom, we found that aerosolized IV lost little infectivity over a wide range of RH, indicating that virus decay is not a barrier to efficient aerosol transmission of IV. Infectivity of H1N1pdm was sustained in aerosols for up to 1 hour at all seven RH conditions tested (**Figures 2.2B and 2.4A**). Based on these observations, we postulated that the lack of RH-dependent decay in aerosols results from protection conferred by supplementation of the viruses with HBE ECM. We confirmed this idea using bacteriophage  $\phi 6$  in the absence and presence of HBE ECM (**Figures 2.3 and 2.4B**). Complementary studies of IV in stationary droplets, both with exogenous HBE ECM and virus collected from infected HBE cells (**Figure 2.5 and A.4**), similarly recapitulated the protective effect of HBE ECM against RH-dependent decay of viral infectivity.

The protective effect of HBE ECM was concentration-dependent (**Figure 2.5B**), raising the question of how well the media composition represented that of actual aerosols and droplets expelled by an infected host. The total protein concentration in the diluted aerosolization media fell at the lower end of the range reported in different types of respiratory fluid <sup>47</sup>; the exact origin of virus-laden aerosols within the respiratory system and their chemical composition are not known. Although protein is an obvious candidate, it is possible that something other than protein in HBE ECM protects the virus from decay. Even though the concentration of virus in our experimental aerosols and droplets may have been higher than found in

real ones, studies using a series of serially diluted H1N1pdm samples did not indicate a concentration-dependent response to RH, suggesting that viability of respiratory viruses in expelled droplets with much lower viral load is also maintained.

Our observations have substantial implications for understanding transmission of epidemiologically successful seasonal and pandemic IV and other respiratory pathogens, and reaffirm the importance of aerosolized respiratory droplets as vehicles for transmission. Studies in the guinea pig model have shown that airborne IV transmission is deficient at 50% RH, perhaps due to biological inactivation of viruses in aerosols <sup>9</sup>. However, transmission of IV in ferrets is routinely studied at RH close to 50%, resulting in 100% transmission efficiency of the H1N1pdm viruses <sup>43,48</sup>. Our data indicate that exhaled viruses likely have not been inactivated at this RH, although whether guinea pig respiratory secretions similarly protect viruses as HBE ECM is unknown. We have also shown that aerosol gravitational settling within our rotating drum increases as RH increases (**Figure A.2**), which is consistent with a reanalysis of the Lowen *et al.* guinea pig study that concluded RH effects on aerosol size and transport can explain the results without invoking airborne virus viability <sup>49</sup>. At higher RH, much less evaporation and shrinkage of aerosolized respiratory droplets occurs, resulting in more of them settling out of the air and contaminating fomites <sup>5</sup>. Therefore, RH-dependent biological inactivation of IV in aerosols and droplets may not significantly affect the efficiency of airborne transmission, suggesting a more substantial role for physical factors that act directly on the droplet vehicle.

Our novel use of HBE ECM to recapitulate physiologically relevant expelled aerosols and droplets has revealed that the classical paradigm regarding the decay of IV at mid-range RH may not accurately represent the processes that occur in nature. These data support the notion that IV may have evolved to exploit host protective barriers to support efficient airborne transmission via creation of a stable microenvironment for the viruses released into the air. While the ECM of the respiratory tract may change during IV infection, our observation that viruses propagated in HBE cells were also protected from RH-mediated decay (**Figure A.4**) suggests that uninfected HBE ECM acts as a reasonable surrogate to study this effect.

Our findings raise a host of questions for future investigation. Studies are required to identify the contribution of other factors, including temperature and virus strain background, that may also affect viability of IV in aerosols and contribute to the seasonal emergence of IV. Characterization of the composition of respiratory droplets emitted by infected hosts will identify the factors that confer protection of IV from environmental decay. Specifically, investigation into the structure and composition of respiratory secretions is needed. Rheological characterization of viscoelasticity and other physical properties of mucus has enhanced understanding of disease pathology at mucosal surfaces<sup>50</sup>. Similar studies of the HBE mucosal matrix will identify host factors affecting stability of IV released from the respiratory tract. Further investigation of the kinetics of virus decay in aerosols and droplets is also needed. Prior studies have shown that decay is most rapid within the first ~15 minutes after aerosolization [Noti et al., 2013; Harper, 1961; Schaffer et al., 1976]. The way we conducted our experiments in the rotating drum, our “unaged” aerosols spanned a range of ages from near-zero to several minutes, during which some decay, possibly RH-dependent, might have occurred.

The results of our studies have important implications for the control of airborne IV transmission. Based on our observations, we recommend a combination of increased air exchange rates coupled with filtration or UV irradiation of recirculated air, as well as regular disinfection of high-touch surfaces to minimize transmission. Utilization of personal protective equipment that reduces inhalation exposure to infectious aerosols, such as N95 respirators, may be recommended to healthcare professionals in high risk situations. The results of this study have substantial implications for understanding transmission of epidemiologically successful seasonal and pandemic IV and other respiratory pathogens, and reaffirm the importance of aerosolized respiratory droplets as vehicles for transmission.

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## Tables and Figures

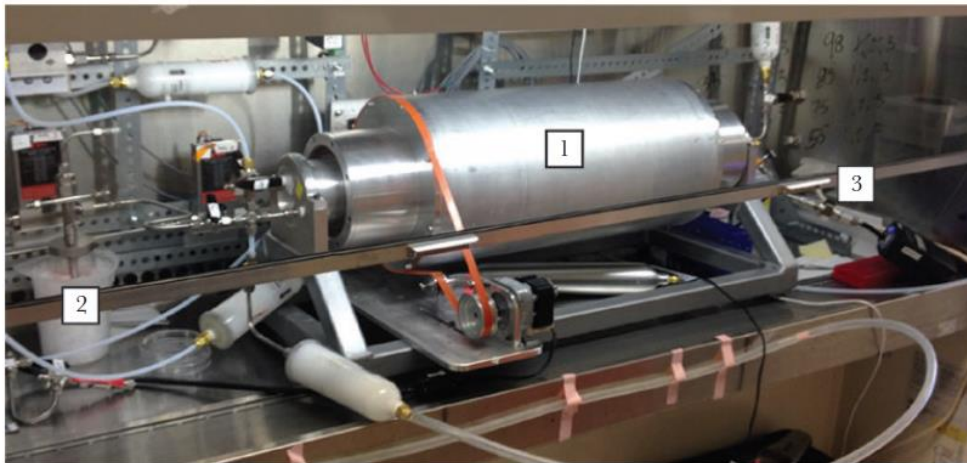
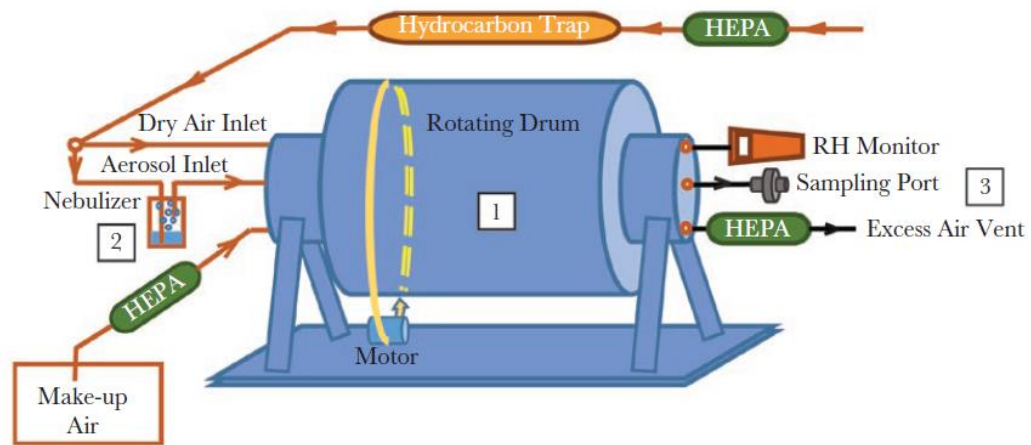


Figure 2.1 Design of a controlled RH rotating drum for the aerosolization of influenza viruses. Schematic (top) and photograph (bottom) of the tunable RH rotating drum. The rotating drum (1) is pre-conditioned to the desired RH prior to aerosolization of the virus. Bulk virus solution is kept on ice and aerosolized via a nebulizer (2) into the drum. The drum is sealed during incubation of the viral aerosols at each specified RH. Viral aerosols are extracted through the sampling port (3) onto a gelatin filter using a pump at 2 L/min for 15 minutes. The gelatin filter is dissolved in warm media to allow for titration of infectious virus.

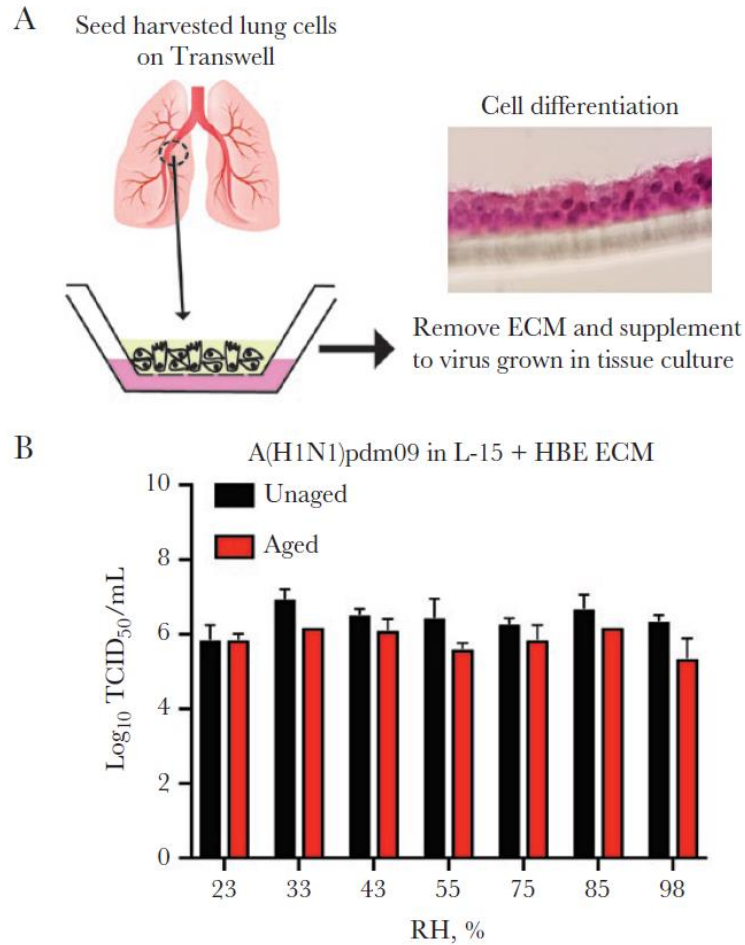


Figure 2.2 Influenza virus maintains infectivity in fine aerosols at all RH conditions. A) Schematic representing preparation of the virus in physiological aerosolization media including extracellular material (ECM) produced by primary human bronchial epithelial (HBE) cells. Inset is an H&E (hematoxylin and eosin) stained image of HBE cells demonstrating the three-dimensional culture. B) Infectivity of aerosolized H1N1pdm supplemented with HBE ECM from uninfected cells in L-15 tissue culture media. The amount of virus pre- and post- 1 hour aging in aerosols was determined by TCID<sub>50</sub> assay on MDCK cells. Data represent the mean  $\pm$  standard deviation of three independent biological replicates, exclusive of 85% RH, which was done twice.

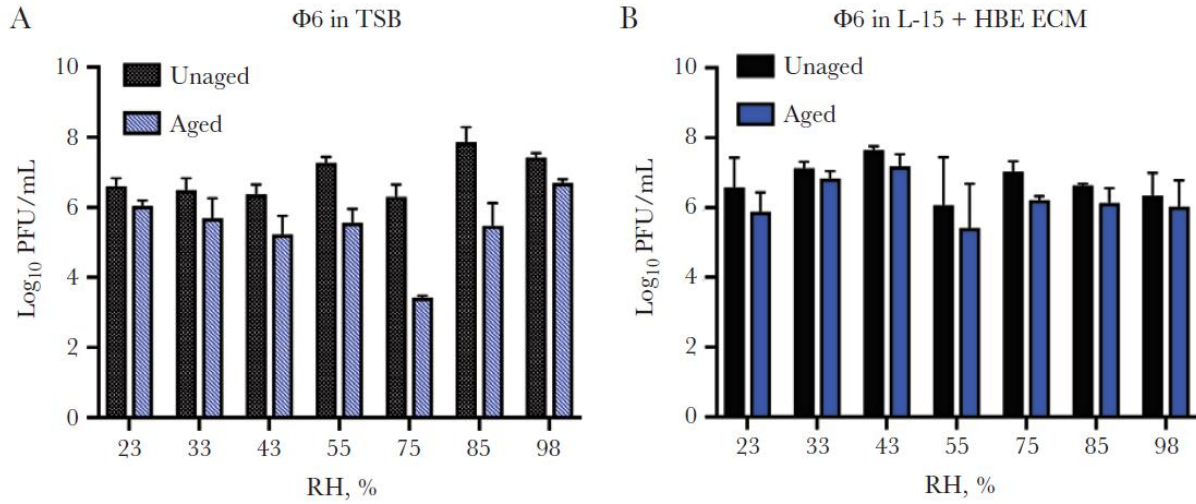


Figure 2.3 Presence of HBE ECM protects  $\phi 6$  from decay at mid-range RH. A) Infectivity of aerosolized  $\phi 6$  bacteriophage in tryptic soy broth (TSB) was tested at a range of RH within the rotating drum. B) As with H1N1pdm,  $\phi 6$  was aerosolized in media containing HBE ECM for comparison of virus titer in unaged and aerosols aged for 1 hour. The amount of virus pre- and post- aging was determined by plaque assay. Data represent the mean  $\pm$  standard deviation of three independent biological replicates.

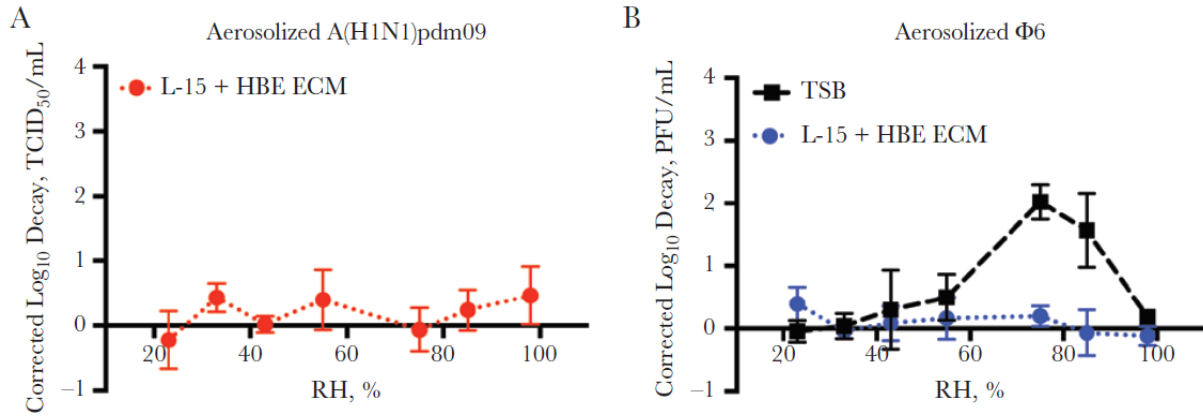


Figure 2.4 Viral decay corrected for physical loss of aerosols within the rotating drum. A) Log<sub>10</sub> decay of H1N1pdm aerosolized with HBE ECM was calculated as the difference in log<sub>10</sub> titer between aged and unaged samples at each RH. A mass balance equation was applied to correct for physical loss of aerosols due to gravitational settling and dilution. B) Log<sub>10</sub> decay of  $\phi 6$  in traditional laboratory media (black) and HBE ECM (blue) demonstrates protection from decay at 75% and 85% RH.

