Although most of the information presented in the Journal *refers to situations within the United States, environmental health and protection know no boundaries. The* Journal *periodically runs International Perspectives to ensure that issues relevant to our international constituency, representing over 60 countries worldwide, are addressed. Our goal is to raise diverse issues of interest to all our readers, irrespective of origin.*

Inactivation of Surface Viruses by Gaseous Ozone

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Abstract

Environmental surfaces may be contaminated with viruses and contribute to their transmission. Concerns have arisen in trying to control viruses because of an increasing incidence of viral infections. Ozone is considered to be a promising method to inactivate viruses on surfaces. In this investigation, the effects of ozone concentration, contact time, different capsid architecture of viruses, and relative humidity (RH) on inactivating viruses by ozone were evaluated. The authors observed that the survival fraction of viruses on surfaces decreased exponentially with increasing ozone dose. Viruses required ozone doses of 20–112 min(mg/m^3) (contact time [min] multiplied by ozone concentration [mg/m^3]) for 90% inactivation and 47–223 min(mg/m^3) for 99% inactivation. The ozone dose for 99% inactivation was two times higher than for 90% inactivation. The required ozone concentration at 85% RH was lower than at 55% RH. In summary, ozone should be an effective method for reducing the viral number between 1 and 3 logs on surfaces.

Introduction

Viruses are obligate parasites and can be pathogenic to humans and animals. Environmental surfaces may be contaminated with viruses and contribute to their transmission. For surfaces to serve as vehicles of viral disease, the contaminating virus must be able to survive in association with the surface, and there must be a successful transfer to the host. Some viruses can transfer from an object contaminated with nasal secretion to fingers that touch the object and subsequently touch the nose or eye. In recent decades, enteroviruses and SARS coronavirus (SARS CoV) are major public health issues. Recent evidence has shown that polymerase chain reaction-positive (PCR-positive) swab samples of enterovirus and SARS CoV were recovered from frequently touched surfaces in occupied patient rooms and nursing stations at hospitals (Booth et al., 2005; Dowell et al., 2004). These observations invite speculation about the possible role of environmental surfaces in the spread of either or both of these viruses. For surface disinfection, ethylene oxide gas has been used for over 40 years to sterilize medical instruments in hospitals. Ethylene oxide, however, was recognized as a potential mutagenic, reproductive, neurologic, and fire and explosion hazard to workers (Recio et al., 2004). Therefore, the potential use of ozone may be timely and safe for disinfection of medical instruments.

To reduce risk from virus-contaminated surfaces, many control techniques, such as heating (Ferenczy, Bergeron, & Richart, 1989), ultraviolet germicidal irradiation (UVGI) (Sharp, 1939; Wyckoff, 1931), disinfectants (Rabenau, Kampf, Cinatl, & Doerr, 2005), and ozone (Foarde, VanOsdell, & Steiber, 1997) have been evaluated. Among these techniques, ozone is known to decrease the viral load (Berrington & Pedler, 1998) and has been used extensively for the disinfection of drinking water and municipal wastewater (Lazarova et al., 1998; Shin & Sobsey, 2003). The mechanism by which ozone inactivates viruses is still not well understood. Viruses may react either directly with molecular ozone or indirectly with the radical species formed when ozone decomposes (Kim, Yousef, & Chism, 1999). Additionally, ozone could react with amino acids, proteins, protein functional groups, and nucleic acids very rapidly (Langlais, Reckhow, & Brink, 1991), causing virus inactivation. Therefore, viruses could be inactivated by ozone acting on the protein structure of a virus capsid or on nucleic acids of viruses.

Early research on ozone applications focused mainly on inactivating viruses in water (Lazarova et al., 1998; Shin & Sobsey, 2003). Studies demonstrated that relatively low ozone concentration (less than 1 mg/L) and short contact time (1 min) are sufficient to inactivate 99% of viruses, such as rotaviruses, parvoviruses, feline calicivirus, and hepatitis A virus (Shin & Sobsey, 2003; Thurston-Enriquez, Haas, Jacangelo, & Gerba, 2005). Ozone concentrations, pH value, water temperature, residence time, mixing degree, and organic compounds were observed to influence virus susceptibility to ozone. These studies found that ozone would effectively inactivate viruses in water.

