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Field trial assessing the antimicrobial decontamination efficacy of gaseous ozone in a public bus setting

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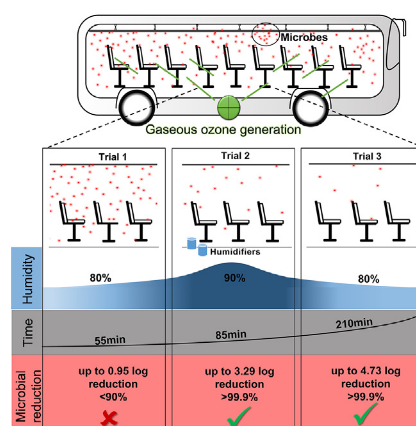
HIGHLIGHTS

- Disinfection practices are important to contain the spread of SARS-CoV-2.
- Gaseous ozone is an effective disinfectant that can be safely applied in confined spaces such as public transport.
- Increase in duration of gaseous ozone exposure and in relative humidity correlated with improved disinfection efficacy.

GRAPHICAL ABSTRACT



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ABSTRACT

The widespread COVID-19 pandemic caused by the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) necessitated measures aimed at preventing the spread of SARS-CoV-2. To mitigate the risk of fomite-mediated transmission, environmental cleaning and disinfection regimes have been widely implemented. However, conventional cleaning approaches such as surface wipe downs can be laborious and more efficient and effective disinfecting technologies are needed. Gaseous ozone disinfection is one technology which has been shown to be effective in laboratory studies. Here, we evaluated its efficacy and feasibility in a public bus setting, using murine hepatitis virus (a related betacoronavirus surrogate) and the bacteria *Staphylococcus aureus* as test organisms. An optimal gaseous ozone regime resulted in a 3.65-log reduction of murine hepatitis virus and a 4.73-log reduction of *S. aureus*, and decontamination efficacy correlated with exposure duration and relative humidity in the application space. These findings demonstrated gaseous ozone disinfection in field settings which can be suitably translated to public and private fleets that share analogous characteristics.

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1. Introduction

The Coronavirus disease 2019 (COVID-19) pandemic has caused almost 500 million infections and about 6.1 million deaths as of April 2022 (WHO, n.d.). Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of coronavirus disease (COVID-19), is known to be spread through close contact, inhalation of contaminated aerosols in poorly ventilated settings, and through fomite-mediated transmission where persons come in physical contact with infectious droplets deposited on surfaces (CDC, n.d.). Reports of cases linked only through shared spaces in Singapore and in other countries, suggest the likelihood of aerosol-mediated or fomite-mediated transmission (Goh, n.d.; Pung et al., 2020). Studies have also shown that individuals can get contaminated through snot-oral transmission from virus droplets found on surfaces (Xie et al., 2020). To reduce disease spread and severity, vaccination programmes have been rolled out across the globe. Various countries have also implemented public and environmental health measures targeted at reducing disease transmission, including case isolation and quarantine, improving ventilation of enclosed premises, and enhancing environmental cleaning and disinfection regimes, among others.

To mitigate risks of fomite-mediated transmission of SARS-CoV-2, several techniques for disinfection and sanitization of surfaces have been proposed. The most common method is surface wipe-downs using disinfectants effective against coronaviruses, such as sodium hypochlorite and hydrogen peroxide solutions (Cheng et al., 2022; NEA, n.d.). However, as such conventional approaches are laborious and time consuming, scalable and automated technologies have also been increasingly introduced. Examples include different types of ultraviolet light (UV) (Kumar et al., 2021) such as ultraviolet germicidal irradiation (UVGI) (Hamzavi et al., 2020), heat inactivation (Kampf et al., 2020), hydrogen peroxide vapor (HPV) (Schwartz et al., 2020), self-disinfecting materials and surfaces (Castaño et al., 2021), and ozone (Zucker et al., 2021). Most of these technologies have been under review and rapidly adapted due to the pandemic as a response for public spaces disinfection (Blanco et al., 2021), yet efficacy data are still lacking, especially of their use in specific settings and conditions.