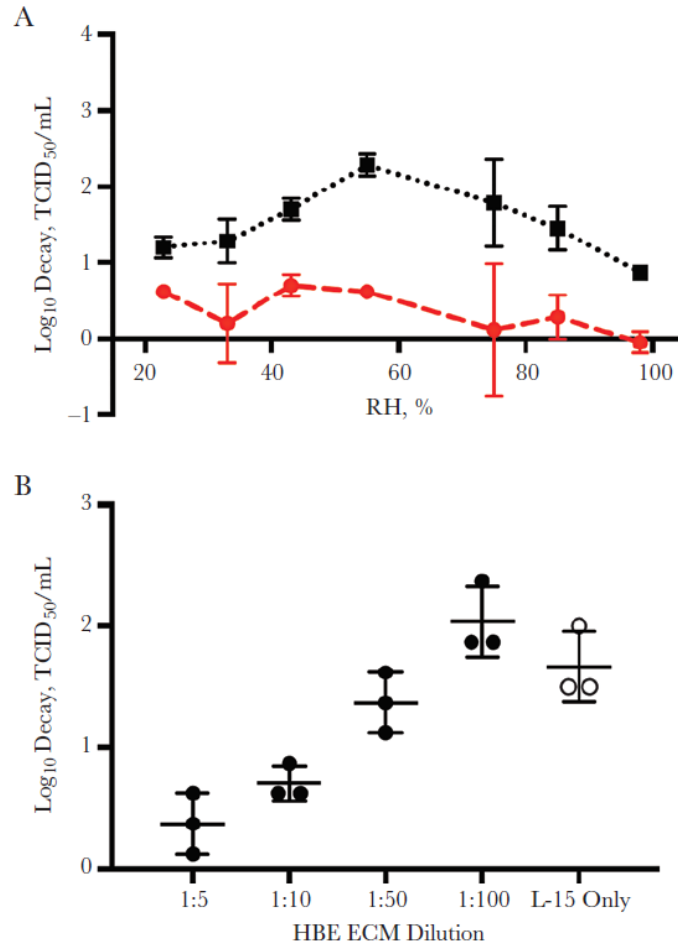


Figure 2.5 Exogenous HBE ECM protects influenza virus from decay in stationary droplets. The viability of 2009 H1N1pdm was tested in stationary droplets at a range of different RH conditions in a controlled RH chamber. Virus samples were compared with and without exogenous HBE ECM. A) RH-dependent decay of 2009 H1N1pdm with (red) and without (black) exogenous HBE ECM after 1 hour. Data represent mean  $\pm$  standard deviation (N = 3). B) Protection from decay of the 2009 H1N1pdm virus in droplets is dependent upon the concentration of HBE ECM. Virus was prepared in the indicated HBE ECM dilutions in L-15 tissue culture medium. Droplets were incubated at 55% RH for 1 hour. Individual data points are shown with error bars indicating standard deviation and are representative of at least two biological replicates.

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### **3. Humidity-Dependent Decay of Viruses, But Not Bacteria, in Aerosols and Droplets Follows Disinfection Kinetics**

Kaisen Lin and Linsey C. Marr\*

Department of Civil and Environmental Engineering, Virginia Tech, 418 Durham Hall, Blacksburg,  
Virginia 24061, United States

\*Corresponding author

Mailing address: Department of Civil and Environmental Engineering, Virginia Tech, 418 Durham Hall,  
Blacksburg, Virginia 24061, United States

E-mail: lmarr@vt.edu

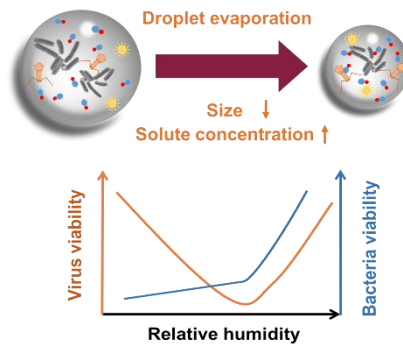
Key words: Relative humidity, disease transmission, viability, droplets evaporation, solute concentration

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## **Abstract**

The transmission of some infectious diseases requires that pathogens can survive (i.e., remain infectious) in the environment, outside the host. Relative humidity (RH) is known to affect the survival of some microorganisms in the environment; however, the mechanism underlying the relationship has not been explained, particularly for viruses. We investigated the effects of RH on the viability of bacteria and viruses in both suspended aerosols and stationary droplets using traditional culture-based approaches. Results showed that viability of bacteria generally decreased with decreasing RH. Viruses survived well at RHs lower than 33% and at 100%, whereas their viability was lower at intermediate RHs. We then explored the evaporation rate of droplets consisting of culture media and the resulting changes in solute concentrations over time; as water evaporates from the droplets, solutes such as sodium chloride in the media become more concentrated. Based on the results, we suggest that inactivation of bacteria is influenced by osmotic pressure resulting from elevated concentration of salts as droplets evaporate. However, the inactivation of viruses is governed by the cumulative dose of solutes, or the product of concentration and time, as in disinfection kinetics. These findings emphasize that evaporation kinetics play a role in modulating the survival of microorganisms in droplets.

# TOC Art



### 3.1 Introduction

Infectious diseases, such as lower respiratory infections, diarrheal diseases, and tuberculosis, cause millions of deaths each year, particularly in lower income countries<sup>1,2</sup>. Some infectious diseases exhibit a strong seasonal cycle<sup>3-6</sup>. For example, influenza epidemics typically peak during the winter season in temperate regions and during the rainy season in tropical regions<sup>7-9</sup>. Researchers have proposed that the greater persistence of infectious influenza viruses under cool, dry conditions or very humid conditions contributes to seasonality of the disease. Certain other human respiratory diseases, both bacterial and viral, also exhibit higher incidence in the winter<sup>10,11</sup>. The temporal pattern has been attributed to several factors, such as seasonality in the survival of pathogens outside their hosts, host behavior, and host immune function<sup>6,12</sup>. Since environmental factors, especially humidity and temperature, also have seasonal cycles, there have been numerous efforts to investigate their roles in shaping the seasonality of influenza and other infectious diseases<sup>13-19</sup>.

Studies with animal models (e.g., ferrets, mice, and guinea pigs) have revealed the importance of humidity and temperature on the transmission of influenza<sup>20-22</sup>. Further studies have compared the impacts of temperature, absolute humidity, and relative humidity (RH) on influenza virus survivability, transmission, and incidence<sup>23-26</sup> and have shown that RH is an important factor in influenza survival and transmission. At the same time, intervention studies have demonstrated that it is possible to reduce the incidence of respiratory infections by manipulating RH to a level that is less favorable for the survival and transmission of certain viruses in the built environment<sup>27-29</sup>.

As successful transmission requires that pathogens remain viable before infecting susceptible individuals, the observed relationship between RH and disease transmission raises the question of how RH affects the survival of pathogens in the environment, after they are expelled from infected individuals. Studies have revealed a complex relationship between RH and the viability of airborne bacteria, with seemingly contradictory results attributed to differences in bacterial species, aerosolization media, and experimental setup<sup>30-32</sup>. For example, some studies have reported that Gram-negative bacteria suspended in 2% Bacto-Tryptose plus 1% dextrose have higher decay rates at either intermediate or high RH,<sup>33</sup> while

another study has shown that *Pasteurella* spp., a Gram-negative rod, suspended in brain-heart infusion broth has a lower decay rate at high RH<sup>34,35</sup>. Gram-positive bacteria, however, generally have elevated decay rates at intermediate RH.<sup>36</sup> Results on the relationship between RH and the viability of viruses are more consistent. Most viruses seem to survive best at both low (<40%) and extremely high (>90%) RH, while some experience increased decay at intermediate RH<sup>23,37,38</sup>. However, the underlying mechanism for the observed patterns remains unknown.

Infectious diseases may transmit via several modes, including aerosols, which remain suspended in air long enough to be inhaled, and large droplets, which may deposit directly on a susceptible person or may deposit on an inanimate object, called a fomite, before being transferred. When aerosols or droplets containing respiratory fluid and microorganisms are expelled into unsaturated air (i.e., RH<100%), they evaporate, either partially so that the water vapor pressure at the aerosol or droplet surface equilibrates with ambient conditions, or fully. The resulting loss of water causes changes in concentrations of solutes, such as sodium chloride and protein, and changes in pH and other properties<sup>24</sup>. These changes alter the microenvironment in which microorganisms are immersed and can influence their decay. Many investigations to date have assumed that evaporation of respiratory droplets to the equilibrium state occurs nearly instantaneously, in less than a second<sup>39</sup>, leaving actual evaporation kinetics and the resulting dynamics in chemistry largely overlooked<sup>40</sup>.

To gain insight into the mechanism of humidity-dependent inactivation (i.e., decay or loss of infectivity) of pathogens in the environment, we have investigated the viability of three bacteria and two bacteriophages that are models for common pathogens in suspended aerosols and stationary droplets at various RHs. We then explore the evaporation rates of droplets and estimate the change in solute concentrations over time. Finally, we propose explanations for the observed patterns of bacteria and virus inactivation as a function of RH in suspended aerosols and stationary droplets, based in part on disinfection kinetics.

## 3.2 Materials and Methods

### 3.2.1 Bacteria and viruses

*Escherichia coli* (ATCC 15597), used as a model for Gram-negative, enteric, bacterial pathogens, was cultivated in Miller's LB medium containing 10g/L NaCl overnight at 37 °C. *Mycobacterium smegmatis* (kindly provided by Dr. Joseph Falkinham at Virginia Tech), a surrogate for *Mycobacterium tuberculosis*, was cultivated in Middlebrook 7H9 broth for 48 h at room temperature. *Bacillus subtilis* (ATCC 6051), used as a model for Gram-positive bacteria, was cultivated in ATCC 3 nutrient broth overnight at 37 °C. The concentrations of *E. coli*, *M. smegmatis*, and *B. subtilis* in culture reached  $10^{10}$ ,  $10^{10}$ , and  $10^8$  colony-forming units (CFU)/mL, respectively, after incubation.

Two bacteriophages, MS2 (ATCC 15597-B1), a model for non-enveloped viruses that is widely used in environmental engineering studies, and  $\Phi 6$  (kindly provided by Dr. Paul Turner at Yale University), a model for enveloped viruses including influenza virus<sup>41-44</sup>, were propagated as previously described<sup>45</sup>. Cells and debris were removed from the virus suspension by filtering it through a 0.22  $\mu\text{m}$  cellulose acetate membrane. MS2 was suspended in LB medium and  $\Phi 6$  in TSB medium, and the virus stocks were stored at 4 °C. Both virus stocks had a concentration of  $10^{10}$ - $10^{11}$  plaque-forming units (PFU)/mL. The characteristics of microorganisms used in this study are summarized in **Table B.1**.

### 3.2.2 Viability of bacteria and viruses in suspended aerosols

The viability of bacteria and viruses in suspended aerosols was studied in a 27-L aluminum rotating drum, as detailed in our previous study<sup>38</sup>. Briefly, bacterial and virus suspensions were aerosolized by a 3-jet Collison nebulizer (BGI MRE-3) at a pressure of 40 psi, and the aerosols were introduced into the drum. The targeted RH inside the drum was achieved by adjusting the flow rates of aerosol, dry air, and saturated air. A RH probe (HP-22A; Rotronic) monitored RH continuously in the drum. The temperature inside the drum remained at room temperature ( $22 \pm 1$  °C) throughout all experiments. The viability of bacteria was tested at 20%, 40%, 60%, 80% and 100% RH, while the viability of viruses was tested at 23%, 33%, 43%, 55%, 75%, 85% and 100% RH; the latter set of values was intended to match conditions in our previous

study of influenza virus<sup>38</sup>. The drum rotated at a speed of 2.5 rpm, which was optimized to minimize physical loss of the aerosols.

Once RH reached equilibrium, a pre-exposure aerosol sample was collected on a 37-mm gelatin filter mounted in an aluminum filter holder at a flowrate of 2 L/min (AirChek XR5000; SKC) for 20 min. The drum was then sealed, and aerosols were incubated for 1 h. A post-exposure aerosol sample was collected following the same procedure as for the pre-exposure sample. Filters were dissolved in 5-mL of pre-warmed liquid medium. Bacteria samples were quantified immediately after sample collection, whereas virus samples were stored at -80°C before quantification.

### **3.2.3 Viability of bacteria and viruses in stationary droplets**

The viability of bacteria and viruses in stationary droplets was studied in an environmental chamber (5518; Electro-Tech Systems) at room temperature ( $22 \pm 1$  °C) and the same RH values listed above for aerosols, except that 98% RH was used instead of 100% RH since saturated conditions in the environmental chamber led to condensation on its surfaces. Ten 1- $\mu$ L droplets of bacterial or virus suspension were spotted on a 6-well, polystyrene cell culture plate (SIAL0516; Sigma) with a 0.1-10- $\mu$ L pipette. Droplets were incubated for 1 h, after which bacteria and viruses were collected by washing, including pipetting up and down several times, with 500  $\mu$ L of culture medium. Control samples containing 1 mL of bacterial or virus suspension in a sealed 1.5-mL microcentrifuge tube were incubated inside the environmental chamber during each experiment.

### **3.2.4 Relative viability**

Bacteria were quantified immediately after sample collection using a standard dilution plating technique<sup>46</sup>. A 100- $\mu$ L aliquot of bacteria sample was first diluted in serial tenfold steps in growth medium, and a 100- $\mu$ L aliquot of each dilution was then plated on their corresponding growth agars (LB agar for *E. coli*, Middlebrook 7H10 agar for *M. tuberculosis*, and 3 nutrient agar for *B. subtilis*) and incubated at their growth temperatures. Colony numbers were counted after 1 d for *E. coli* and *B. subtilis*, and 2 d for *M. smegmatis*. Virus samples were quantified by plaque assay<sup>45</sup>. All experiments were performed in triplicate.

Relative viability, which is the ratio of the microorganism's post-exposure concentration,  $C_{post-exposure}$ , to its pre-exposure concentration,  $C_{pre-exposure}$ , was calculated as shown in **eq 1** to report bacterial and viral decay.

$$\text{Relative Viability} = \frac{C_{post-exposure}}{C_{pre-exposure}} \quad (1)$$

For bacteria and viruses in suspended aerosols, relative viability was further corrected for physical loss of aerosols in the chamber so that it reflected biological decay only, as described in our previous study<sup>38</sup>. The physical loss coefficient and dilution coefficient for bacteria and viruses at different RHs are listed in **Table B.2**.

### 3.2.5 Enrichment of solutes in evaporating droplets

A microbalance (MSE3.6P; Sartorius) was placed inside the environmental chamber to study the evaporation rate of LB and TSB droplets at the same RHs as in the viability experiment. Ten 1- $\mu$ L droplets were spotted on a microscope cover glass (12-545-M; Fisher Scientific), and droplet mass was recorded continuously for 1 h at 1-min intervals. As the solutes were not volatile, their concentrations were expected to increase in a predictable way as droplet volume decreased. The enrichment factor, which is the fold increase in solute concentrations in droplets, was calculated according to **eq 2**:

$$\text{Enrichment factor} = \frac{V_0}{V_t} = \frac{m_0}{m_t} \quad (2)$$

where  $V_0$  and  $V_t$  are the droplet volumes and  $m_0$  and  $m_t$  are the droplet masses at  $t=0$  and  $t$ , respectively. This equation assumes that the density of the solution remains constant.

The cumulative dose of a potentially harmful solute experienced by a microorganism was calculated as the sum of the product of the concentration and time, as shown in **eq 3**:

$$\text{Cumulative dose} = \sum_0^T C_i \cdot \Delta t \quad (3)$$

where  $C_i$  is the solute concentration at the  $i$ th measurement.  $C_i$  is the lesser of the product of the initial solute concentration and the enrichment factor (i.e., the calculated concentration of the solute given a certain amount of water loss), and the solubility of the solute (i.e., the maximum possible concentration); and  $\Delta t$  is the interval between two measurements, which equals 1 min in this study.

### 3.2.6 Viability of MS2 in concentrated bulk solution

To test the hypothesis that cumulative dose drives the decay of viruses, MS2 was incubated in bulk LB broth of different strengths for various periods of time, producing a wide range of cumulative doses. Concentrated LB broths were made by dissolving 25 g, 250 g, and 500 g of Miller's LB power into 1 L of distilled water to make 1X, 10X, and 20X LB broth, respectively. Subsequently, 90  $\mu$ L of MS2 stock was diluted with 8.91 mL of concentrated LB broth in 15 mL culture tubes. The mixtures were then incubated at room temperature for 6 h, 1 d, and 7 d, after which 200  $\mu$ L of the suspension was collected for further quantification.

### 3.2.7 Statistical analysis

Differences in microorganism concentrations and viability were assessed using a paired sample t-test with a significance level of 0.05. Linear, polynomial, and logistic regression analysis was performed with JMP Pro 14 to assess the correlation between the cumulative dose and log-transformed relative viability of MS2 in bulk solution.