In contrast to water, little research has been done on inactivation of surface microorganisms by ozone. Our previous study demon-

strated that ozone could be used for reduction by 1 to 2 logs of *E. coli* and yeast on agar (Li & Wang, 2003). *Bacillus subtilis* and *Penicillium citrinum* spores, however, needed an extremely high ozone dose of 3,200 min(mg/m^3)(contact time [min] multiplied by ozone concentration [mg/m^3]) for 1 log reduction (Li & Wang, 2003). Vegetative organisms are more susceptible than spore-forming organisms to the inactivation effect of ozone, and bacterial spores are usually less susceptible than fungal spores (Li & Wang, 2003). The inactivation effect of ozone has been found to increase as relative humidity (RH) increases (Foarde, VanOsdell, & Steiber, 1997; Li & Wang, 2003). Previous investigations have shown that a microorganism's susceptibility to ozone is highly dependent on the microorganism's species. Very little data is available, however, regarding inactivation of viruses on surfaces by ozone.

The purpose of this study was to use ozone for virus inactivation on surfaces. Bacteriophages were used as models because they are safe to handle. Because ozone mainly causes capsid protein damage, viruses with different capsid protein architecture were assessed. The phages used in this study were MS2 (ssRNA), ϕX174 (ssDNA), ϕ6 (dsRNA), and T7 (dsDNA). MS2, ϕX174, ϕ6, and T7 phages are composed of 180, 60, 120, and 415 molecules of capsid protein, respectively. The effects of ozone concentration, contact time, architecture of capsid protein, and RH on virus survival were under evaluation. The results of this study could be useful to public health workers in disinfecting the surfaces in hospitals or other high risk areas.

Methods

Test Viruses

In medical and environmental virology applications, bacteriophages have been widely used as suitable surrogates for mammalian viruses (Dileo, Vacante, & Deane, 1993; Lytle, Budacz, Keville, Miller, & Prodouz, 1991; Maillard, Beggs, Day, Hudson, & Russell, 1994). In this study, the tested viruses were four different bacteriophages: ssRNA (MS2, American Type Culture Collection [ATCC] 15597-B1), ssDNA (ϕX174, ATCC 13706-B1), dsRNA (ϕ6 with envelope lipid, ATCC 21781-B1), and dsDNA (T7, ATCC 11303-B1). The host bacteria were *E. coli* F-amp (ATCC 15597) for MS2, *E. coli* CN-13 (ATCC 13706) for ϕX174, *E. coli* 11303 (ATCC 11303) for T7, and *Pseudomonas syringae* (ATCC 21781) for ϕ6. The bacteriophages used in this study have been used as indicators of poliovirus, enterovirus, enveloped viruses, and human immunodeficiency virus (Dileo, Vacante, & Deane, 1993; Lytle, Budacz, Keville, Miller, & Prodouz, 1991; Maillard, Beggs, Day, Hudson, & Russell, 1994).

In our study, a high titer stock of bacteriophages (10^9–10^{10} PFUs/mL [plaque-forming units/mL]) was prepared by plate lysis and elution (Sambrook & Russell, 2001). To allow the phage to attach to the host, the bacteriophages were mixed with their own respective host. First, 5 mL of molten top agarose (containing only 0.7 % agarose) was added to a sterile tube of infected bacteria. The media for phage cultivation were as follows: Luria-Bertani Agar (Difco Laboratories, 244520) for MS2; Nutrient Agar (Difco Laboratories, 213000) with 0.5% NaCl, for ϕX174; Trypticase Soy Agar (Difco Laboratories, 236950) for T7; and NBY Agar (containing Nutrient Broth, Yeast extract, K_2HPO_4, KH_2PO_4 and $MgSO_4 \bullet 7H_2O$) for ϕ6. Second, the contents of the tube were mixed by gentle tapping for five seconds and poured onto the center of a labeled agar plate. Finally, the plate was incubated for 24 hours either at 37°C for coliphages or at 26°C for ϕ6. After cultivation, 5 mL SM buffer (containing NaCl, $MgSO_4 \bullet 7H_2O$, Tris, and gelatin) was pipetted onto a plate that showed confluent lysis. The plate was then slowly rocked for 40 minutes and the buffer was transferred to a tube for centrifugation at 4,000 *g* for 10 minutes. After the supernatant was removed, the remaining phage stock was kept at -80°C. According to our preliminary results (data not shown), virus infectivity could be maintained for 24 hours at 4°C. For ozone experiments, the virus titers were determined by plaque assay, and the virus suspension was stored at 4°C within 24 hours.