Ozone has been extensively used in killing bacteria (Aydogan and Gurol, 2006; Knobling et al., 2021; Huang et al., 2012; Megahed et al., 2018; Selma et al., 2008; Sharma and Hudson, 2008), fungi, and molds (Mohamed and Barbara, 2021) and in inactivating viruses (Pekovic and Kacimi, 2015; Lee et al., 2021; Tanaka et al., 2009; Hudson et al., 2007; Brié et al., 2018; Dubuis et al., 2020); its antimicrobial properties and effectiveness have been well documented in literature (Table S1). Ozone technology has been applied in water and wastewater treatment (Wang et al., 2020), air disinfection (Grignani et al., 2021a), food sanitation (Brodowska et al., 2018) and even as a novel medical treatment for various illnesses (Manjunath et al., 2021). Ozone can disrupt surface proteins and/or membrane receptors, compromising the infectivity of the pathogen by reacting with amino acids and functional groups of protein (Tseng and Li, 2008). Furthermore, ozone can damage the viral envelope and genetic material, due to the direct oxidation of several molecules or the peroxidation of phospholipids and consequent production of reactive oxygen species (Cristiano, 2020). The efficacy of gaseous ozone against enveloped viruses (e.g., coronavirus, influenza virus, mumps virus) has been consistently shown to be effective (Table S1). Nevertheless, its efficacy can vary largely depending on environmental factors in the field, and it is important to demonstrate its effectiveness for each specific use case.

Public transport (such as buses and trains) settings require frequent disinfection as they are often high-footfall facilities with multiple common touch points (such as hand grips, grab poles, windows, seats, etc.) that may serve as a medium for fomite-mediated transmission (Moreno et al., 2021). The survivability of SARS-CoV-2 on non-porous surfaces commonly found in transport carriages such as stainless steel and plastic for up to 48 h post-deposition (van Doremalen et al., 2020), further necessitates regular disinfection in these settings. This therefore presents an ideal use-case to trial the efficacy of gaseous ozone surface disinfection, as the technique offers ease of disinfection and scalability.

This study aimed to determine the decontamination efficacy of gaseous ozone generated by a gaseous ozone generator in a public bus setting. Adapting our methods from the standard BS EN 17272:2020, we used murine hepatitis virus (a betacoronavirus surrogate) and the bacteria *Staphylococcus aureus* as our challenge organisms. Decontamination efficacy correlated with exposure duration and relative humidity in the application space, with an optimal gaseous ozone regime resulting in a 3.65-log reduction of murine hepatitis virus and a 4.73-log reduction of *S. aureus*. These findings provide real-world estimates of the effectiveness of gaseous ozone as a method for decontamination of surfaces, with the findings translatable to spaces that are analogous to buses.

2. Materials and methods

2.1. Study protocol

The trial methodology was adapted from the standard BS EN 17272:2020 – "Chemical disinfectants and antiseptics. Methods of airborne room disinfection by automated process".

2.2. Preparation of host cell line and test organisms

Murine hepatitis virus (MHV; a betacoronavirus) and *Staphylococcus aureus* were used as challenge organisms. MHV, a Risk Group 2 pathogen, has been used as a surrogate for SARS-CoV-2 in many disinfection studies (Boegel et al., 2021), and it is genetically and morphologically related to SARS-CoV-2 (Körner et al., 2020). To prepare the working stock of MHV, virus was subcultured on H2.35 mouse hepatocytes (ATCC CRL-1995™) and maintained in HyClone RPMI 1640 media (Cytiva) with 10 % FBS supplementation and incubated at 33 °C with 5 % CO_2. *S. aureus* (ATCC 25923) was prepared through two successive subcultures. Briefly, *S. aureus* was sub-cultured through streak-plating from a stock culture on tryptone soy agar (TSA). The bacterial plate was incubated at 37 °C overnight for 18 h to 24 h before a single colony was picked for the next subculture. Approval was sought and obtained from the Biosafety Branch (Public Health Group), Ministry of Health, Singapore, for the use of the test organisms in the field trial.

2.3. Preparation of contaminated carriers

Stainless steel disks (Fujitson, rated grade 2B, 301), 4 cm in diameter and 1.2 mm thick were used in this trial. The disks were cleaned with 70 % v/v ethanol and then autoclaved (121 °C, 20 min) prior to use. A 50 μL volume of MHV or *S. aureus* inoculum was evenly spread over the surface of the carrier, covering an internal area 2 cm diameter wide. The carriers were then placed onto petri dishes and into a humidified chamber for drying, with lid opened.