## 3.3 Results

### 3.3.1 Viability of bacteria in aerosols and droplets

We measured the viability of Gram-positive model organism *B. subtilis*, Gram-negative model organism *E. coli*, and *M. smegmatis* in suspended aerosols at RHs of 20%, 40%, 60%, 80% and 100% over an exposure period of 1 h using culture-based approach. As shown in **Figure 3.1A**, the relative viability of the three bacterial strains generally decreased with decreasing RH, and there was little loss at high RH ( $\geq 80\%$ ). *M. smegmatis* and *B. subtilis* decayed by less than 1  $\log_{10}$  unit across all RHs. The viability of *B. subtilis* was lowest at 20% RH and increased monotonically with RH, while the pattern was similar for *M. smegmatis* except that viability was lower at 80% RH than at 60% or 100% RH. *E. coli* decayed more than the other two strains did at RHs of 60% and lower. In fact, viable *E. coli* concentration at 20% RH was below the detection limit (0.625 CFU/L of air) after exposure, and greater than 1- $\log_{10}$  reduction was observed at 60%

RH. The post-exposure concentrations of *E. coli* and *M. smegmatis* were higher, though not significantly, than their pre-exposure concentrations at 100% RH.

Similar to the results in aerosols, bacteria in stationary droplets survived better at higher RHs, as shown in **Figure 3.1B**. Very little bacterial decay was observed at 80% RH and higher for all bacterial strains. However, their viability at 60% RH and lower was reduced and varied significantly. *B. subtilis* had the most pronounced decay ( $> 3\text{-log}_{10}$  reduction), while *M. smegmatis* and *E. coli* decayed by less than 1  $\log_{10}$  unit.

### 3.3.2 Viability of viruses in aerosols and droplets

We measured the viability of two bacteriophages, MS2 and  $\Phi 6$ , at RHs of 23%, 33%, 43%, 55%, 75%, 85% and 100% over an exposure period of 1 h with plaque assay. In contrast to the mostly monotonic patterns of relative viability vs. RH observed in bacteria, the viability of viruses showed a distinct U-shaped pattern (**Figure 3.2**). There was little to no decay at RHs below 33% and at 100%, whereas more significant decay occurred at intermediate RHs. Specifically, MS2 and  $\Phi 6$  decayed 0.9 and 1.8  $\log_{10}$  units at 55% and 75% RH, respectively.

In droplets, the viability of MS2 was lowest at 55% RH but with a more pronounced decay of 2  $\log_{10}$  units compared to 0.9  $\log_{10}$  in aerosols. The minimum viability of  $\Phi 6$  in droplets occurred at a higher RH (85%) than in aerosols, although the relative viability in aerosols was similar at 75% and 85% RH.

### 3.3.3 Droplet evaporation and solute enrichment

To be able to calculate the concentrations of solutes in droplets over time, we measured the mass of droplets continuously for 1 h as they evaporated at each RH. As shown in **Figure 3.3A**, evaporation of LB droplets was rapid at RHs of 43% and lower, whereas it was slower at higher RH. The droplets completely dried out after 27, 30 and 33 min at 23%, 33% and 43% RH, respectively. Droplets almost fully evaporated, but retained a small amount of liquid at 55% RH. Substantial liquid remained in droplets at RHs above 85%. **Figure B.1** shows example images of droplets during evaporation. These droplets did not contain microorganisms, which could potentially affect the evaporation rate, so we also measured evaporation rates

of LB droplets supplemented with MS2 at selected RHs. Results indicated that the presence of viruses had negligible effect on the rate of droplet evaporation (**Figure B.2**). The evaporation rate of TSB droplets (**Figure B.3**) was similar to that of LB droplets.

**Figure 3.3B** shows the enrichment factors, or the fold increase in concentration, of solutes as droplets evaporated. These values are hypothetical, assuming that the solutes remain dissolved and their concentrations do not reach saturation. The enrichment factors were similar over the first several minutes across all RH levels but started to diverge after ~10 min. At RHs of 43% and below, the enrichment factors climbed steeply in less than 30 min. Once droplets fully evaporated, we assigned an enrichment factor of “undefined,” as there was no bulk water and thus no solution. At higher RHs, evaporation was much slower, and the enrichment factor continued to increase throughout the exposure period. After 1 h, solute concentrations in LB droplets increased 17.7, 2.7, and 1.3 fold at 75%, 85%, and 100% RH, respectively.

In reality, enrichment factors of solutes are limited by their solubility, as their concentrations will not greatly exceed their saturation concentrations, even taking critical supersaturation into consideration<sup>47,48</sup>. **Figure 3.4A** shows the concentration of NaCl, used as an example solute, in evaporating droplets to demonstrate its dynamics during droplet evaporation. Initially, the NaCl concentration was 10 g/L, as found in standard LB media. At low RH, its concentration quickly rose and reached saturation at 21, 26, and 27 min at 23%, 33% and 43% RH, respectively. When droplets desiccated completely at ~30 min, there was no longer any dissolved NaCl. At 55% RH, droplets continued to evaporate during the entire 1-h exposure period, but the NaCl concentration stabilized at 360 g/L, its solubility ignoring the effects of other solutes, after 36 min according to our prediction based on the enrichment factor. At higher RHs, the increase in NaCl concentration was slower due to much slower evaporation. The estimated NaCl concentrations at 1 h were 177, 27, and 12 g/L at 75%, 85%, and 100% RH, respectively. If the experiments were to continue beyond 1 h, we would expect the concentration of NaCl to continue increasing at high RHs.

### 3.3.4 Cumulative dose to viruses

**Figure 3.4B** shows the cumulative dose of NaCl, or the product of its concentration and time, as a function of time at different RHs. At RHs of 43% and below, the cumulative dose increased quickly due to rapid evaporation and the resulting increase in NaCl concentration. Once droplets desiccated around 30 min, the cumulative dose stopped increasing. In contrast, at intermediate RHs, the cumulative dose initially increased more slowly but then grew rapidly since the NaCl concentration remained high for an extended period of time. An extended duration of high NaCl concentration led to the highest cumulative dose of NaCl occurring at 55% RH, the same RH at which the relative viability of MS2 was lowest. At higher RHs, evaporation was much slower, resulting in a moderate increase in NaCl concentration and the lowest cumulative dose of NaCl.

## 3.4 Discussions

Our results show that the viability of bacteria and viruses in suspended aerosols and droplets is RH dependent, varying by over three orders of magnitude for bacteria and up to two orders of magnitude for viruses. These results suggest that environmental conditions have the potential to influence transmission of certain pathogens by affecting their viability while they are transmitting between hosts. Whereas bacteria survived better at humid conditions than dry conditions, viruses survived best at both low and extremely high RHs while experiencing greater decay at intermediate RHs. The difference in viability patterns suggests that different mechanisms may dominate the humidity-dependent decay of bacteria and viruses.

### 3.4.1 Viability of bacteria

Our results agree well with previous observations for survival of bacteria as a function of RH<sup>34,35</sup>, and conflicting results can probably be attributed to differences in aerosolization media, exposure time, bacteria species, and culture techniques<sup>30,32</sup>. The culture media for the three bacterial strains in our study all contain salt as essential component for bacterial growth. While the initial salt concentration in culture media is ideal for bacteria growth, the elevated salt concentration resulting from droplet evaporation could be harmful for bacteria. High salt concentrations can induce an osmotic pressure that inhibits bacterial growth and causes

bacterial inactivation<sup>49</sup>. Studies have reported that the critical salt level for *E. coli* in LB broth and other bacteria in their growth media is around 2.5% to 4%<sup>50,51</sup>, which is achieved in evaporating droplets after approximately 1 h of exposure at 80% RH. This is consistent with our observation of minimal loss in viability at 80% and higher RHs, but not at low and intermediate RHs. At low RH, droplets lose more water than at high RH, resulting in a higher salt concentration that is more harmful to bacteria. To confirm that inactivation took place mainly while the droplets were evaporating and not after they had dried out, we conducted a separate experiment in which we monitored the relative viability of bacteria at several time points after the droplets dried completely. At 20% RH, the droplets dried out after 20 min, and we measured viability at 1 h, 2 h, and 6 h. We found that bacteria decayed much faster in evaporating droplets that were still wet; the inactivation rate was at least two orders of magnitude smaller after the droplets had dried out (**Figures B.4 and B.5**). This result agrees with the conclusion of a review that dried bacteria and viruses can survive for days to months<sup>52</sup>. Thus, the loss of viability is largely controlled by conditions experienced while liquid is still present.

We observed that *E. coli* survived better in droplets than in aerosols at RHs of 60% and lower, whereas the reverse was true for *B. subtilis*. The reason for this difference is not known. It is possible that *B. subtilis* in droplets, but not in aerosols, entered the viable but not culturable state during the exposure period, or that the opposite was true for *E. coli*. Experimental artifacts could also explain this behavior, although we attempted to minimize bias. For example, the recovery of *B. subtilis* in dried droplets from the culture plate surface might have been much lower than for *E. coli*.

### **3.4.2 Viability of viruses**

Many previous studies have reported that certain viruses, including various strains of influenza, undergo greater decay at intermediate RHs than at lower or higher RHs<sup>23,31,37,53–57</sup>. Although there have been attempts to explore how RH affects the viability of viruses, the underlying mechanism is still unclear. In this study, we observed similar U-shaped patterns between viability and RH for MS2 and  $\Phi$ 6, consistent with findings reported in the literature for many non-enveloped and enveloped viruses. Osmotic pressure

has been shown to cause shrinkage and loss of vaccine activity of inactivated influenza virus, but it has not been directly linked with the inactivation of viable viruses<sup>58</sup>. Noue *et al.* has shown that when norovirus, a non-enveloped virus, is fully inactivated at 55% RH, its viral RNA remains detectable while the binding capacity of its capsid is compromised<sup>59</sup>. These results suggest that humidity-driven inactivation compromises the capsid structure of non-enveloped viruses, while the effect on the genome is inconclusive.

As our results indicate that the cumulative dose of solutes and decay of MS2 both peak at 55% RH, we hypothesize that exposure of the virus to continuously increasing solute concentrations in evaporating droplets leads to accumulation of damage to the virus structure and ultimately inactivation. There is a wealth of evidence that viruses can survive for days or more on dry surfaces<sup>52,60,61</sup>, so we presume that inactivation took place mainly while the droplets were still wet. Enveloped viruses are generally more vulnerable than non-enveloped viruses in the environment<sup>62,63</sup>. However, we observed that the magnitude of decay is similar between the enveloped virus  $\Phi 6$  and non-enveloped virus MS2. More importantly, we observed a U-shape pattern in viability against RH in both viruses, suggesting a common inactivation mechanism. Solutes in media might act on shared viral structures between the two types of viruses, such as the capsid, to cause inactivation. The proposed mechanism of inactivation is consistent with the idea of disinfection kinetics, where the product of the concentration of disinfectant and time, or CT, is the determinant of inactivation of microorganisms.

To test this hypothesis, we measured the viability of MS2 in bulk LB medium at varying broth concentrations (1X, 10X, and 20X) and varying durations of exposure (6 h, 1 d, 7 d). Since the experiment was conducted in 9 ml of bulk solution in a sealed 15-ml vial with minimal opportunity for evaporation, solute concentrations remained essentially unchanged throughout the experiment. In this case, the cumulative dose is simply the product of the initial medium concentration and the exposure time. The duration of the experiment in bulk solution was much longer than 1h in order to yield a range of cumulative doses matching those in droplets. As shown in **Figure 3.5A**, the viability of MS2 decreased with increasing concentration of the medium and exposure time, respectively, and there was a strong negative correlation between the log-transformed relative viability of MS2 and cumulative dose of LB broth ( $p=0.0012$ ), as

shown in **Figure 3.5B**. Thus, it appears that increased viral decay at intermediate RHs is caused by exposure to a higher cumulative dose of solutes at these conditions. Future studies focusing on the functional and structural integrity of viruses are required to gain a more complete mechanistic understanding of the effects of RH and solute concentrations on viral decay.

While the correlation between the cumulative dose of LB medium and MS2 viability highlights the overall effect of media on viability of viruses, it does not identify the specific composition of aerosols and droplets that is responsible for inactivation. Assuming NaCl to be the culprit, we compared the inactivation rate of MS2 in evaporating droplets and in bulk concentrated LB as a function of NaCl dose and found that decay was faster in evaporating droplets (0.2 vs 0.02 log<sub>10</sub> decay in droplets vs. bulk per 1000 g L<sup>-1</sup> min of NaCl). The composition of aerosols and droplets that carry microorganisms is usually very complex, as they originate from sources such as respiratory tracts, seawater, and wastewater. For example, LB, one of the media tested in this study, consists of NaCl, tryptone, and yeast extract. The evolution of each component's concentration in evaporating aerosols and droplets is not exactly the same as that of NaCl (**Figure 3.4A**), due to differences in their initial concentration and solubility. Other components might have different cumulative dose patterns in evaporating droplets and bulk solutions and thus induce different magnitudes of viral decay. Furthermore, each component is likely to affect the viability of microorganisms differently. One component might dominate lethality, while others could have moderate or even protective effects. Our recent study revealed that human bronchial epithelial cell wash has a protective effect on aerosolized influenza viruses, possibly due to proteins<sup>38</sup>.

In addition to the components that were originally present in aerosols and droplets, there might be others that could migrate into aerosols and droplets from the surrounding environment. For example, oxidants such as ozone, hydroxyl radical, or hydrogen peroxide could diffuse into aerosols and droplets from ambient air; such compounds are known to inactivate viruses by breaking down the genome and capsid<sup>64,65</sup>. In the context of disinfection for drinking water treatment, hypochlorous acid, singlet oxygen, and chlorine dioxide have been shown to inactivate viruses by damaging either their genome or their ability to bind to a host cell<sup>66</sup>. Components of solid materials may dissolve into droplets that have deposited on

surfaces. Once they migrate into aerosols and droplets, the concentration of these molecules or ions could increase as aerosols and droplets evaporate.

### **3.4.3 Aerosols vs. droplets**

The sub-micron aerosols and 1- $\mu$ L droplets studied here are relevant to aerosol transmission and indirect transmission of infectious diseases, respectively. The proposed mechanisms of bacterial and viral decay illuminate the importance of evaporation kinetics of aerosols and droplets in disease transmission. Here, we report the evaporation kinetics of 1- $\mu$ L droplets, which fully evaporated within ~30 min when RH was below 43%. At intermediate and high RHs, their evaporation rate was slower, and droplets did not desiccate. However, we did not characterize the evaporation kinetics of suspended aerosols due to the greater technical challenge. According to the literature, evaporation kinetics of aerosols vary significantly depending on the composition of aerosols<sup>67,68</sup>. Pure water aerosols fully evaporate on the timescale of seconds<sup>39</sup>, while it takes minutes to hours for aerosols with high viscosity, such as secondary organic aerosols, to lose half of their mass<sup>69</sup>. We expect aerosols consisting of culture media to evaporate on the timescale of minutes because they are composed of organic compounds. This relatively slow evaporation process allows the salt concentration and cumulative dose to evolve differently at different RH conditions and could facilitate the observed patterns in the viability of bacteria and viruses in aerosols.

To our best knowledge, this is the first study directly comparing the viability of microorganisms as a function of RH in both suspended aerosols and stationary droplets. Three of the microorganisms tested here, specifically the two viruses and *M. smegmatis*, had similar trends in inactivation and magnitude of decay in both aerosols and droplets. Thus, in certain cases, droplets could be used as a surrogate for aerosols in studies of viability, an attractive option because stationary droplets are much easier to handle.

### **3.4.4 Media composition**

We have shown that RH impacts the viability of microorganisms via two steps: (1) RH controls the evaporation kinetics of droplets and causes changes in solute concentrations, and (2) the elevated solute concentrations and cumulative exposure to them cause decay of bacteria and viruses. Differences in media

composition are known to affect the viability of microorganisms in aerosols and droplets<sup>37,38</sup>. Although the medium differed by microorganism in the present study, the trends in viability vs. RH were similar between the two bacteria and similar between the two viruses. The present work focuses on explaining the inactivation kinetics of bacteria and viruses in aerosols and stationary droplets using the recommended growth media for each microorganism. A separate study on the effects of individual components of the media at physiologically and environmentally relevant concentrations on microorganism viability is ongoing.

In addition to differences in media composition, the evaporation rates of LB and TSB (media for MS2 and  $\Phi 6$ ) were slightly different (**Figure B.3**). After 1 h at 55% RH, TSB droplets fully evaporated, whereas LB droplets still retained some liquid. The differences in evaporation kinetics as well as media composition could explain why the minimum relative viability of these two viruses occurred at different RHs.