Surface Test System

Gelatin-based Medium

In previous studies (Berrington & Pedler, 1998; Foarde, VanOsdell, & Steiber, 1997), only bacteria and fungi were evaluated for surface inactivation by ozone. The assessed surface was blood agar. In another previous study (Abad, Pinto, & Bosch, 1994), different kinds of surface compositions to which viruses were adsorbed may have caused the viruses to lose their infectivity. Therefore, the stability of virus infectivity on the evaluated surface is very important. In our study, we used a gelatin-based medium as the tested surface. Gelatin is a protein source and solidifying agent used for preparing microbiological culture media. The smooth surface of a gelatin-based medium offers a more effective means of growth for preserving viral infectivity. Moreover, the gelatin-based medium could be directly dissolved for further quantification by plaque assay and without the elution procedure. In our preliminary results, virus infectivity was observed to stay the same for at least two hours at 55% and 85% RH (with coefficient of concentration variation below 20%).

We used a gelatin-based medium composed of LB (Luria-Bertani) broth with 7% gelatin. A diluted culture of virus stock solution (0.1 mL) was spread on the surface of gelatin-based medium plates and then dried for 20 minutes in laminar flow. The virus concentration in each plate was 10^8 PFUs/mL. After ozone exposure, the gelatin-based media were dissolved at 37°C for 10 minutes. Then, viral samples in the liquid phase were subjected to plaque assay for coliphages at 37°C and for ϕ6 at 26°C.

RH Regulation Unit

A humidified gas stream was generated by passing pure compressed air through a humidity saturator. The water vapor content (i.e., RH) in the gas stream was adjusted by changing the flow-rate ratio of humidified gas stream to dry gas stream, and measured with a hygrometer (Testo, Sekunden-Hygrometer 601) placed in the sampling chamber. For evaluating the effect of RH, the humidified gas stream was heated by adding a dry gas stream to reach the medial (RH 55%) or humid condition (RH 85%) at 25°C–28°C.

Ozone Exposure Unit

The exposure chamber was approximately 23 liters in volume (inside diameter of 14 cm and 38 cm in height). The ozone was generated from an ozone generator (OZ1PCS-V/SW, Ozotech Inc., Yreka, CA) with pure oxygen at 3 L/min. Ozone levels were measured by an ozone analyzer (model 401, Advanced Pollution Instruments, San Diego, CA) with a detection limit of 1.0 ppb. In our study, experiments were performed at least in triplicate for each set of conditions for ozone concentration (0.6, 0.9, and 1.2 ppm), exposure time (5, 10, 15, 20, 30, 40, 60, 90, and 120 min), RH (55% and 85%), and tested virus (MS2, ϕX174, T7, and ϕ6). For all viral samples (both ozone-exposed and ozone-unexposed), the observed incubation time period was 24 hours. The virus survival fraction was calculated as the ratio of the number of plaques forming on the ozone-exposed plates compared to that on the ozone-unexposed control plates. The test system was located in a chemical hood so that the exhausted gas was vented outside.

Survival Fraction of Viruses vs. Ozone Exposure

The ozone dose to which a virus was exposed was defined as the product of ozone concentration on the virus and the contact time (Ct). The survival fraction is a ratio that represents the virus concentration after ozone exposure; *K* factors were obtained from the exponential decay model as follows.

$$Ns/N_0 = e^{-KCt}$$

where

Ns = Concentration of surface virus surviving after exposure to ozone (PFUs/mL);

N_0 = Concentration of surface virus unexposed to ozone (PFUs/mL);

C = Ozone concentration (mg/m^3), (mg/m^3) = (ppm x 48 g/mol) / 24.45 (L/mol) at 25°C, 1 atm;

t = Ozone contact time (min); and

K = Virus susceptibility factor ($min[mg/m^3]$).