2.4. Tissue culture infectious dose 50 % (TCID50) assay

Tissue culture flat-bottom 96-well plates were seeded with H2.35 cells at a density of 2×10^5 cells per well, one day prior to the tests. Post-decontamination regime, 1 mL of each recovered sample was serially diluted 10-fold, and 50 μL of each dilution was added into 5 replicate wells. The plates were then incubated at 33 °C with 5 % CO_2 for three days. Cytopathic effects were determined by visualizing under a light microscope. The cell cultures were subsequently stained with crystal violet, and virus titer was determined by TCID50/mL were quantified based on the Spearman and Kärber method.

2.5. Bacterial enumeration assay

Following exposure, the spiked carriers were transferred to a sterile urine container containing 10 mL of tryptone salt broth (TSB). The container was capped and sealed with parafilm, placed in a sonicating waterbath (Elma P 30H) and sonicated at 37 kHz at 100 % power for 5 min. 1 mL of the TSB was recovered for bacterial enumeration using the drop-plate method. Briefly, the TSB was serially diluted 10-fold to 10^{-8}

with PBS, and 10 μL of each dilution was deposited onto a marked quadrant of a TSA agar plate. A total of 5 drops were deposited for each dilution, and the agar plate was incubated at 37 °C for 24 h. The dilution that had 0–30 isolated colonies within each drop was used to determine the CFU recovered from each carrier.

2.6. Virestorm ozone generator

The Virestorm V5 ozone generator generates ozone gas from oxygen in ambient air and breaks down the generated ozone gas back into oxygen. This is accomplished through the combination of a first stage high-powered (average working power is approximately 1000 watts) atmospheric-pressure plasma generator in a dense dielectric barrier discharge configuration to generate the gaseous ozone and a second stage miniaturized high-efficiency catalytic converter using a proprietary blend of catalysts designed to accelerate the decomposition of ozone gas into oxygen.

2.7. Phase 1 - trial location and set-up

Phase 1 of the trial was conducted at a bus depot, with support from Singapore Bus Service Transit (SBS Transit) and the Land Transport Authority (LTA) of Singapore. Two double-deck buses were used for this trial. Each bus was an air-conditioned 80-seater with internal dimensions of 11 m (L) × 2.5 m (W) × 2.5 m (H), approximate volume of 57.96 m^3 for lower deck, 11.5 m (L) × 2.5 m (W) × 1.8 m (H), approximate volume of 51.89 m^3 for upper deck. One bus was subjected to gaseous ozone while the second served as a control (not exposed to gaseous ozone) (Fig. S1).

Two spiked carriers were placed at each corner, and on all 4 corners of both decks (2 carriers per corner × 4 corners × 2 decks = 16 carriers total). For the corners where carriers were close to the ceiling, 1 carrier with the inoculated side faced the wall (vertically) and 1 carrier faced the ceiling (horizontally); while at the corners where carriers were close to the floor, 1 carrier faced the wall (vertically) and 1 carried faced the floor (horizontally) (Fig. 1A). The same setup was performed in the control bus. The inoculated carriers in the control and test buses were secured with clip stands attached to Styrofoam blocks (Fig. 1B). All carriers were located about 50 cm from the wall and ceiling/floor (Fig. 1B). MHV spiked carriers were tested in the first cycle, and then removed after the decontamination cycle. Following that, the *S. aureus* inoculated carriers were then tested using the same layout in a second decontamination cycle.