Furthermore, organic aerosols tend to form a glassy layer, which is a highly viscous semi-solid or solid state outer layer of aerosols, at the air-liquid interface as they evaporate<sup>70</sup>. This glassy layer slows further evaporation by inhibiting mass transfer between liquid and air. Solute concentrations in evaporating organic aerosols typically increase more slowly than in inorganic aerosols at the same RH. This effect may help microorganisms survive in organic aerosols because it will take longer to elevate solute concentrations to levels that cause lethal effects on bacteria and viruses.

Lastly, the pH of media may also play a role in microorganisms' viability in aerosols and droplets. If the pH favors the aggregation of bacteria and viruses<sup>71</sup>, a process that depends on their isoelectric points (**Table B.1**), the microbes could be protected from inactivation. However, the presence of a pH gradient in aerosols and droplets<sup>72</sup> complicates this process, especially when the spatial distribution of microorganisms in aerosols and droplets is not clear. Future research on the effects of pH on the viability of bacteria and viruses in aerosols and droplets is recommended.

### 3.4.5 Limitations

While this study provides new insight into the inactivation of bacteria and viruses in droplets, it does not conclusively identify the mechanisms of inactivation. Based on the observations in this study, we have proposed different mechanisms of inactivation of bacteria and viruses in droplets. However, the pattern of viability vs. RH is somewhat similar for both types of microorganisms at intermediate and high RH: viability decreases with decreasing RH. Thus, it is possible that the same mechanism applies to both bacteria and viruses at intermediate and high RH, whereas a different mechanism applies at low RH, where patterns in viability diverge. It is also possible that multiple mechanisms act simultaneously, such as osmotic pressure and oxidative stress, but that one or the other dominates depending on the type of microorganism in question. Additionally, we elected to study viability in a relatively simple system of the recommended medium for each microorganism. Future studies should consider more physiologically and/or environmentally relevant media.

In summary, we have described the effects of RH on the viability of bacteria and viruses that are surrogates for important pathogens in both suspended aerosols and stationary droplets. Bacterial viability was highest at high RH and lowest at low RH. That viruses maintained viability at both low RH and extremely high RH, while decaying at intermediate RH, is consistent with the observed seasonality of certain infectious diseases, such as influenza, for which incidence is highest in the wintertime in temperate regions, when indoor RH is low, and during the rainy season in tropical regions, when RH is very high.<sup>7</sup> We also explored droplet evaporation kinetics and the evolution of solute concentrations in evaporating droplets. We concluded that osmotic pressure, which results from elevated salt concentration as aerosols and droplets evaporate, dominates inactivation of bacteria. However, the inactivation of viruses is linked to the cumulative dose of a harmful substance in solution. Our study has provided new, mechanistic insight into the effect of RH on the viability of pathogens in the environment and infectious disease transmission.

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## Tables and Figures

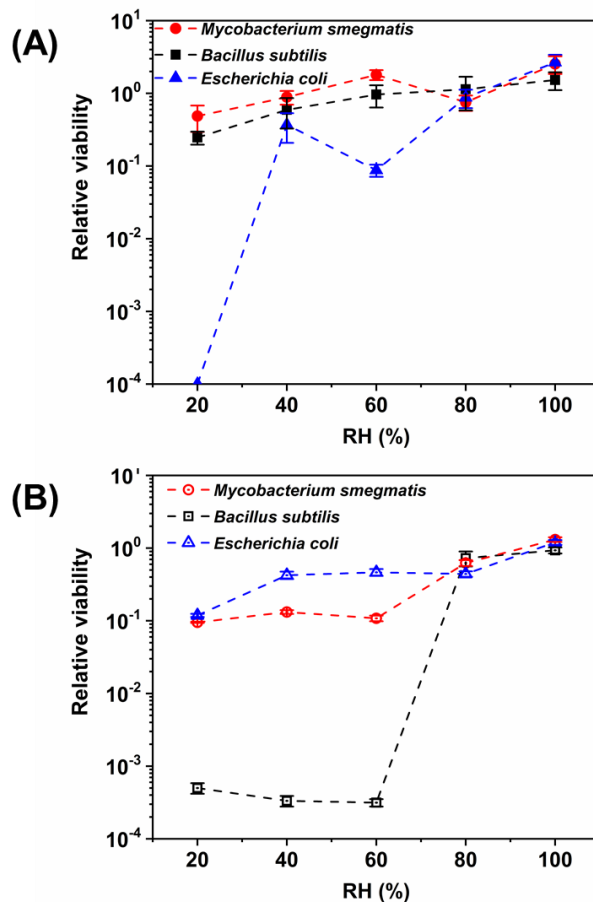


Figure 3.1 Relative viability of three bacterial strains, determined by culturing, as a function of RH in suspended aerosols and stationary droplets (mean  $\pm$  s.d. of triplicates). (A) Relative viability of *M. smegmatis* in Middlebrook 7H9 broth (red), *B. subtilis* in 3 nutrient broth (black), and *E. coli* in LB broth (blue) in aerosols after 1 h at 20%, 40%, 60%, 80% and 100% RH. The relative viability of *E. coli* in aerosols at 20% RH was below the detection limit of  $10^{-5}$ . (B) Relative viability of bacteria after 1 h in 1- $\mu$ L stationary droplets.

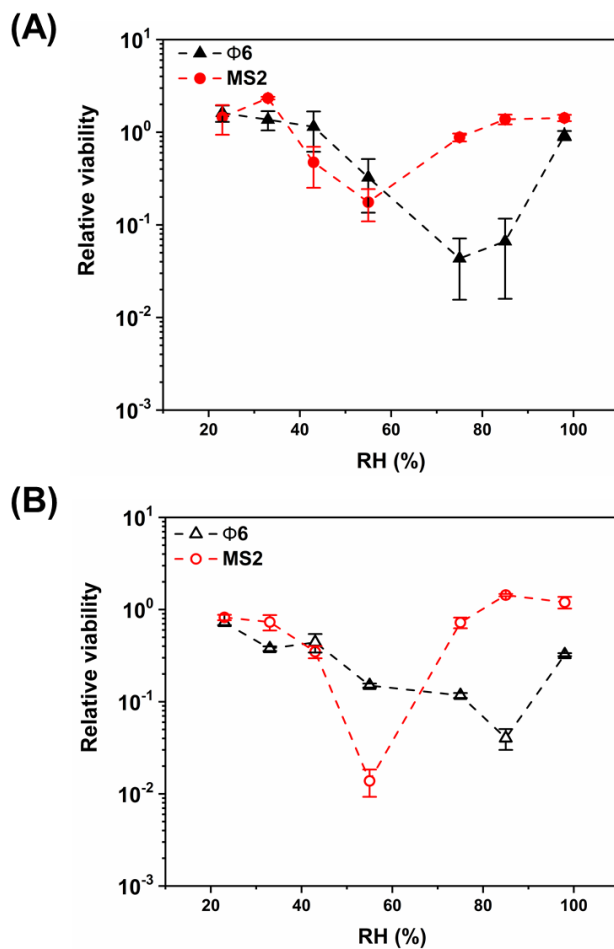


Figure 3.2 Relative viability of two viruses, determined by plaque assay, as a function of RH in suspended aerosols and stationary droplets (mean  $\pm$  s.d. of triplicates). (A) Relative viability of  $\Phi 6$  in TSB broth (black) and MS2 in LB broth (red dot) in aerosols after 1 h at 23%, 33%, 43%, 55%, 75%, 85% and 100% RH. (B) Relative viability of viruses after 1 h in stationary droplets.

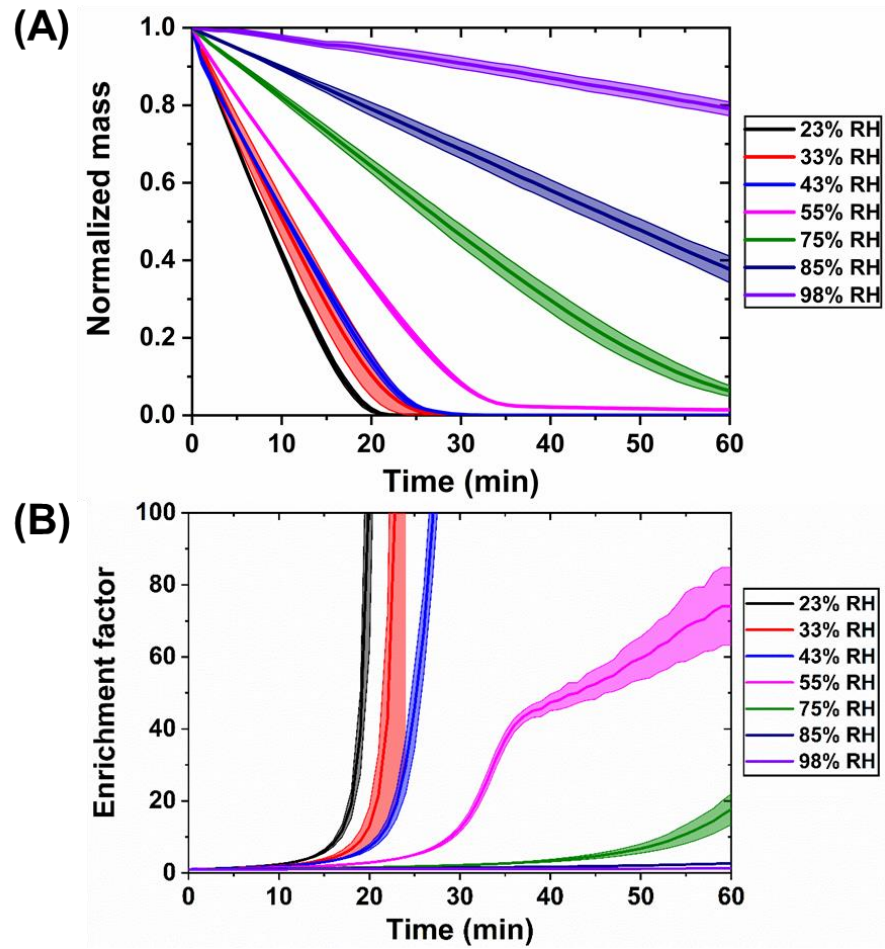


Figure 3.3 Change in droplet weight and solute concentration at various RH during 1 h exposure. (A) Normalized mass of plain (without microorganisms) LB droplets. (B) Enrichment factors of solutes in plain LB droplets. Dark lines show the average of triplicates at each RH, and the shaded bands show the standard deviation.

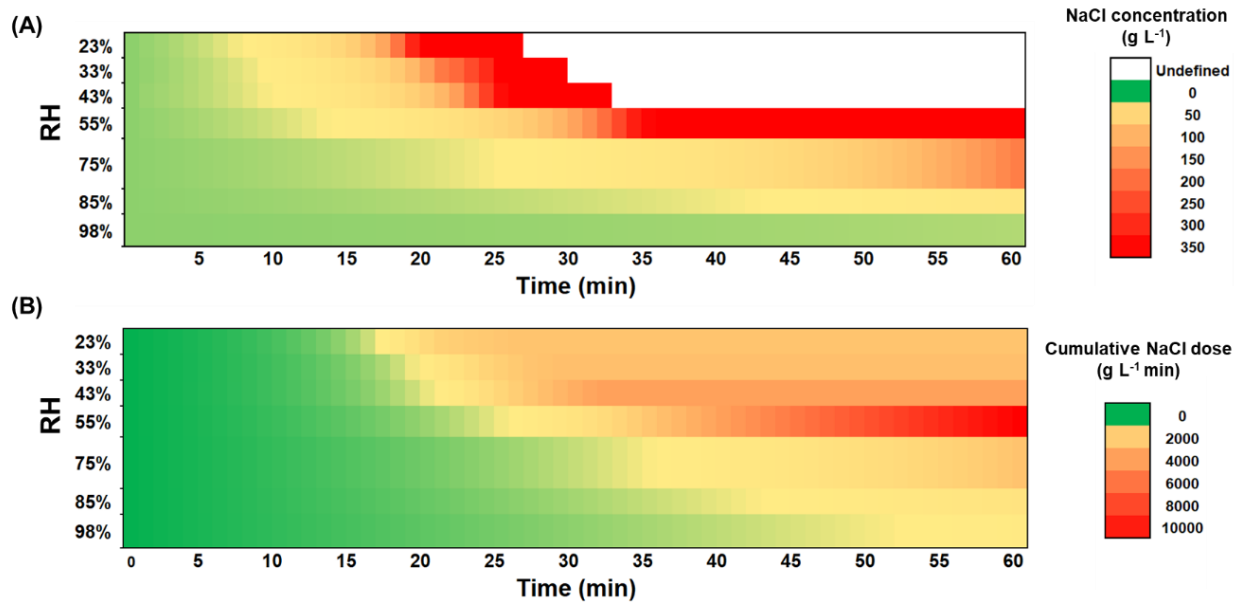
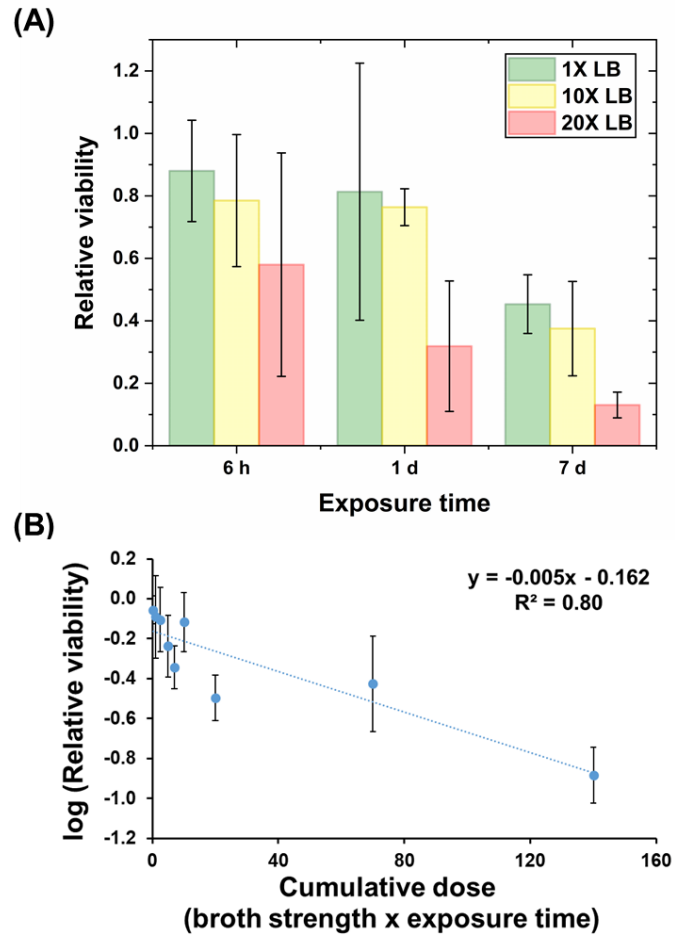


Figure 3.4 Solute concentration and cumulative dose to microorganisms as a function of time, using NaCl as an example. (A) Concentration of NaCl in LB droplets at each RH. The initial concentration of NaCl in LB broth was 10 g/L. (B) Cumulative NaCl dose to microorganisms in evaporating droplets. The values represent the average from three independent experiments.



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## **4. Survival of Viruses in Droplets as a Function of Relative Humidity, pH, and Salt, Protein, and Surfactant Concentrations**

Kaisen Lin, Chase R. Schulte, and Linsey C. Marr\*

Department of Civil and Environmental Engineering, Virginia Tech, 418 Durham Hall, Blacksburg,  
Virginia 24061, United States

\*Corresponding author

Mailing address: Department of Civil and Environmental Engineering, Virginia Tech, 418 Durham Hall,  
Blacksburg, Virginia 24061, United States

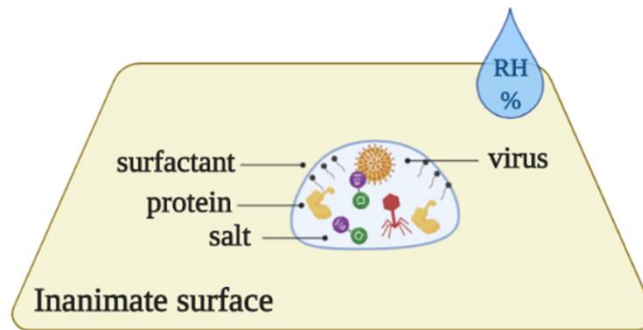
E-mail: [lmarr@vt.edu](mailto:lmarr@vt.edu)

Key words: MS2, Phi6, virus, bacteriophage, media, persistence, viability

## **Abstract**

The survival of viruses in droplets is known to depend on their chemical composition. However, the effect of each component on the viability of viruses has not been extensively explored. We investigated the effects of salt, protein, surfactant, and droplet pH on the viability of viruses in stationary droplets at 20%, 50%, and 80% relative humidity (RH) using a culture-based approach. Results showed that viability of MS2, a non-enveloped virus, was generally higher than that of  $\Phi 6$ , an enveloped virus, in droplets after 1 hour. The chemical composition of droplets greatly influenced virus viability. Specifically, the survival of MS2 was similar in droplets at different pH values, but the viability of  $\Phi 6$  was significantly reduced in acidic and basic droplets compared to neutral ones. The presence of bovine serum albumin (BSA) protected both MS2 and  $\Phi 6$  in droplets. The effects of sodium chloride and surfactant sodium dodecyl sulfate varied by virus type and RH level. Meanwhile, RH affected the viability of viruses as previously shown: viability was lowest at intermediate to high RH. The results demonstrate that the viability of viruses is determined by the chemical composition of carrier droplets, especially pH and protein content, and environmental factors. These findings emphasize the importance of understanding the chemical composition of carrier droplets in order to predict the persistence of viruses contained in them and provide insight into infectious disease transmission.