Statistics

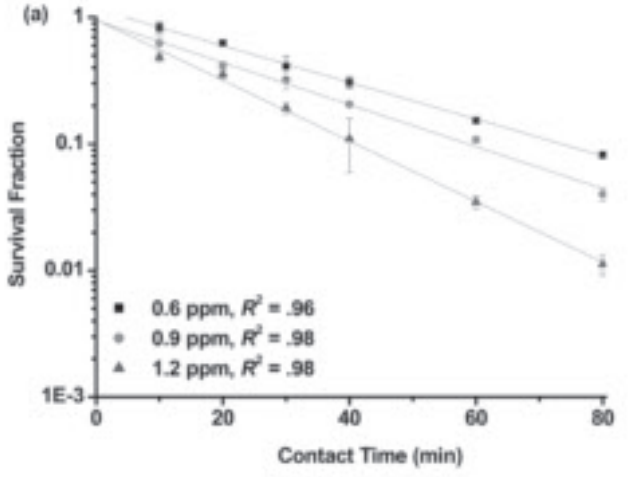
Exponential log of the survival fraction versus ozone dose for each experiment was used to perform regression analysis on the data for each virus. R^2-values were obtained by regression analysis. Generation of regression curves and prediction of the doses required for 90% and 99% viral reduction were accomplished by including data points from all experiments for each virus. Comparisons of survival fraction among the viruses were performed using *t*-test to evaluate statistically significant differences.

Results

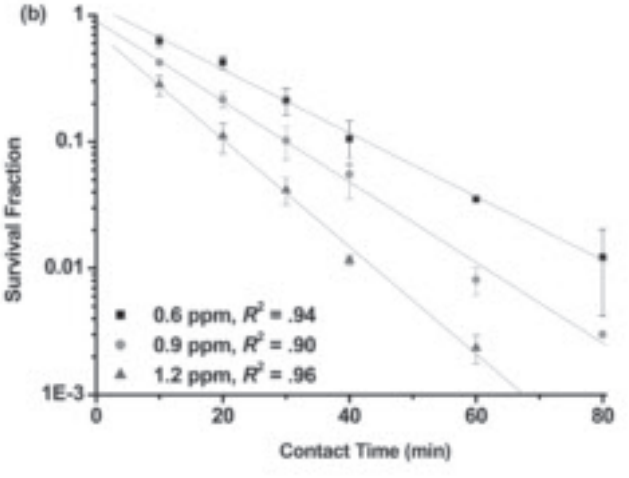
In our study, the effects of ozone dose and RH on four selected bacteriophages (ssDNA [ϕX174], ssRNA [MS2], dsDNA [T7], and dsRNA [ϕ6]) were evaluated. The figures show the log reduction of four bacteriophages at different ozone exposure levels. At 55% RH, to obtain 90% viral reduction at ozone concentration of 0.6 ppm (Figures 1a, 2a, 3a, and 4a), ϕ6 required ozone contact time of 22 minutes; ϕX174, 46 minutes; MS2, 73 minutes; and T7, 100 minutes. When ozone concentration increased from 0.6 ppm to 0.9 ppm (50%), the required ozone contact time decreased 20%–55% (from 65 min to 17 min). When ozone concentration increased from 0.6 ppm to 1.2 ppm (100%), the required ozone contact time decreased 38%–85% (from 44 min to 7 min) for the same 90% reduction. For 99% viral reduction at the ozone concentration of 0.9 and 1.2 ppm, the required contact time for 99% reduction was twice as long as for 90%

FIGURE 1

Survival Fraction of MS2 on Surface Exposed to Ozone at RH 55%



Survival Fraction of MS2 on Surface Exposed to Ozone at RH 85%



Survival Fraction of MS2 on Surface Exposed to Ozone at Different Ozone Doses ($min[mg/m^3]$)