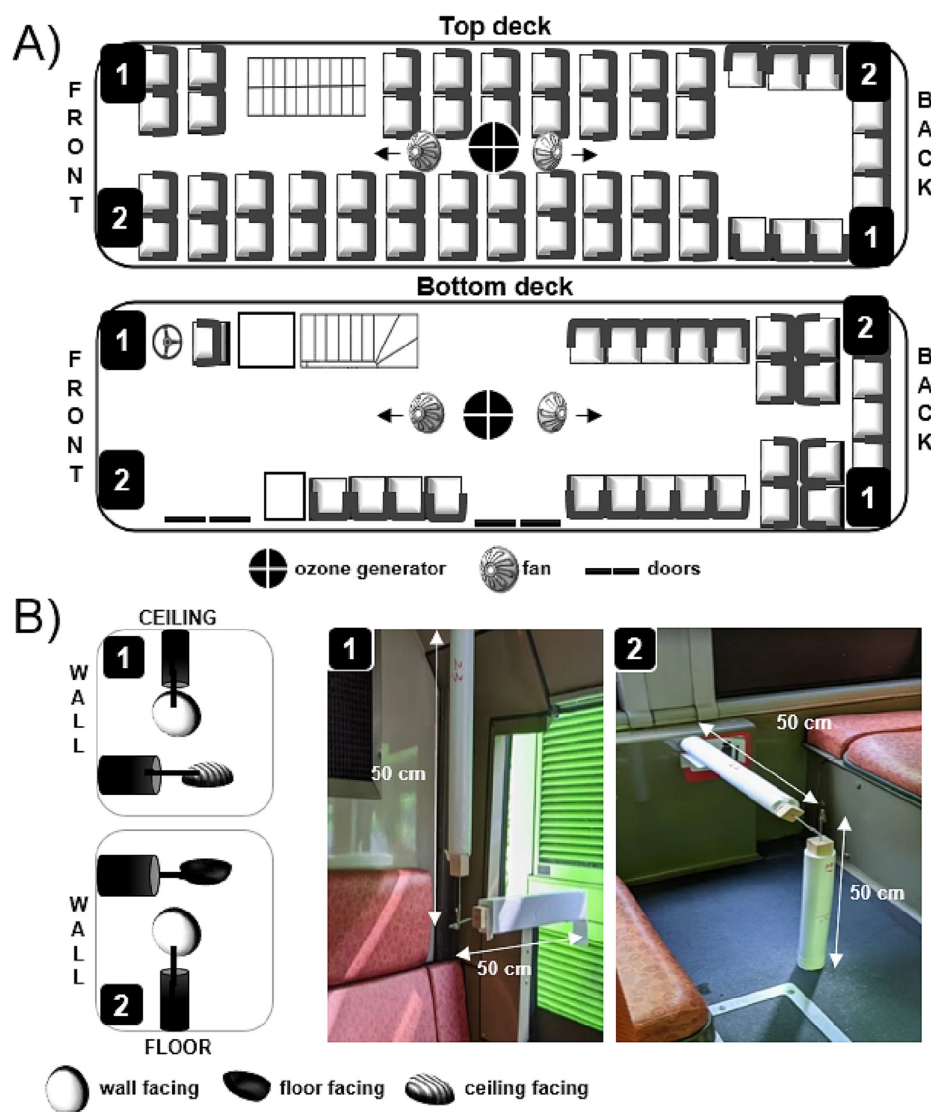

Fig. 1. Experimental set-up in a double decker bus in Phase 1. A) Sixteen carriers were spiked with MHV or *S. aureus*, 8 carriers in the top deck and 8 carriers in the bottom deck. Along with positions of the gaseous ozone generators and fans for circulation. The same set-up was performed in the control bus, but without the generator and fans. B) Schematic diagram showing the positioning of the contaminated carriers, with the contaminated side facing either the wall, floor, or ceiling.

2.8. Phase 1 - efficacy test conditions

The decontamination cycle consisted of 15 min of gaseous ozone generation, aiming to achieve a gaseous ozone concentration of between 13 and 18 ppm as the ozone sensors (EcoSensors, USA) were calibrated to accurately measure up to 20 ppm. This was followed by an approximately 40 min for ozone degeneration (Fig. S2). During the test, gaseous ozone concentration, temperature and humidity were monitored. At the step of ozone degeneration, the ozone concentration inside the bus was continuously monitored until levels were below 0.5 ppm, before the bus was immediately ventilated by opening the bus doors with an outward-facing ventilation fan. Following which, operators were allowed to enter the bus to retrieve the carriers. A cycle was run for each organism separately; the first cycle with MHV, followed by a second cycle with *S. aureus*.

2.9. Phase 2 - trial location and set-up

Phase 2 of the trial was conducted in a well-ventilated private parking area. Two single-decked buses (one control bus and one test bus) were used for the second trial. Each bus was an air-conditioned 53-seater with internal dimensions of 10.5 m (L) × 2.5 m (W) × 2 m (H), with an approximate internal volume of 52.5 m^3, similar to one deck of the double-decker buses used in Phase 1 of the trial (Fig. S3).