**TOC Art**



## 4.1 Introduction

Pathogenic organisms, including bacteria, viruses, fungi, protozoa, and helminths, cause infections that are responsible for substantial morbidity and/or mortality <sup>1</sup>. For example, it is estimated that influenza has caused 9 million to 45 million illnesses and 12,000 to 61,000 deaths annually since 2010 in the United States <sup>2</sup>. Some diseases rely on the spread of pathogenic organisms in the environment, from infected hosts to susceptible hosts via aerosol, direct contact, fecal-oral, and/or fomite routes. Successful transmission requires that the pathogen survive, or maintain its infectivity, while it is in the environment. Studies on the persistence of pathogenic organisms in solutions, on material surfaces, and in the air have shown that the survival of pathogenic organisms varies by strain, composition of the surrounding media, and environmental factors <sup>3-12</sup>.

Aerosol transmission and fomite transmission are important routes for the spread of many infectious diseases, such as influenza, tuberculosis, and measles <sup>13</sup>, which cause millions of cases of illness every year. Pathogens can be released from a host in aerosols (particles small enough to remain airborne) and droplets (larger particles that quickly deposit onto surfaces). Aerosols and droplets are complex systems due to their varying size, high surface-to-volume ratio, and spatially heterogeneous composition. Pathogens that are immersed in droplets and aerosols experience a dynamic, highly variable microenvironment that affects their viability <sup>14</sup>, as illustrated in **TOC Art**.

The composition of the fluid that constitutes aerosols and droplets may greatly impact the viability of pathogens. Often, aerosols and droplets emitted from different sources have distinct chemical and physical properties. For example, fluids expelled by infected patients when they exhale, talk, cough, or sneeze contain high levels of proteins and are usually viscous <sup>15</sup>, while sea spray aerosols contain more salts and organic compounds <sup>16</sup>. Because each component might have a different effect on a pathogen, its survival in aerosols and droplets that are generated from different sources may vary <sup>17</sup>. However, for simplicity, most studies have evaluated viability in culture media. Extending these results to more environmentally or physiologically relevant fluids requires an understanding of how specific media components affect pathogens.

The viability of pathogens in aerosols and droplets is also affected by environmental factors, such as temperature<sup>18,19</sup>, humidity<sup>17,20–26</sup>, and ultraviolet radiation<sup>27–29</sup>. Temperature and ultraviolet radiation primarily affect the viability of bacteria and viruses by destroying their structural integrity. Other studies have shown that the viability of bacteria and viruses in both aerosols and droplets depends on relative humidity (RH)<sup>24–26</sup>. We have proposed that this relationship is mediated by changes in the physicochemical properties of aerosols and droplets as they evaporate to equilibrate with ambient RH<sup>13,30</sup>.

Lastly, pathogens can be inactivated by applying chemicals (e.g., cleaning products or disinfectants) on surfaces and in air during cleaning activities in healthcare facilities and at home<sup>31,32</sup>. Certain chemicals are known to alter bonds in genetic material and proteins, lyse cell membranes, and disrupt cell metabolism<sup>33</sup>. This practice is usually very effective and can reduce disease transmission.

Viruses are responsible for diseases such as influenza, hepatitis, SARS, and many cases of gastroenteritis. Media composition is known to affect virus viability in aerosols and droplets<sup>20</sup>, but the effects of individual components have not been extensively studied in a controlled manner. With respect to specific components of the media, our prior study showed that airborne bacteriophage  $\Phi 6$  decayed  $\sim 2 \log_{10}$  units in tryptic soy broth (TSB) after 1 h exposure to 75% RH, whereas the phage did not decay in human epithelial bronchial (HBE) cell wash under the same conditions<sup>24</sup>. We suspect that proteins in HBE cell wash might protect  $\Phi 6$ , but the exact causes of the observed difference remain unknown. A more complete understanding of the effect of individual media components on the inactivation of viruses in environment is needed. Addressing this question may also enable advances in understanding the mechanism of pathogen inactivation in the environment.

In this study, we manipulated the concentrations of several common media components, including salt, protein, and surfactant, as well as pH, over environmentally or physiologically relevant ranges, while quantifying the viability of model viruses in droplets of the media. To gain insight into the interactions between media composition and RH on the viability of viruses in evaporating droplets, we exposed the virus-containing droplets to low, intermediate, and high RH levels for 1 h and evaluated the reduction in

virus viability. Results from this study will provide information on the effects of specific media components on the decay of non-enveloped and enveloped viruses in droplets.

## 4.2 Materials and Methods

### 4.2.1 Virus Stock

Bacteriophage MS2 (ATCC 15597-B1), a model for non-enveloped viruses that is widely used in environmental engineering studies<sup>34-36</sup>, was propagated as previously described<sup>37</sup>. Briefly, *E. coli* (ATCC 15597) was inoculated in Miller's LB medium and incubated overnight at 37 °C. Fifty microliters of MS2 stock, 450 µL of *E. coli* liquid culture, and 4.5 mL of LB soft agar were mixed and overlaid on LB agar plates. Plates were incubated at 37 °C for 24 h before the top layer of soft agar was collected in LB medium. The mixture of soft agar and LB medium was shaken at 100 rpm at 37 °C for 2 h. The mixture was then centrifuged at 5000 rpm for 5 min, and the supernatant was filtered through a 0.22 µm cellulose acetate membrane to remove cells and debris. The filtrate was collected as virus stock and stored at 4 °C.

Bacteriophage Φ6 (kindly provided by Dr. Paul Turner at Yale University), a model for enveloped viruses including influenza virus<sup>7,36,38,39</sup>, was propagated using the same method described above, except that the bacterial host was *Pseudomonas syringae*, which grows at 25 °C. Determined by plaque assay, the concentrations of virus stocks were 10<sup>10</sup>-10<sup>11</sup> plaque-forming units (PFU)/mL.

### 4.2.2 Preparation of Virus Suspension

For testing the effect of different components of media on virus viability, solutions containing various amounts of salt, protein, and surfactant were prepared. Specifically, a 100 g/L NaCl stock solution was prepared by adding 100 g of sodium chloride (Fisher Scientific) to ultrapure water (Barnstead Nanopure; Thermo) to a final volume of 1 L. Aliquots of the solution were then diluted with ultrapure water to produce working solutions with the concentrations shown in **Table 4.1**. Stock solutions of bovine serum albumin (BSA) (Sigma), a protein derived from cows and often used as a standard in lab experiments, and sodium dodecyl sulfate (SDS) (Sigma), an anionic surfactant used in many cleaning and hygiene products, were prepared similarly and diluted.

Virus suspensions at the targeted concentrations of salt, protein, surfactant were prepared right before experiments were conducted. Virus stock and the working solutions were mixed at a ratio of 1:100. Specifically, 50  $\mu\text{L}$  of virus stock was diluted with 4.95 mL of the working solution of interest in 15 mL centrifuge tubes and vortexed for 30 seconds. Virus stock and ultrapure water, which had an initial pH of 5.5, were mixed at the same ratio. The pH of the mixture was adjusted to 4.0, 7.0, and 10.0, respectively, with 0.1 M hydrochloric acid and 0.1 M sodium hydroxide and was measured using a pH meter (Orion Versa Star; Thermo). The amount of ions introduced to the solutions to adjust the pH, shown in **Table C.1**, was much lower than that used to make the 1 g/L NaCl working solution. Thus, the change in ionic strength due to pH adjustment should have a negligible effect on virus inactivation.

#### **4.2.3 Viability of Viruses in Droplets**

The viability of viruses in droplets was studied in an environmental chamber (5518; Electro-Tech Systems) at room temperature ( $22 \pm 1$  °C). For each virus suspension, droplets were exposed at low, intermediate, and high RH levels of 20%, 50%, and 80%, respectively. The targeted RH inside the environmental chamber was achieved by vaporizing ultrapure water with a humidifier or passing air through a desiccator. Fifteen minutes after the RH reached equilibrium, ten separate 1- $\mu\text{L}$  droplets of virus suspension were spotted on a 6-well, polystyrene cell culture plate (SIAL0516; Sigma) with a 0.1-10- $\mu\text{L}$  pipette. Droplets were incubated for 1 h, after which viruses were collected in 500  $\mu\text{L}$  of LB medium by pipetting up and down several times. Samples were stored at -80 °C immediately after collection until they were quantified by plaque assay. Control samples containing 10  $\mu\text{L}$  of virus suspension in a sealed 1.5-mL microcentrifuge tube were incubated inside the environmental chamber during each experiment and collected in 500  $\mu\text{L}$  of culture medium after 1 h.

#### **4.2.4 Plaque Assay and Relative Viability**

Virus samples were quantified by plaque assay as described previously<sup>37</sup>. Briefly, 10-fold serial dilutions of the collected samples were prepared. Fifty microliters of the serial dilutions, 450  $\mu\text{L}$  of liquid culture of bacterial host, and 4.5 mL of soft agar were mixed and poured over agar plates. Plates were incubated at

the bacterial host's growth temperature for 24 h. The number of plaques on plates was counted, and the virus concentration in the samples were calculated accordingly.

The change in infectious viral concentration after 1 h of exposure was expressed as relative viability. Relative viability was calculated as the ratio of the post-exposure viral concentration,  $C_{post-exposure}$ , to the pre-exposure concentration,  $C_{pre-exposure}$ , as shown in **eq 1**.

$$\text{Relative Viability} = \frac{C_{post-exposure}}{C_{pre-exposure}} \quad (1)$$

#### 4.2.5 Statistical Analysis

All experiments were performed in triplicate. The relative viability of viruses was expressed as mean  $\pm$  standard deviation. One-way ANOVA and a post-hoc analysis with Tukey's HSD test were performed to determine significant differences ( $P < 0.05$ ) in the relative viability of bacteriophages among different levels of media composition and among RH levels, respectively. Two-way ANOVA was performed to determine the interaction effect between media compositions and RH.

### 4.3 Results

#### 4.3.1 Salt

The viability of MS2 and  $\Phi 6$  was examined in droplets composed of different compositions at RHs of 20%, 50%, and 80% by plaque assay. As shown in **Figure 4.1A**, the effect of sodium chloride on MS2 viability was RH-dependent. MS2 decayed more in 35 g/L NaCl droplets than in 0 and 1 g/L NaCl droplets at 20% RH, while the pattern was opposite at 80% RH. At 80% RH, the viability of MS2 was significantly higher in droplets containing NaCl than in those without it, suggesting that NaCl had a protective effect at this RH condition. At 50% RH, the relative viability of MS2 was lower than at the other RHs and was similar across all NaCl levels.

The enveloped virus,  $\Phi 6$ , was generally more susceptible than the non-enveloped one, MS2. Thus, the relative viability of  $\Phi 6$  is shown on a log scale, whereas that of MS2 is shown on a linear scale. The relative viability of  $\Phi 6$  was less than 10% in droplets containing NaCl at all RHs after 1 h (**Figure 4.1B**).

At 20% RH, the relative viability was significantly lower in droplets containing NaCl, at concentrations of both 1 and 35 g/L compared to 0 g/L. At 50% RH, the relative viability was lowest in 1 g/L NaCl droplets, while it was significantly higher in droplets containing 0 and 35 g/L NaCl. At 80% RH, the relative viability was significantly higher in droplets containing 1 g/L NaCl compared to 35 g/L NaCl.

### 4.3.2 pH

The relative viability of MS2 in droplets at pH values of 4.0, 7.0, and 10.0 (i.e., acidic, pH-neutral, and basic) was generally similar at any one RH level (**Figure 4.2A**). At 20% RH, MS2 survived better, although not statistically significantly, in pH-neutral droplets than in more acidic or more basic droplets. There were slight, but not significant, differences in viability across pH at the other two RHs. Regardless of pH, viability was significantly lower at 50% RH compared to the other RHs.

The relative viability of  $\Phi 6$  differed significantly by pH, while the patterns in viability were similar across all three RHs (**Figure 4.2B**). At a pH of 4.0, no viable  $\Phi 6$  was detected in either the control solution or the evaporating droplets after 1 h, suggesting a strong inactivation effect of acidic conditions on  $\Phi 6$ . At a pH of 10.0, the virus decayed by  $\sim 1-3 \log_{10}$  units depending on RH, while the virus survived best in pH-neutral droplets (7.0), in which it decayed by  $\sim 1-2 \log_{10}$  units depending on RH. At both these pHs, relative viability was greater at 20% RH compared to the two higher RHs.

### 4.3.3 Protein

Similar to salt, the effect of protein on MS2 was also RH-dependent (**Figure 4.3A**). The relative viability of MS2 decreased slightly as the concentration of BSA increased in droplets at 20% RH. However, at RHs of 50% and 80%, the relative viability was higher in the presence of BSA. At 50% RH, the viability of MS2 was reduced by only 7% in droplets containing 100  $\mu\text{g/mL}$  BSA after 1 h, a significantly lower loss than the  $>80\%$  reduction in droplets that did not contain any BSA. At 80% RH, there was no decay in droplets containing BSA, regardless of its concentration, suggesting that BSA has a protective effect on the viability of MS2.

Similar to its effect on MS2, BSA protected  $\Phi 6$  from inactivation in droplets at intermediate and high RHs (**Figure 4.3B**). At 20% RH, the relative viability of  $\Phi 6$  was similar in droplets with and without BSA. However, at 50% RH, the relative viability of  $\Phi 6$  was significantly higher in droplets containing 1000  $\mu\text{g}/\text{mL}$  BSA than in droplets with 0 or 100  $\mu\text{g}/\text{mL}$  BSA. At 80% RH, the presence of BSA, regardless of its concentration, reduced the decay of  $\Phi 6$  in droplets, suggesting its protective effect on viruses in droplets again.

#### **4.3.4 Surfactant**

The relative viability of MS2 in droplets with different surfactant concentrations is shown in **Figure 4.4A**. MS2 generally survived better when SDS was present in droplets, and relative viability increased with SDS concentration at 20% and 80% RH. MS2 incurred no decay in droplets containing 10  $\mu\text{g}/\text{mL}$  SDS, whereas it at least lost 25% viability in droplets containing no SDS. The relationship between viability and SDS concentration differed at 50% RH, at which MS2 survived best in droplets with 1  $\mu\text{g}/\text{mL}$  SDS, but decayed most in droplets containing 10  $\mu\text{g}/\text{mL}$  SDS. As shown in **Figure 4.4B**, SDS did not significantly affect the viability of  $\Phi 6$  in droplets at RHs of 20%. However, 10  $\mu\text{g}/\text{mL}$  SDS induced a significantly higher inactivation of  $\Phi 6$  at RHs of 50% and 80%; no viable  $\Phi 6$  was recovered from droplets containing 10  $\mu\text{g}/\text{mL}$  SDS at high RH after 1 h.

#### **4.3.5 Relative Humidity**

U-shaped patterns in the viability of MS2 against RH were observed in droplets composed of salt and surfactant, as well as in pH-adjusted droplets. Specifically, the relative viability of MS2 was lowest at 50% RH, while it was significantly higher at RHs of 20 and 80%. A different pattern was observed for the viability of MS2 in droplets containing protein, in which the relative viability of MS2 generally increased as RH increased. Two-way ANOVA indicated that there was a main effect of RH, but not salt or pH, on the viability of MS2 in droplets. In droplets composed of protein and surfactant, both RH and droplet composition (i.e., protein and surfactant) had a statistically significant effect on the relative viability of

MS2. Meanwhile, there was an interaction effect between RH and droplet composition on the viability of MS2 in droplets containing salt, protein, and surfactant, but not between RH and the pH of droplet media.