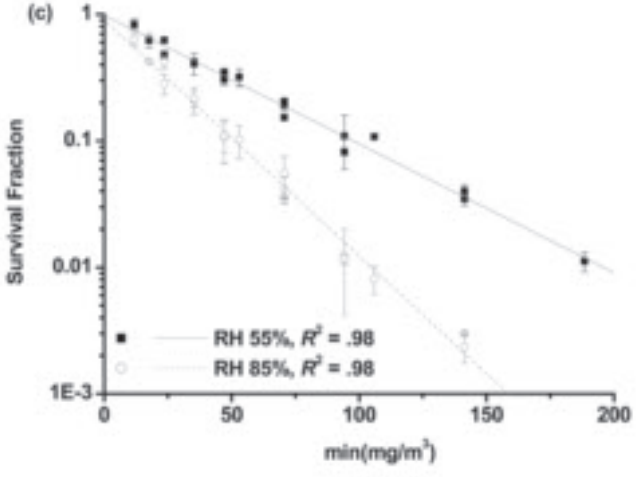
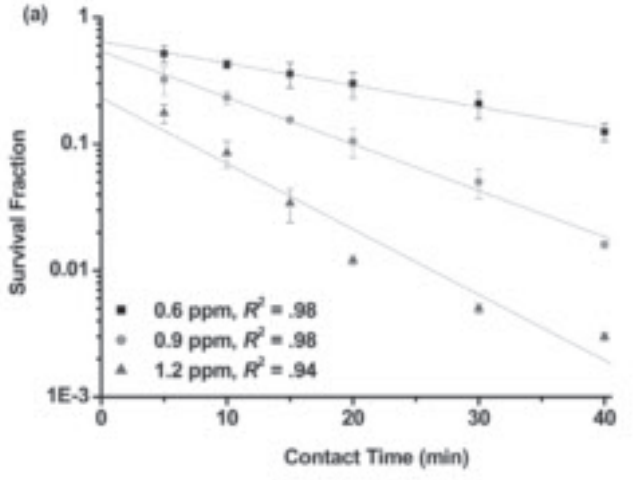
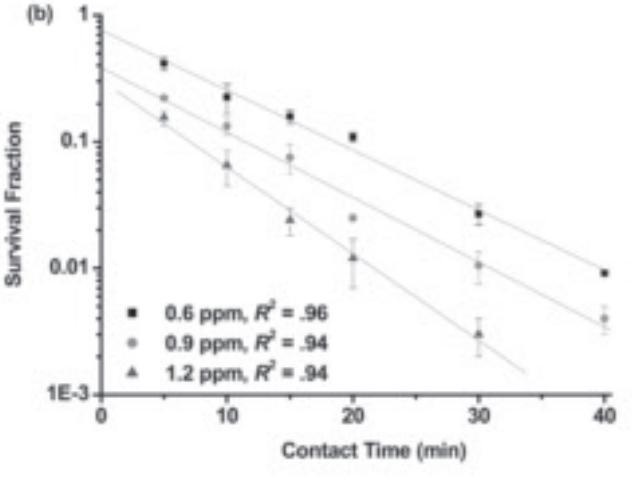


FIGURE 2

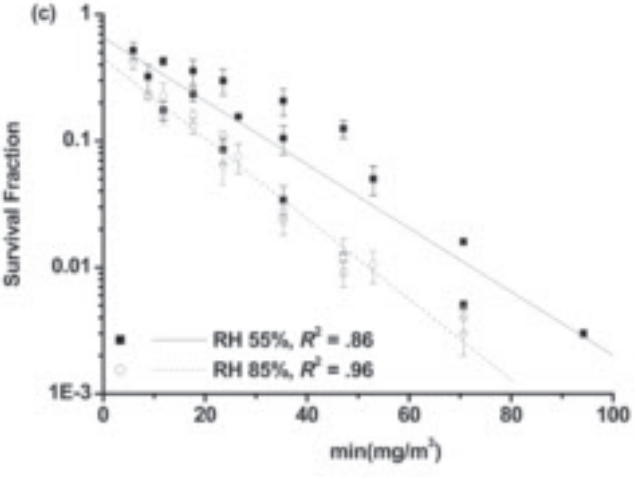
Survival Fraction of ϕX174 on Surface Exposed to Ozone at RH 55%



Survival Fraction of ϕX174 on Surface Exposed to Ozone at RH 85%



Survival Fraction of ϕX174 on Surface Exposed to Ozone at Different Ozone Doses (min[mg/m³])



reduction of all four viruses. Moreover, our results also indicated that the ozone contact time for both 90% and 99% viral reduction of MS2 and T7 was approximately two to five times longer than ϕX174 and ϕ6 ($p < .05$). At 85% RH, to obtain 90% viral reduction at the ozone concentration of 0.6 ppm (Figures 1b, 2b, 3b, and 4b), ϕ6 required ozone contact time of 18 minutes; ϕX174, 19 minutes; MS2, 42 minutes; and T7, 70 minutes. For 99% viral reduction, ϕ6 required ozone contact time of 36 min; ϕX174, 40 minutes; MS2, 82 minutes; and T7, 126 minutes. We observed that the required ozone contact time at 85% RH was 1.2 to 2.4 times shorter than at 55% RH for the same 90% and 99% reduction.