In this phase, carriers spiked with either MHV or *S. aureus* were placed in the buses together and run in the same decontamination cycle. A total of 16 spiked carriers (8 × MHV, 8 × *S. aureus*) were used in each bus (Fig. 2A). Twenty ultrasonic humidifiers (Xiaomi Mijia Humidifier 4 L, CAT# MJJSQ02LX) were also placed along the aisle in each bus for humidification. (Fig. 2A and C).

2.10. Phase 2 - efficacy test conditions

Two experimental conditions were tested in Phase 2: the first considered the antimicrobial effect of ozone with increased humidity, and the second considered the antimicrobial effect of prolonged exposure to ozone. The decontamination cycle for the first experiment consisted of 20 min of ozone generation (to achieve a concentration of between 13 and 18 ppm), 15 min of humidification to increase the relative humidity to above 90 % concentration, 10 min of incubation, and approximately 40 min for ozone degeneration (Fig. S4.A). The cycle for the second trial comprised 20 min of ozone generation, 150 min (2.5 h) of incubation, and approximately 40 min for ozone degeneration (Fig. S4·B).

3. Results

Prior to conducting the efficacy trial, we conducted gaseous ozone leak tests to assess the safety of the ozone disinfection trial (S1.1). Additionally, drying tests (S1.2) and residual efficacy tests (S1.3) were performed to confirm the validity of the laboratory test method. Details about these prequalification tests can be found in the Appendix S1. Pre-Qualification Tests.

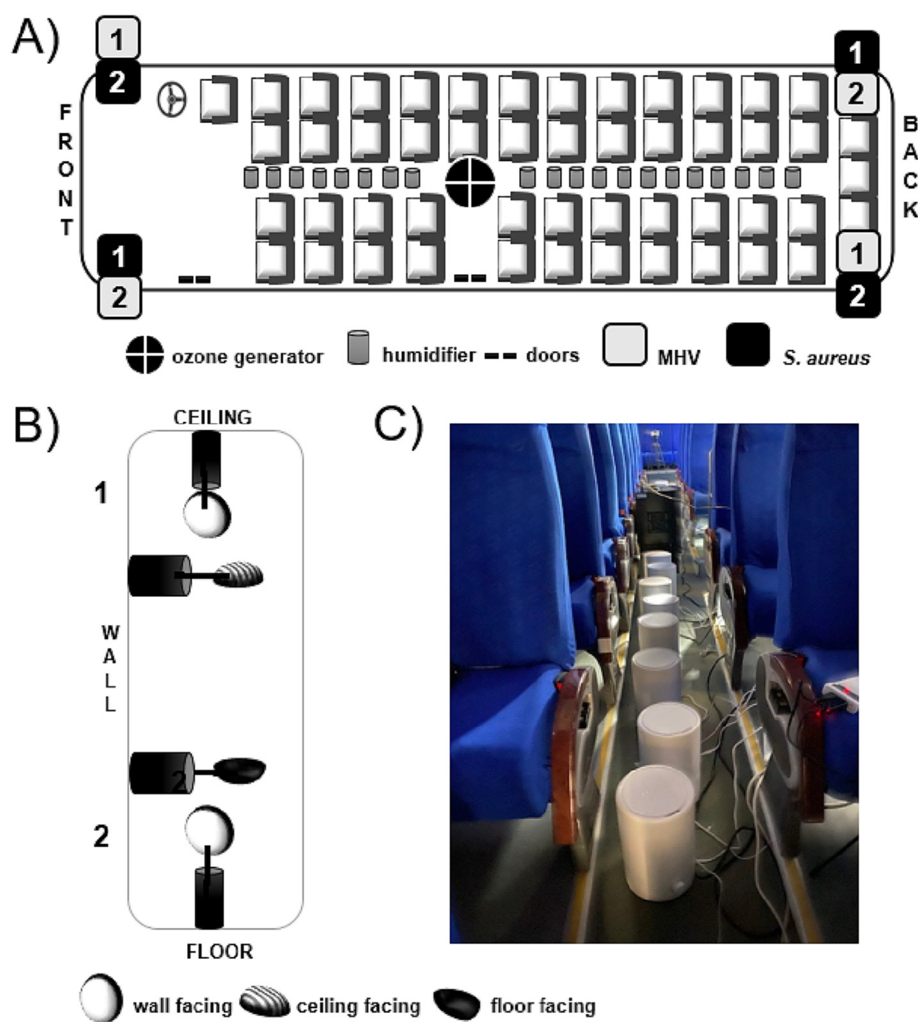

Fig. 2. Experimental set-up in a single deck bus in Phase 2. A) Sixteen carriers were spiked with MHV (8 carriers) or *S. aureus* (8 carriers). Along with positions of the gaseous ozone generator and humidifiers. The same set-up was done in the control bus, but only without the ozone generator. B) Schematic showing the positioning of the contaminated carriers, with the contaminated side facing either the wall, floor, or ceiling. C) Photograph of the humidifiers set-up in both control and test buses.