The effect of RH on the viability of  $\Phi 6$  was different from that on MS2. Instead of U-shaped patterns, the viability of  $\Phi 6$  generally decreased as RH increased in droplets containing salt and surfactant, and in pH-adjusted droplets. The pattern was slightly different for droplets containing protein due to the protective effect from BSA, which made the patterns more U-shaped. Statistical analysis suggests that there was a main effect of RH on the viability of  $\Phi 6$  in droplets composed of surfactant. There was an interaction effect between RH and salt, protein, and pH on the survival of  $\Phi 6$ , respectively.

#### **4.4 Discussion**

Our results show that the chemistry of carrier droplets has significant impacts on the viability of both non-enveloped and enveloped viruses. The results suggest that the chemical composition of carrier droplets can influence the stability of viruses once they are released into the environment. While salt, pH, and surfactant reduced the viability of viruses at most RH conditions, protein provided some protection against virus decay in droplets. The effect of chemical composition was coupled with RH, which suggests the importance of exploring the effects of droplets' chemical composition and environmental factors simultaneously in investigating the survival of viruses in the environment.

##### **4.4.1 Salt**

Sodium chloride promoted the inactivation of viruses at low RH, but it did not affect, and sometimes even reduced, the decay of viruses at intermediate and high RHs. These seeming conflicting results can be explained by two distinct mechanisms. Firstly, previous studies have reported that NaCl inactivates viruses, although the mechanism of inactivation was not explicitly identified<sup>40,41</sup>. Our results confirmed the inactivation effect through the observation of enhanced decay of MS2 and  $\Phi 6$  in droplets containing NaCl at 20% RH. On the other hand, studies have suggested that viruses tend to generate large aggregates in solutions with high salt concentration<sup>42-45</sup>. The formation of large virus aggregates increases virus stability in such environments<sup>43</sup>. Although other studies have demonstrated that media containing low levels of salt

(e.g., initial concentration of 1 g/L NaCl in our droplets) does not effectively facilitate the formation of virus aggregates<sup>46,47</sup>, we speculate that virus aggregation would be enhanced in evaporating droplets. This is because the salt concentration increases in evaporating droplets as they lose water, especially when they are close to desiccation, while the decrease in droplet volume brings viruses into contact with one another. We hypothesize that the increased relative viability of MS2 and  $\Phi 6$  in droplets containing sodium chloride at 80% RH is due to the formation of virus aggregates. Further studies are needed to provide direct evidence supporting this hypothesis.

The observed RH-dependent effect of salt on the viability of viruses suggests that the relative contribution of the abovementioned mechanisms may vary at different RH conditions. The evaporation kinetics of droplets at various RHs seems to play an important role. Droplets evaporate rapidly at low RH condition, desiccating within 15 minutes at 20% RH, whereas the evaporation process is much slower at high RH. It is plausible that at low RH, droplets quickly desiccate before considerable amount of virus aggregates are generated, in which case the inactivation effect of NaCl dominates and results in enhanced decay of viruses. Conversely, at high RH, droplet evaporation is much slower, allowing viruses to form aggregates and thus protecting viruses from inactivation. Again, additional investigation is needed to test this hypothesis.

#### **4.4.2 pH**

Our results demonstrate that pH affects the stability of MS2 and  $\Phi 6$  differently in droplets. MS2 survived equally well in acidic, pH-neutral, and basic droplets, whereas  $\Phi 6$  survived best in pH-neutral droplets and decayed more in acidic or basic droplets. Previous studies have reported that viruses in bulk solutions are sensitive to pH<sup>48,49</sup>. Both non-enveloped and enveloped viruses are generally more susceptible in acidic and basic solutions than in pH-neutral solutions<sup>48</sup>. At extreme pHs, viruses decay due to the denaturing of surface proteins and the hydrolysis of the viral genome<sup>41,50</sup>. However, MS2 appears to be fairly insensitive to pH. In a previous study, a moderate decay rate of  $\sim 0.5 \log_{10}$  unit per day was observed for MS2 in bulk solutions at pH values of 4 and 10, whereas MS2 retained its viability when the solution was pH-neutral.

The effect of pH on the viability of enveloped viruses is generally more noticeable than its effect on non-enveloped viruses<sup>48</sup>, consistent with our observation for the pronounced decay of  $\Phi 6$  in acidic solutions across all RH levels. Besides the protein denaturing effect, the fusion of envelope viruses' membrane structure caused by extreme pH is another mechanism that inactivates viruses<sup>41</sup>. Low-pH treatment is widely used in monoclonal antibody purification processes to inactivate viruses because of its reliable performance (e.g.,  $> 4 \log_{10}$  decay) on the inactivation of enveloped viruses<sup>51</sup>.

In addition to the inactivation effect induced by pH, the dynamic change in pH of evaporating droplets can also affect virus survival. Although the pH of all virus suspensions was adjusted to the target pH at the beginning of experiments, the pH is likely to change dynamically as droplets evaporate. The loss of water will enrich ions, such as  $H_3O^+$  and  $OH^-$ , in droplets, which may create pH-gradients inside droplets<sup>52</sup>. Additionally, since droplets were exposed to ambient air, the uptake of  $CO_2$  and formation of carbonic acid may lower the pH of droplets, but determining the extent of this process in evaporating droplets is challenging. Therefore, the pH of droplets is not expected to remain constant at its initial value throughout the experiment. The dynamic change in the pH of evaporating droplets will induce uncertainties on its effect on the survival of viruses. Tools to monitor the real-time pH in evaporating droplets are necessary to fully explain the effect of pH on the viability of viruses in this complex system.

#### **4.4.3 Protein**

The relative viability of MS2 and  $\Phi 6$  was elevated in droplets containing BSA at RHs of 50% and 80%. Previous studies have found that the decay of viruses was greatly reduced in both aerosols and droplets supplemented with human respiratory fluid or fetal calf serum<sup>20,24</sup>; protein may provide a protective effect. For example, influenza virus retained its viability in aerosols across a wide range of RHs after 1 h when the aerosolization media was supplemented with human bronchial epithelia cell wash. Here, we suspended viruses in media containing BSA and observed a similar protective effect. The detailed mechanism by which proteins protect viruses from decay remains unknown. Researchers have proposed that the inactivation of viruses in aerosols and droplets mainly happens at the air-water interface<sup>53,54</sup>. The presence

of proteins in droplets may reduce the solution surface tension, which inhibits viruses from reaching the air-water interface and preserves their viruses<sup>55,56</sup>. Another possible mechanism is that potentially damaging compounds may first act on free proteins in droplets instead of those on the surfaces of viruses. Quantitative information on residual “free protein” in droplets over the course of exposure will be useful to test this hypothesis. It is also possible that proteins in solution may interact with those on the surface of viruses and help stabilize them.

#### **4.4.4 Surfactant**

Surfactants have been reported previously to enhance the inactivation of viruses<sup>57,58</sup>, which is in agreement with our results on  $\Phi 6$ . High concentrations of surfactant are very effective in inactivating enveloped viruses. For example,  $> 4 \log_{10}$  reduction has been reported after 1 h incubation in surfactin solution with a concentration of  $80 \mu\text{M}$ <sup>59</sup>. According to electron microscopy results, the decay mechanism was concluded to be the disintegration of the lipid membrane and partial disintegration of the protein capsid on enveloped viruses. Since the initial concentrations of SDS in our droplets were much lower ( $3.4$  and  $34 \mu\text{M}$ ), the magnitude of virus decay in our study was lower than previously reported.

Since a lipid membrane is present only in enveloped viruses, the effect of surfactant on non-enveloped viruses is generally much weaker than on enveloped viruses<sup>59</sup>. Interestingly, we observed less decay of MS2 in droplets containing SDS, suggesting a protective effect of SDS on the survival of non-enveloped viruses in droplets. Surfactants could protect viruses in a similar manner as proteins. Surfactants are known to strongly affect the surface tension of solutions, especially when the surfactant concentration is below the critical micelle concentration, beyond which micelle starts to form and the surface tension of solutions remains relatively constant. Since the concentration of SDS examined in our study is much lower than its critical micelle concentration ( $8.2 \text{ mM}$ ), the presence of SDS in droplets affect the surface tension and protect viruses from decay by reducing their ability to reach the air-water interface.

#### 4.4.5 Relative Humidity

RH has large impacts on the viability of viruses in droplets, larger than the effect of chemical composition in some cases. We observed U-shaped patterns in the viability of MS2 against RH, and monotonically decreasing relationships between the viability of  $\Phi 6$  and RH, respectively, in droplets of different compositions. We reported previously that the viability of MS2 and  $\Phi 6$  in droplets composed of culture medium follows U-shaped patterns, in which the lowest viability occurs at 55% and 85% RH, respectively<sup>14</sup>. Many other studies have reported a similar pattern: certain viruses undergo greater decay at intermediate RHs than at lower or higher RHs<sup>20,23,24,26,60</sup>. The viability patterns observed in this study for  $\Phi 6$ , decreasing with RH rather than U-shaped, seem to conflict with results in the literature. However, we examined the viability of  $\Phi 6$  between 20% and 80% RH in the current study, in which range the viability of viruses also decreased monotonically in previous studies; we have shown that the minimum viability of  $\Phi 6$  in droplets occurs around 85% RH<sup>14</sup>.

As we concluded in our prior study<sup>14</sup>, RH shapes the viruses' viability patterns mainly by controlling droplet evaporation kinetics, which induce changes in solute concentrations and the resulting cumulative dose of specific compounds to which viruses are exposed. At intermediate RH, the cumulative dose is higher because the solute concentration increases relatively quickly and is then maintained at a high level throughout the experiment. While our previous work focused on viruses in their prescribed culture medium, results of the present study indicate that their viability follows the same pattern in droplets consisting of culture medium diluted 100x in ultrapure water and lacking salt, protein, and surfactant. Components in LB medium that are potentially harmful for viruses, though diluted, can accumulate as droplets evaporate and eventually cause viruses inactivation over time.

While RH is the major factor that determines droplet evaporation kinetics, droplet composition can affect evaporation rates as well. According to weighing experiments, the evaporation kinetics differ when droplets are composed of different chemical compositions. Furthermore, the evaporation rate of droplets is a function of the initial solute concentration and changes with time as solutes become more concentrated.

Droplets containing 1 g/L NaCl evaporated faster than those containing 35 g/L NaCl. A previous study demonstrated that the evaporation rates of droplets containing less than 0.1 M NaCl was almost two times higher than for droplets containing 1M NaCl at  $RH < 60\%$  <sup>61</sup>. The authors concluded that Marangoni flows induced by surface tension gradients, which originated from local peripheral salt enrichment, caused the difference in evaporation rate. Since the evaporation kinetics determines the change in solute concentration and cumulative dose, it is necessary to understand the influences of droplet chemicals compositions and their concentrations on the evaporation rates of virus-containing droplets.

#### **4.4.6 Limitations**

While this study provides novel results on the viability of viruses in evaporating droplets of different compositions over a range of RHs, it does not examine how the surface material upon which viruses are deposited might affect viability. Previous studies have reported that the persistence of viruses in droplets depends on the type of material (e.g., plastic vs. steel) <sup>62</sup>. It is possible that material exchange between surfaces and droplets (e.g., dissolution of metal ions into droplets) leads to accumulation of surface materials in droplets and inactivates viruses. It will be interesting to investigate the effects of the interplay among surface materials, droplet composition, and environment on the survival of viruses in droplets. Additionally, we have focused on the biological inactivation of viruses in droplets but not on their physical behavior, which likely depends on physicochemical characteristics of the droplets. Future studies should be conducted in this area, although pinpointing viruses within droplets is challenging. Results might help explain the protective or inactivation effect of certain media components we observed in this study.

To conclude, we demonstrated that both the chemical composition of droplets and RH strongly affect the viability of non-enveloped and enveloped viruses. The effects of sodium chloride and SDS varied by RH level and virus type. pH does not affect the viability of MS2, but effectively inactivates  $\Phi 6$  in solutions at pHs of 4 and 10. BSA generally preserves the viability of MS2 and  $\Phi 6$  in droplets. We also found that the viability of viruses in droplets of certain compositions is RH-dependent at most conditions. Our results reveal that two factors contribute to the inactivation of viruses in droplets: (1) droplet

evaporation kinetics, which is controlled by RH; and (2) inactivation or protective effects induced by chemicals. Additionally, the physical behavior of viruses, such as forming aggregates and partitioning to the air-liquid interface, resulting from changes in droplets' characteristics may also affect inactivation. Results from our study are meaningful in predicting the persistence of viruses in droplets of various compositions in the environment and infectious disease transmission.

### **Acknowledgements**

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## Tables and Figures

Table 4.1 Chemicals used to make solutions and their concentrations in virus suspensions.

Component	Chemical(s) used	Stock solution concentration	Concentration tested	Relevance
Salt	Sodium chloride	100 g/L	0, 1, 35 g/L	Salinity of seawater: 35 g/L Salinity of surface water: 0.01 to few g/L <sup>63</sup> Lung fluid: ~10 g/L <sup>64</sup>
Protein	Bovine serum albumin	100 mg/mL	0, 100, 1000 µg/mL	Respiratory fluid: 30-8500 ug/mL <sup>23,65-67</sup>
Surfactant	Sodium dodecyl sulfate	100 µg/mL	0, 1, 10 µg/mL	Lung surfactant: up to 1000 µg/mL <sup>68</sup> Surface water: less than 1 µg/mL <sup>69</sup>
pH	Hydrochloric acid, sodium hydroxide	NA	4.0, 7.0, 10.0	Ambient aerosol: 0-4 <sup>70,71</sup> Inorganic aerosol: basic <sup>52</sup> Human respiratory fluid: pH-neutral <sup>72</sup>

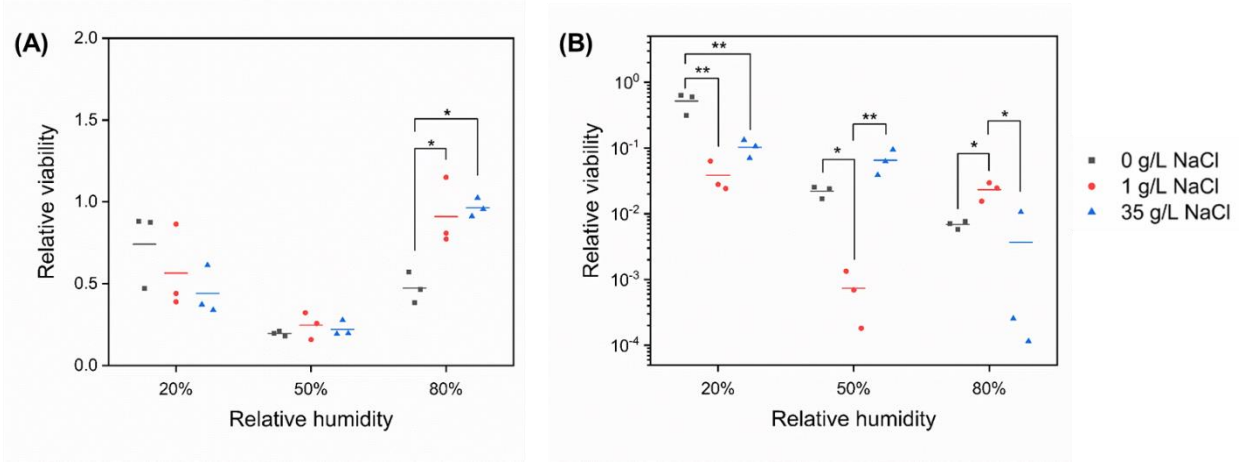


Figure 4.1 Relative viability of bacteriophages (A) MS2 and (B)  $\Phi 6$  in droplets with different initial sodium chloride concentrations after 1 h of exposure to low, intermediate, and high RH (mean  $\pm$  s.d. of triplicates).