We found that survival fraction of all four viruses decreased exponentially with increasing ozone dose (Figures 1c, 2c, 3c, and 4c). At 55% RH, to obtain 90% viral reduction, ϕ6 required ozone dose of 30 min(mg/m³); ϕX174, 32 min(mg/m³); MS2, 96 min(mg/m³); and T7, 112 min(mg/m³). For 99% viral reduction, ϕ6 required ozone dose of 58 min(mg/m³); ϕX174, 72 min(mg/m³); MS2, 194 min(mg/m³); and T7, 223 min(mg/m³). The results clearly indicated that phages T7 and MS2 are less susceptible to ozone than phages ϕX174 and ϕ6 ($p < .05$). At 85% RH, to obtain 90% viral reduction, ϕ6 required ozone dose of 20 min(mg/m³); ϕX174, 22 min(mg/m³); MS2, 50 min(mg/m³); and T7, 80 min(mg/m³). For 99% viral reduction, ϕ6 required ozone dose of 47 min(mg/m³); ϕX174, 53 min(mg/m³); MS2, 104 min(mg/m³); and T7, 191 min(mg/m³). Our results indicated that ozone inactivation of surface viruses depended on ozone dose. Moreover, the required ozone doses at 85% RH were one to two times lower than those found at 55% RH for the same 90% and 99% reduction.

Based on the exponential decay model, the virus susceptibility factors, *K* (expressed in min[mg/mg³]), were found to vary widely. It was observed that the *K* factors of phages ϕX174 and ϕ6 (0.06–0.09) were higher than phages T7 and MS2 (0.01–0.04). This result could be because a more complex capsid could provide protection from ozone. For the four types of viruses, *K* factors at 85% RH (0.02–0.09) were higher than those at 55% RH (0.01–0.07). A higher ozone dose was required to inactivate the viruses at lower RH ($p < .05$). This result could be due to the generation of more radicals from ozone reacting with more water vapor at higher RH, which agrees with results from previous studies (Foarde, VanOsdell, & Steiber, 1997; Li & Wang, 2003).

Discussion

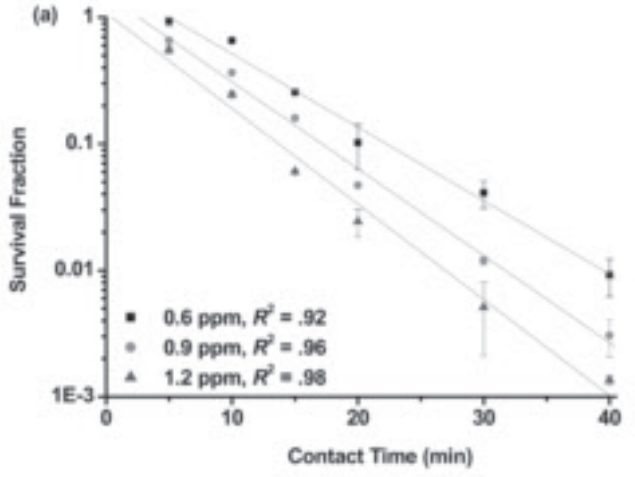
In our study, the order of susceptibility to ozone was ϕ6 > ϕX174 > MS2 > T7. In comparison with simple capsid architecture (ϕX174), more complex viral capsids (MS2 and T7) were less susceptible to ozone ($p < .05$). It has been suggested that viral capsids may be degraded into protein subunits by ozonation (Kim, Gentile, & Sproul, 1980), and viruses with more complex capsids could be less susceptible to ozone. Although ϕ6 has higher molecules of capsid protein than ϕX174, we found that ϕ6 was the most sensitive virus to ozone. This result could be related to the lipid content of ϕ6, which is extremely sensitive to environmental stress (Woolwine & Gerberding, 1995). The enveloped viruses are usually more sensitive to physical and chemical challenges than naked viruses. Male-specific bacteriophage MS2 is often used as an indicator for wastewater disinfection because of its high resistance (Havelaar et al., 1991; Shin & Sobsey, 2003). In our study, however, T7, with a more complex capsid architecture, was less susceptible to ozone than MS2. Therefore, MS2 may not be a suitable indicator for virus inactivation by ozone in air.

In our previous surface investigation of *E. coli*, *Candida famata*, *Bacillus subtilis*, and *Penicillium citrinum*, it was also revealed that survival fraction declines exponentially with ozone dose increase (Li & Wang, 2003). Currently, U.S. EPA recommends using Ct values (ozone dose) as an indicator for viral inactivation in water by ozone (U.S. EPA, 1989). The Ct values of 0.15–0.30 min(mg/L) (contact time [min] multiplied by mg/L) for 2 to 4 logs viral reduction by ozone at 25°C are suggested. Compared to our findings in this study, viruses in water were found to be less susceptible to ozone than those on surfaces.