3.1. Phase 1 - efficacy test

Due to the operational requirement of a quick turn-around time for decontamination, a short decontamination cycle was initially tested. Gaseous ozone concentration was raised to 13–18 ppm (approximately 15 min) with the degeneration cycle initiated once the concentration was reached (Fig. 3A and B). The complete decontamination cycle lasted approximately 1 h. One cycle was performed for MHV contaminated carriers, and a second cycle was performed for the *S. aureus* contaminated carriers.

Thirty-two carriers were spiked with 6.99-log TCID50/carrier of MHV, 16 were placed in the control bus, and 16 were placed in the ozone-exposed bus. The mean recovery of MHV from control carriers was 5.822-log TCID50/carrier. The mean recovery of MHV from ozone exposed carriers was 4.865-log TCID50/carrier (Fig. 3C). Compared to the mean recovery of control carriers, the ozone exposed carriers showed a log reduction of 0.95-log ± 0.16-log SEM (95 % CI – 0.62- to 1.28-log reduction) (Fig. 3C).

Similarly, 32 carriers were spiked with 6.74-log CFU/carrier *S. aureus*, 16 placed in the control bus, and the remaining 16 placed in the ozone exposed bus. The mean recovery of *S. aureus* from control carriers was 6.59-log CFU/carrier, and 5.98-log CFU/carrier from ozone exposed carriers (Fig. 3D). Compared to the mean recovery of control carriers, the ozone exposed carriers showed a log reduction of 0.61-log ± 0.09-log SEM (95 % CI – 0.41- to 0.80-log reduction) (Fig. 3D).

3.2. Phase 2 - efficacy test

Following lower than expected efficacy data in the first phase, the second phase of trials expanded on investigating additional experimental parameters that could influence disinfection efficacy of gaseous ozone.

The first trial investigated the role of a 10 min incubation with increased humidity in disinfection efficacy of gaseous ozone. After the gaseous ozone concentration of 13–18 ppm was achieved (in approximately 25 min) (Fig. 4A), humidifiers were activated for approximately 16 min (Fig. 4B), during this time the ozone concentration was fluctuating likely due to the fine water droplets interfering with the ozone sensor (sensor was rated for 25–75 % relative humidity). After the relative humidity crossed >90 % both the ozone generator and the humidifiers were switched off (Fig. 4B). A 10 min incubation time was allowed before the ozone degeneration cycle was switched on. For the duration of the experiment, the temperature within both control and test buses were between 27 and 29 °C.

The test and control carriers were spiked with a 7.8-log TCID50/carrier of MHV or 6.3-log CFU/carrier of *S. aureus*.

The mean recovery of MHV from control carriers was 4.17-log TCID50/carrier. The mean recovery of MHV from ozone exposed carriers was 1.46-log TCID50/carrier (Fig. 4C), and when compared to the mean recovery of control carriers, the ozone exposed carriers showed a MHV log reduction of 2.71-log ± 0.19-log SEM (95 % CI – 2.29- to 3.13-log reduction) (Fig. 4C).

The mean recovery of *S. aureus* from the control carriers was 5.82-log CFU/carrier while the recovery from ozone-exposed carriers was 2.52-log CFU/carrier, demonstrating a log reduction of 3.29-log ± 0.51-log SEM (95 % CI – 2.18- to 4.40-log reduction) (Fig. 4D). These results suggested that increased humidity improved antimicrobial efficacy of gaseous ozone, as compared to the results obtained from Phase 1 of the study.

A second experimental condition in phase 2 investigated the effect of increased exposure duration (2.5 h) on disinfection efficacy. The gaseous ozone concentration was maintained at 13–18 ppm (Fig. 5A). For the duration of the decontamination cycle, the temperature in both control and test buses were between 29 and 31 °C, with relative humidity (ambient) maintained at approximately 80 %.