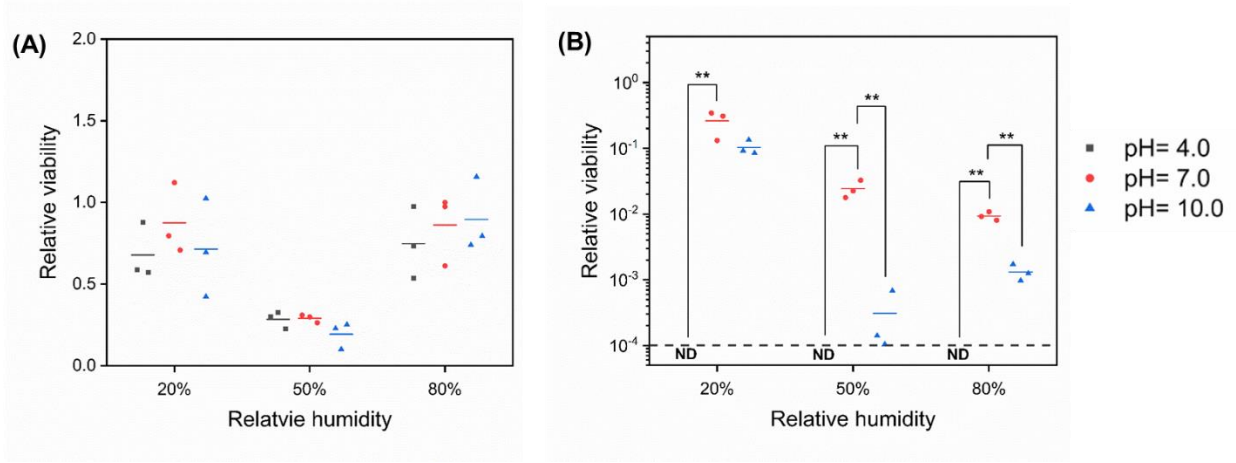


Figure 4.2 Relative viability of bacteriophages (A) MS2 and (B)  $\Phi 6$  in droplets with different initial pH values after 1 h of exposure to low, intermediate, and high RH (mean  $\pm$  s.d. of triplicates). The dark gray dash line indicates the detection limit ( $10^{-4}$ ) of plaque assay. \*ND: no viable virus was detected.

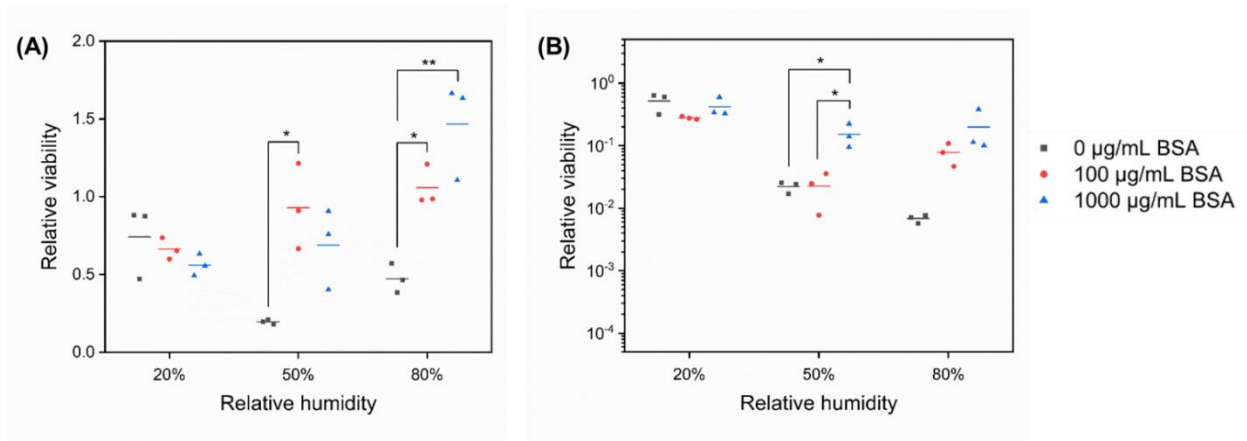


Figure 4.3 Relative viability of bacteriophages (A) MS2 and (B)  $\Phi$ 6 in droplets with different initial protein concentrations after 1 h of exposure to low, intermediate, and high RH (mean  $\pm$  s.d. of triplicates).

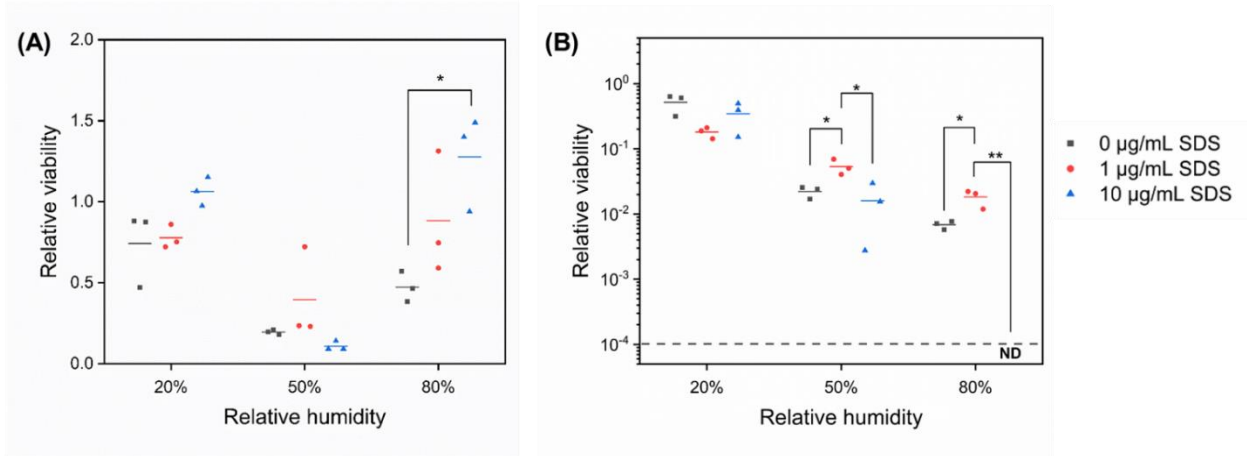


Figure 4.4 Relative viability of bacteriophages (A) MS2 and (B) Φ6 in droplets with different initial surfactant concentrations after 1 h of exposure to low, intermediate, and high RH (mean ± s.d. of triplicates. ND, not detected). The dark gray dash line indicates the detection limit ( $10^{-4}$ ) of plaque assay. \*ND: No viable virus was detected.

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## 5. Conclusions

Successful transmission of infectious diseases requires pathogens to retain their viability while they are transferring from a host to susceptible individuals. For most respiratory diseases, pathogens must be able to remain viable in the environment, either in suspended aerosols or in droplets on material surfaces, in order to spread. There is a large knowledge gap regarding the survival of pathogens in the environment as well as their inactivation mechanisms in both suspended aerosols and stationary droplets. This dissertation is structured around three main components to fill this knowledge gap: investigating the relationship between the viability of pathogens and RH, explaining the inactivation mechanisms of pathogens in droplets, and exploring the effects of media composition on the viability of pathogens.

The main contribution of this work is novel data on the survival of bacteria and viruses in aerosols and droplets of different compositions over a wide range of RH conditions. Our results indicate that both RH and the composition of surrounding media affect the viability of bacteria and viruses in suspended aerosols and stationary droplets. By characterizing the evaporation kinetics of droplets and estimating the resulting changes in solute concentrations, we have also developed new insight into the humidity-dependent inactivation of bacteria and viruses. Inactivation of viruses is related to cumulative exposure, in terms of dose or the product of concentration and time, to an as-yet unidentified compound in evaporating droplets. We conclude that the inactivation of bacteria is governed by osmotic pressure, while the inactivation of viruses follows disinfection kinetics.

### 5.1 Outcomes of Research Objectives #1

*Investigate the viability of bacteria and virus in aerosols and droplets at various RH.*

We have assessed the effects of RH on the viability of bacteria and viruses that are surrogates for important pathogens in both suspended aerosols and stationary droplets. Bacterial viability was highest at high RH and lowest at low RH. Two viruses, MS2 and Phi6, suspended in their culture media, maintained viability at both low RH and extremely high RH while decaying at intermediate RH. This observation is consistent with the observed seasonality of certain infectious diseases, for which incidence is highest in the wintertime in temperate regions and during the rainy season in tropical regions <sup>1</sup>. However, the viability of influenza virus, which was suspended in a more physiologically relevant fluid containing respiratory mucus, was independent of RH.

We confirmed that bacteria and viruses are able to survive for an hour or more under certain conditions and thus maintain their ability to infect susceptible individuals via the aerosol, droplet, and fomite transmission routes. Results from this study can be used to guide intervention practices for controlling the spread of infectious diseases by manipulating RH to a level that inhibits the survival of pathogens of interest in the indoor environment <sup>2-4</sup>, among other strategies.

## **5.2 Outcomes of Research Objectives #2**

*Explain the observed patterns in microorganisms' viability against RH.*

This work explained the observation of a U-shaped relationship between virus viability and humidity, in which survival is lowest at mid-range RHs. We demonstrated that the inactivation of bacteria and viruses is related to dynamic changes in solute concentrations that occur as droplets evaporate. We concluded that osmotic pressure, which results from elevated salt concentrations as aerosols and droplets evaporate, dominates inactivation of bacteria. However, the inactivation of viruses is linked to the cumulative dose of a harmful substance in solution. This study provides new, mechanistic insight into the effect of RH on the viability of pathogens in the environment and infectious disease transmission. The results emphasize the importance of characterizing the

evaporation kinetics of aerosols and droplets in understanding the inactivation of immersed pathogens.

### **5.3 Outcomes of Research Objectives #3**

*Determine the effects of droplets media components on the viability of viruses.*

This work provides information on how different chemical constituents of media affect the viability of viruses in droplets. Protein protects viruses in droplets, whereas acidic and basic solutions inactivate viruses. The effects of sodium chloride and surfactant depend on RH and the strain of virus. Results from this work can be used to predict the environmental persistence of viruses, when information on chemical composition of carrier droplets and environmental conditions is available.

### **5.4 Recommendations for Future Work**

Chapter 3 investigated the viability of bacteria and viruses in aerosols and droplets at various RHs and explained their inactivation mechanisms in droplets based on evaporation kinetics. While pathogen-containing aerosols are expected to evaporate and reach equilibrium with ambient RH as do droplets, the exact evaporation kinetics of aerosols was not determined in this work due to the challenges in characterizing changes in aerosol size in real time. The evaporation kinetics of aerosols and droplets are likely to be different<sup>5-7</sup>, meaning that there would be differences in the changes in solute concentrations. We recommend future research to measure the evaporation kinetics of aerosols and examine whether the proposed inactivation mechanisms for droplets also applies to aerosols.

Due to challenges in acquiring physiologically and environmentally relevant media, in particular respiratory fluid from the bronchioles, our study was limited to the individual effects of common media components on the viability of pathogens in droplets. However, the chemical

composition of human respiratory fluids and environmental media is very complicated and in many cases not fully known<sup>8</sup>. Even a trace amount of some species may have a substantial impact on the viability of pathogens. As the understanding of the chemical composition of physiological and environmental fluids improves in the future, the viability of pathogens in such media should be investigated to better simulate their survival in the environment.

This work evaluated the effects of RH and media composition on the viability of bacteria and viruses by culturing and plaque assay, respectively. While it provides novel data on the survival of pathogens in the environment, a detailed explanation at the molecular level on how pathogens are inactivated in aerosols and droplets is still lacking. We recommend future studies that explore bacterial and viral structure and function to gain insights on the exact mechanism by which RH and media components inactivate microorganisms. Molecular techniques can provide additional information regarding the infectivity of pathogens, including identifying bacteria in the viable but non-culturable state<sup>9</sup> and determining pathogens' binding ability to host receptors.

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## APPENDIX A. SUPPLEMENTAL INFORMATION TO CHAPTER 2

### Calculation of $\log_{10}$ decay of infectivity corrected for physical loss of aerosols

Total loss of infectious virus during aging and sampling was due to physical loss, dilution, and biological inactivation, which was also assumed to follow first-order kinetics. The instantaneous concentration of infectious virus at 1 hour, or 60 minutes ( $C_{60}$ ), was a function of the initial, unaged concentration ( $C_{unaged}$ , determined by  $TCID_{50}$ ) and the physical loss and biological inactivation ( $k_b$ , units of  $\text{min}^{-1}$ ) coefficients, as shown in equation 1. The concentration of virus collected over 15 minutes after 1 hour of aging ( $C_{aged}$ , determined by  $TCID_{50}$ ) was approximated by equation 2, where the concentration at 15/2 minutes was assumed to be representative for the time-integrated filter sample. Solving these two equations simultaneously yielded  $C_{60}$  and  $k_b$ . The instantaneous concentration of infectious virus after aging ( $C_{60'}$ ), corrected for physical loss during this period, was calculated according to equation 3. The decay in virus concentration was calculated using equation 4. The physical loss coefficient  $k_p$  and dilution coefficient  $k_d$  are plotted in Figure A.2, and the values of  $k_b$  for each RH are summarized in Table A.2.

$$C_{60} = C_{unaged} \times e^{-(k_p+k_b) \times 60} \quad (1)$$

$$C_{aged} = C_{60} \times e^{-(k_p+k_d+k_b) \times (\frac{15}{2})} \quad (2)$$

$$C_{60'} = C_{60} / e^{-k_p \times 60} \quad (3)$$

$$\text{Decay in virus concentration} = \log_{10} \left( \frac{C_{unaged}}{C_{60'}} \right) \quad (4)$$

Table A.1 RH (%) data collected during the drum experiment.

RH (%)	Replicate	Post-equilibration	Pre-aging	Post-aging
23	1	21.2	21.0	20.9
	2	23.2	23.3	23.7
	3	22.7	22.5	23.0
33	1	-	32.3	31.7
	2	-	34.9	35.4
	3	33.2	33.2	31.5
43	1	44.4	44.8	47.8
	2	41.8	42.7	44.3
	3	42.1	42.0	44.0
55	1	54.4	55.0	58.1
	2	51.4	51.4	53.1
	3	51.7	52.5	56.0
75	1	71.9	72.6	74.8
	2	76.7	74.1	77.7
	3	73.5	73.5	74.5
85	1	84.9	83.7	89.9
	2	82.8	82.0	79.1
98 <sup>a</sup>	1	100	-	-
	2	-	-	-
	3	-	-	-

<sup>a</sup>RH data at the 98% condition was not collected for the duration of the experiment because extended exposure to high humidity would damage the RH monitor. Virus solution was aerosolized for an extended period to assure RH inside the drum reached 100% RH.

Table A.2 Virus biological inactivation coefficient (kb, min<sup>-1</sup>) at each RH condition.

Aerosolization Medium	RH (%)	Rep 1	Rep 2	Rep 3
L-15 (+) HBE ECM	23	-0.0085	-0.0256	0.0085
	33	0.0079	0.0165	0.0250
	43	0.0035	-0.0050	0.0035
	55	-0.0047	0.0209	0.0294
	75	-0.0167	0.0089	0.0003
	85	0.0177	0.0006	NA
	98	0.0348	0.0178	0.0007
TSB (+) HBE ECM	23	0.032	0.0203	0.0217
	33	-0.0007	0.0000	-0.0035
	43	0.0155	-0.0039	-0.0018
	55	-0.0012	0.0210	-0.0009
	75	NA	0.0032	0.0120
	85	NA	0.0074	-0.0126
	98	-0.0102	0.0012	-0.0041
TSB (-) HBE ECM	23	0.0059	-0.0044	-0.0064
	33	-0.0048	0.0100	-0.0007
	43	0.0002	-0.0053	0.0393
	55	0.0105	0.0115	0.0353
	75	0.0883	0.0774	0.0670
	85	0.0748	0.0340	0.0717
	98	0.0112	0.0049	0.0053

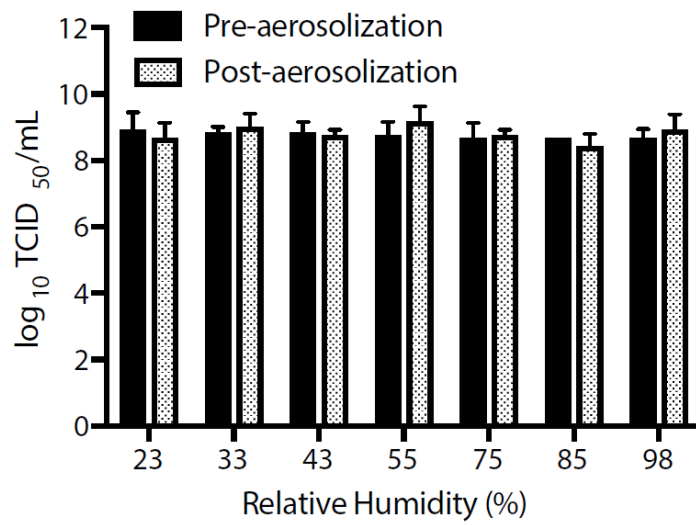


Figure A.1 H1N1pdm did not lose infectivity after aerosolization. Raw titer of the bulk virus solution pre- and post-aerosolization into the RH drum was determined using TCID<sub>50</sub> assay on MDCK cells. No change in virus titer was observed. Data represent three independent experiments at each RH (85% RH was done twice).