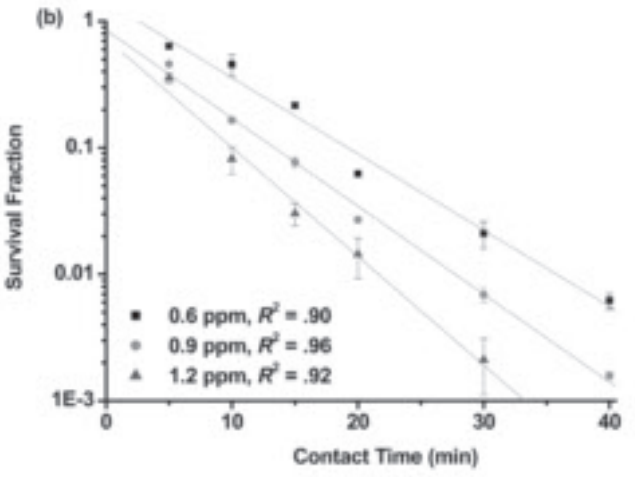
Compared to bacteria and fungi, the ozone doses for 90% viral reduction were found to be similar to those for *E. coli* (100 min[mg/m^3]), but much lower than for yeast (416 min[mg/m^3]), *P. citrinum* (1,177 min[mg/m^3]), and *B. subtilis* (4,974 min[mg/m^3]) (Li & Wang, 2003). These findings revealed that virus susceptibility to ozone is similar to fragile bacteria but is higher than fungi spores, bacteria, and endospores. The higher susceptibility of surface viruses indicated that ozone reacts more readily with capsid proteins than with lipids of the cell membrane (Komanapalli & Lau, 1998). The differences in the viral capsid protein and bacterial cell membrane may result in the various microorganisms' susceptibility to ozone (Mudd, Leavitt, Ongun, & McManus, 1969; Pryor, Das, & Church, 1991). In a previous evaluation (Tseng & Li, 2006), ozone doses required for

FIGURE 3

Survival Fraction of ϕ6 on Surface Exposed to Ozone at RH 55%



Survival Fraction of ϕ6 on Surface Exposed to Ozone at RH 85%



Survival Fraction of ϕ6 on Surface Exposed to Ozone at Different Ozone Doses (min[mg/m^3])

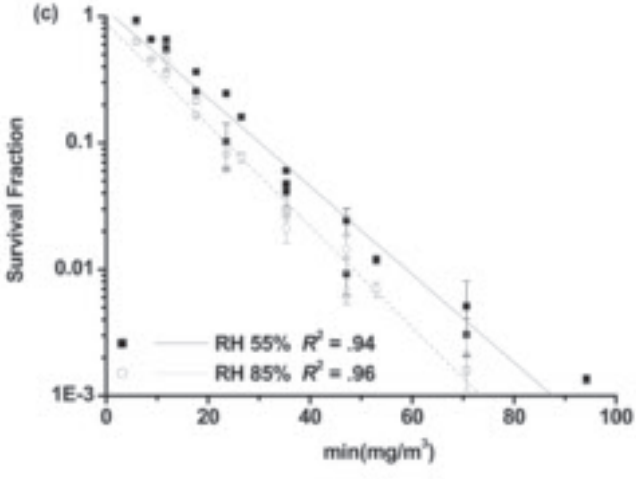
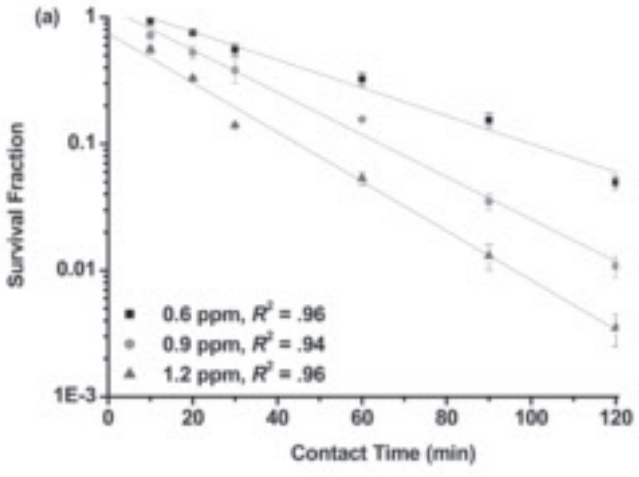
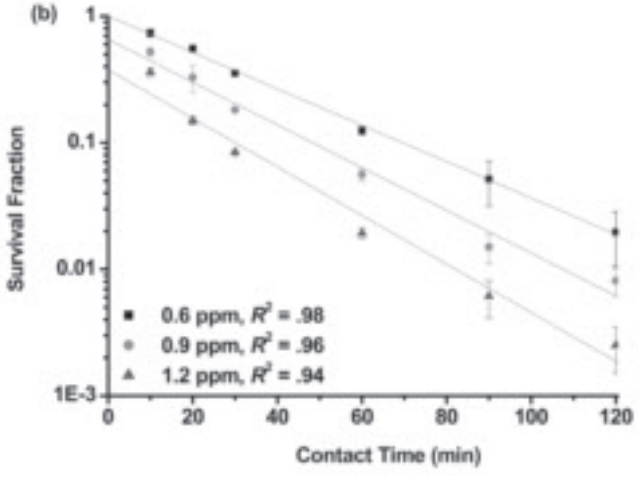