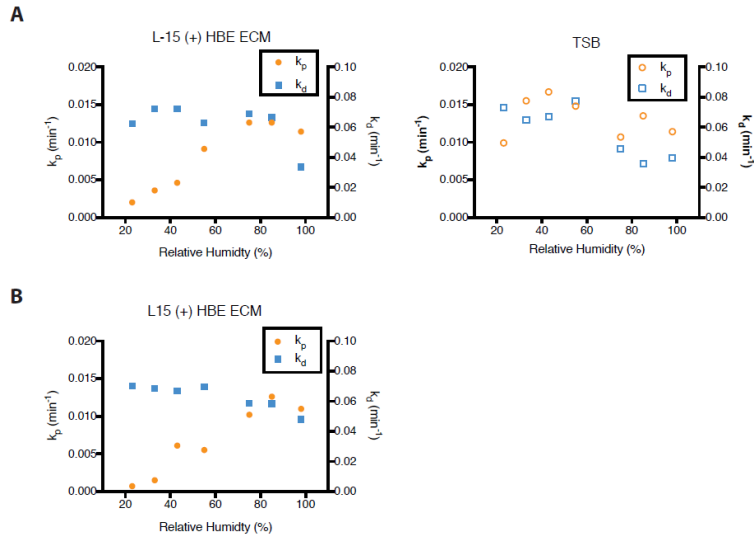


Figure A.2 Physical loss coefficient ( $k_p$ ) and dilution coefficient ( $k_d$ ) at each RH condition. Aerosol volume concentration was measured using a scanning mobility particle sizer and an aerodynamic particle sizer during both unaged and aged sample collection at each RH.  $k_p$  and  $k_d$  were determined by fitting measurement results to a first-order decay model. A)  $k_p$  and  $k_d$  measured using L-15 + HBE ECM (left) and TSB (right) aerosolization media used for  $\Phi 6$  and B) L-15 + HBE ECM aerosolization media used for H1N1pdm.

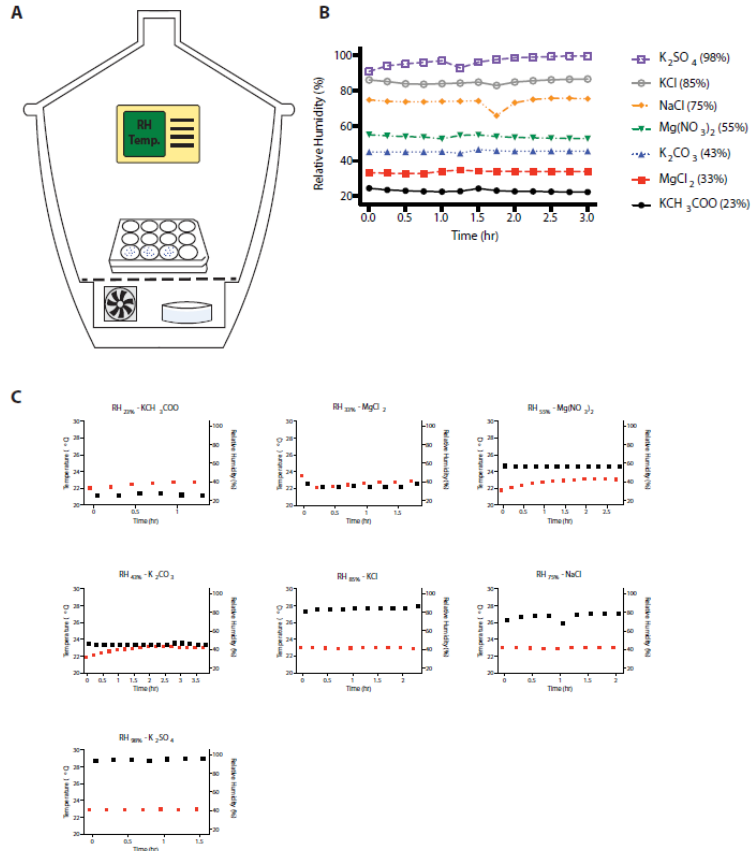


Figure A.3 Controlled RH chamber to assay virus infectivity in stationary droplets. A) The RH chamber was housed within a sealed glass desiccator. RH was maintained by the placement of an aqueous saturated salt solution in the bottom of the chamber. 1  $\mu$ L droplets of virus are spotted into the wells of a tissue culture dish, in triplicate, and incubated in the pre-equilibrated chamber for 1 hour. B) Range of RH conditions generated in the RH chamber using different aqueous saturated salt solutions. C) RH and temperature data collected during equilibration of the chamber and chamber experiments at each RH condition. Data were collected using an Onset HOBO temperature/RH logger.

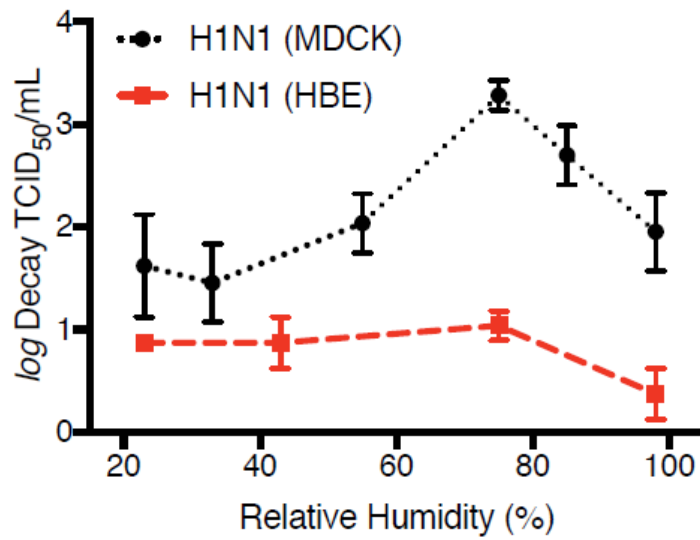


Figure A.4 Influenza virus collected from HBE cells is resistant to RH-dependent decay. Viruses propagated in MDCK (black) and HBE (red) cells were exposed to a range of RH conditions in stationary droplets for 2 hours. MDCK propagated H1N1 experiences more decay, particularly at mid-range RH, than the HBE propagated virus. Data represent mean  $\pm$  standard deviation of at least two independent biological replicates.

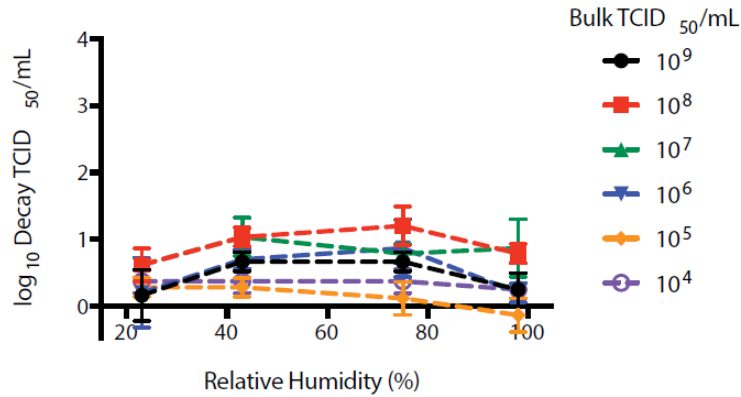


Figure A.5 Stability of 2009 H1N1pdm in stationary droplets is not dependent upon virus concentration. Serial dilutions of 2009 H1N1pdm + HBE ECM were exposed to 23%, 43%, 75%, and 98% RH in stationary droplets within the RH chamber. Decay of the viruses at each RH was calculated compared to the titer of equivalent bulk solution controls incubated at room temperature outside of the RH chamber. Experiments were performed in triplicate. Data points represent mean  $\pm$  standard deviation, and are representative of two independent biological replicates.

## APPENDIX B. SUPPLEMENTAL INFORMATION TO CHAPTER 3

Table B.1 Characteristics of bacteria and viruses examined in this study.

<b>Microorganisms</b>	<b>Category</b>	<b>Size</b>	<b>Isoelectric Point</b>	<b>Medium</b>
<i>Escherichia coli</i>	Gram-negative bacteria	2 $\mu\text{m}$ $\times$ 0.25-1 $\mu\text{m}$	5.6	LB
<i>Mycobacterium smegmatis</i>	Gram-positive bacteria	3-5 $\mu\text{m}$ $\times$ 0.25-1 $\mu\text{m}$	9.5	TSB
<i>Bacillus subtilis</i>	Gram-positive bacteria	4-10 $\mu\text{m}$ $\times$ 0.25-1 $\mu\text{m}$	3.2	3 nutrient broth
Bacteriophage MS2	Enveloped bacteriophage	20 nm	3.9	LB
Bacteriophage Phi6	Unenveloped bacteriophage	80 nm	6.5	TSB

Table B.2 Physical loss coefficient ( $k_p$ ) and dilution coefficient ( $k_d$ ) of aerosols containing different microbes at each RH condition in the rotating drum.

<b>Microorganisms</b>	<b>RH</b>	<b><math>k_p</math> (<math>\text{min}^{-1}</math>)</b>	<b><math>k_d</math> (<math>\text{min}^{-1}</math>)</b>
Gram-negative bacteria	20%	0.011	0.056
	40%	0.014	0.061
	60%	0.020	0.058
	80%	0.018	0.057
	100%	0.015	0.058
Gram-positive bacteria	20%	0.010	0.061
	40%	0.013	0.059
	60%	0.014	0.053
	80%	0.014	0.050
	100%	0.018	0.046
Viruses	23%	0.010	0.073
	33%	0.016	0.063
	43%	0.017	0.067
	55%	0.015	0.078
	75%	0.011	0.046
	85%	0.013	0.036
	100%	0.011	0.040

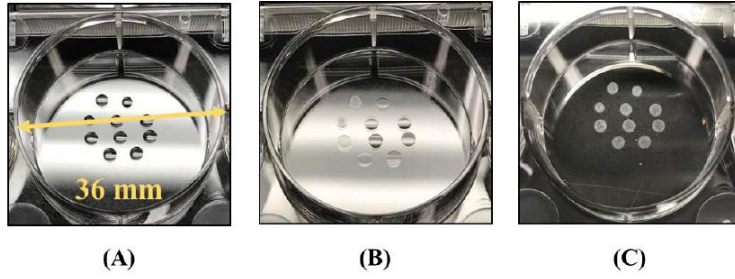


Figure B.1 Photos of evaporating droplets composed of plain LB broth at 40% RH. (A) Droplets just after spotting on the surface of a 6-well culture plate. (B) Partially evaporated droplets. (C) Desiccated droplets.

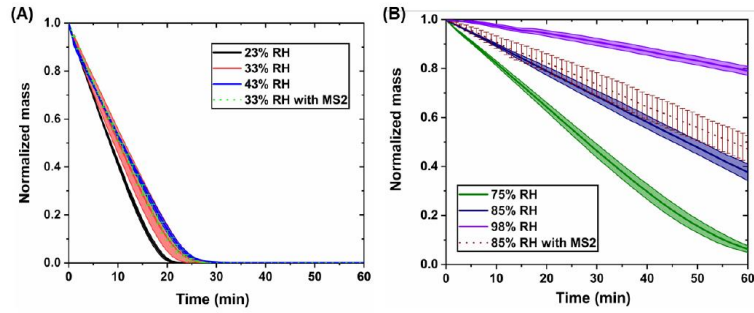


Figure B.2 Normalized mass of evaporating droplets composed of plain LB media and droplets supplemented with MS2 at (A)  $RH < 50\%$  and (B)  $RH > 50\%$ . For droplets composed of plain LB media, dark lines show the average of triplicates at each RH, and the shaded bands show the standard deviation. For droplets supplemented with MS2, dots show the average of triplicates, and the error bars show the standard deviation.

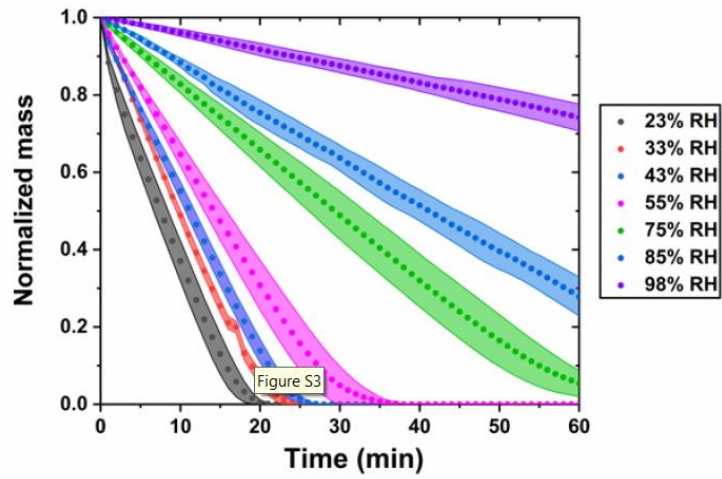


Figure B.3 Normalized mass of evaporating droplets composed of plain TSB media. Dots show the average of triplicates at each RH, and the shaded bands show the standard deviation.

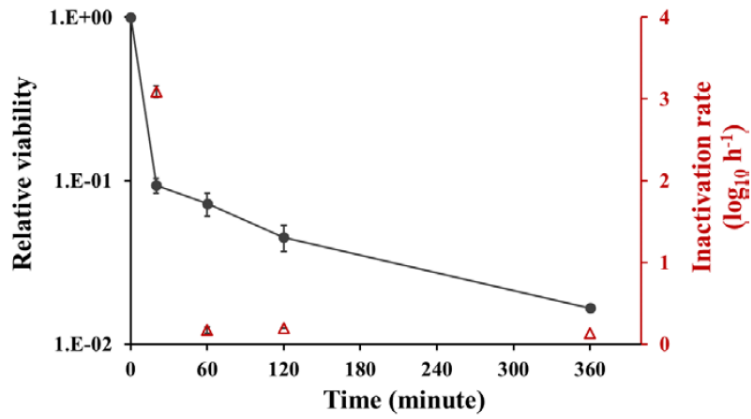


Figure B.4 Relative viability and inactivation rate of *E. coli* in stationary droplets of LB after 20, 60, 120, and 360 minutes at 20% RH. Droplets completely dried in 20 minutes. Black dots represent the relative viability of *E. coli* at each time point (left axis, mean  $\pm$  s.d. of triplicates). Red triangles represent the inactivation rate of *E. coli* between 0 and 20, 20 and 60, 20 and 120, and 20 and 360 minutes, respectively (right axis, mean  $\pm$  s.d. of triplicates).

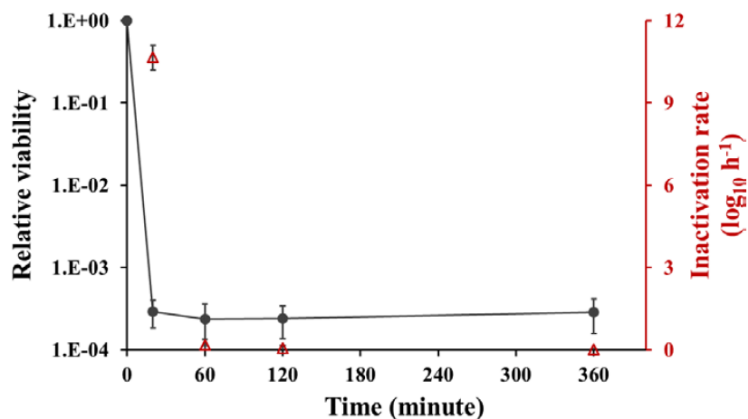


Figure B.5 Relative viability and inactivation rate of *B. subtilis* in stationary droplets of 3 nutrient broth after 20, 60, 120, and 360 minutes at 20% RH. Droplets completely dried in 20 minutes. Black dots represent the relative viability of *B. subtilis* at each time point (left axis, mean  $\pm$  s.d. of triplicates). Red triangles represent the inactivation rate of *B. subtilis* between 0 and 20, 20 and 60, 20 and 120, and 20 and 360 minutes, respectively (right axis, mean  $\pm$  s.d. of triplicates).

## APPENDIX C. SUPPLEMENTAL INFORMATION TO CHAPTER 4

Table C.1 The adjusted pH of solution and equivalent ion strength added to solution after pH adjustment.

<b>Solution</b>	<b>Target pH</b>	<b>pH of solution after adjustment</b>	<b>Equivalent Na<sup>+</sup> or Cl<sup>-</sup> ion strength introduced by pH adjustment (mM)</b>
MS2 suspension	4.0	3.82	0.15
	7.0	7.16	0.07
	10.0	9.63	1.00
Φ6 suspension	4.0	4.19	0.29
	7.0	6.69	0.00
	10.0	9.82	1.20
1 g/L NaCl	NA	NA	17.09