FIGURE 4

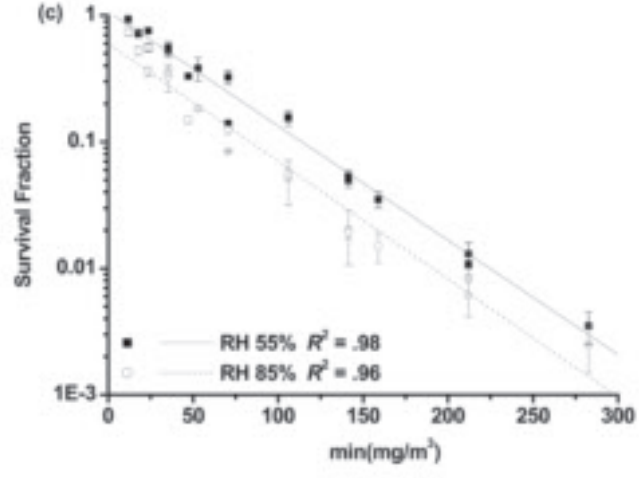
Survival Fraction of T7 on Surface Exposed to Ozone at RH 55%



Survival Fraction of T7 on Surface Exposed to Ozone at RH 85%



Survival Fraction of T7 on Surface Exposed to Ozone at Different Ozone Doses (min[mg/m^3])



airborne viruses were much lower than for surface viruses. Furthermore, the 90% viral reduction dose for viruses on surfaces was 64 times higher for MS2, 45 times higher for ϕX174, 75 times higher for ϕ6, and 56 times higher for T7 than for airborne viruses. These differences could be because surface viruses may be aggregated (Galasso, 1965), therefore, a higher dose is necessary to inactivate them.

In comparison with other methods, one needs to use care when using UVGI to inactivate surface viruses. It is known that microorganisms can grow in crevices, and UVGI can not completely penetrate these hidden areas. Ozone, however, may penetrate these areas. Ethylene oxide gas is commonly used to sterilize medical instruments in hospitals, but it may be harmful to humans. The potential use of ozone may be timely and safe for disinfection of medical instruments. Two concerns arise in using ozone for disinfection: the deleterious effect of ozone exposure to surfaces and harmful side effects to humans. Exposure to ozone via inhalation may induce peripheral vasoconstriction (Brook et al., 2002). This effect may be problematic for some individuals, particularly the aged in hospitals and nursing homes. In our investigation, the evaluated ozone concentrations were higher than ozone regulation standards (120 ppb, 1 hour average) of the U.S. National Ambient Air Quality Standard, the Taiwan Environmental Protection Agency, and the U.S. Occupational Safety and Health Administration (100 ppb, 8 hour average). It will be necessary to disinfect the surface in a chamber, rather than in open space, to prevent ozone exposure for workers. Since it was found that the survival fraction of viruses declined exponentially with ozone dose increase, we suggest that disinfection of surface viruses should be performed for longer exposure time and at lower ozone levels. In summary, ozone disinfection is a promising technique for surface virus contamination.

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