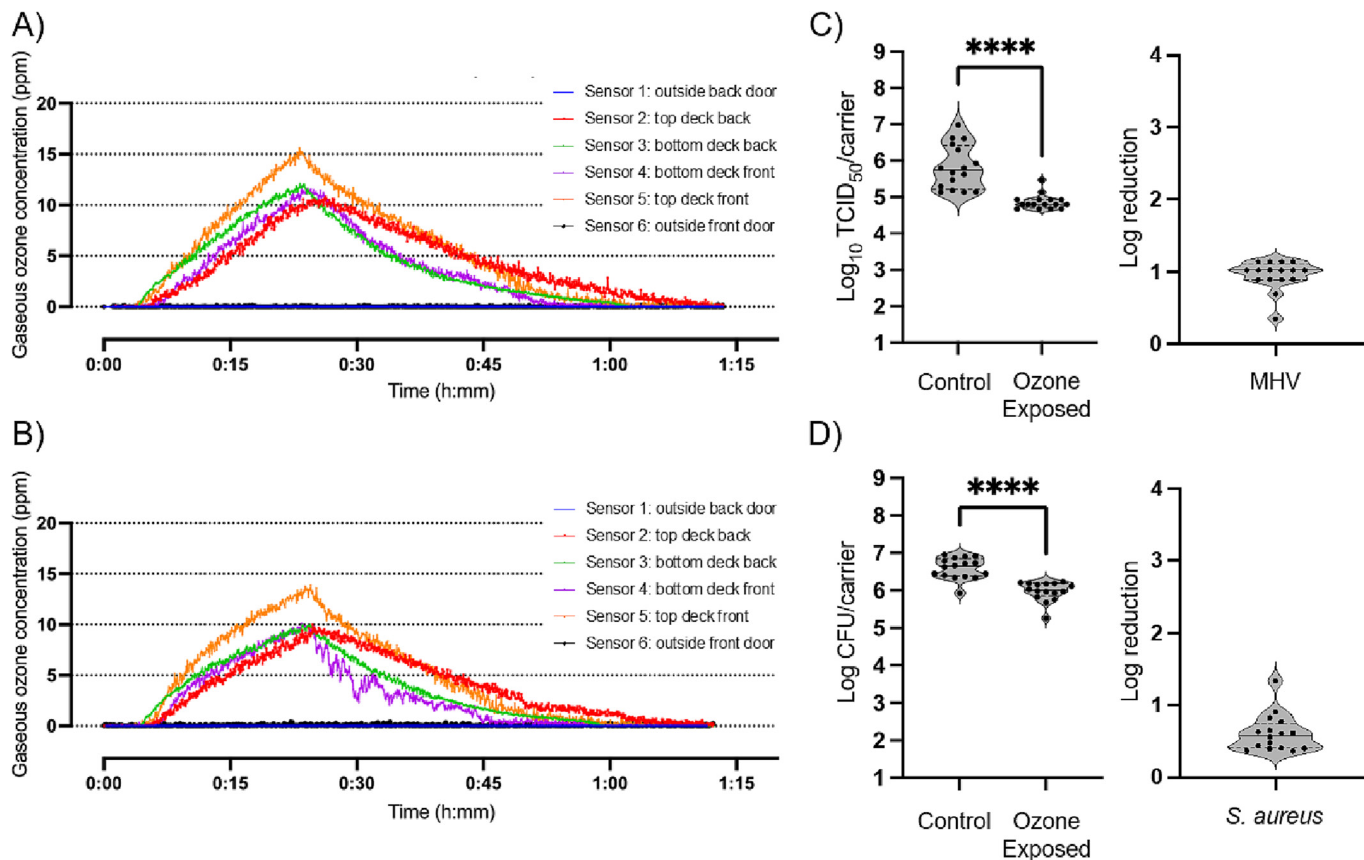

Fig. 3. Phase 1 efficacy test. Measurements of ozone concentration in the A) MHV decontamination cycle, and the B) *S. aureus* decontamination cycle. Recovery of C) MHV and D) *S. aureus* from control and ozone exposed carriers, and their respective log reduction (Median (Solid line) with upper and lower quartiles (dotted lines), *t*-test **** $p < 0.0001$).

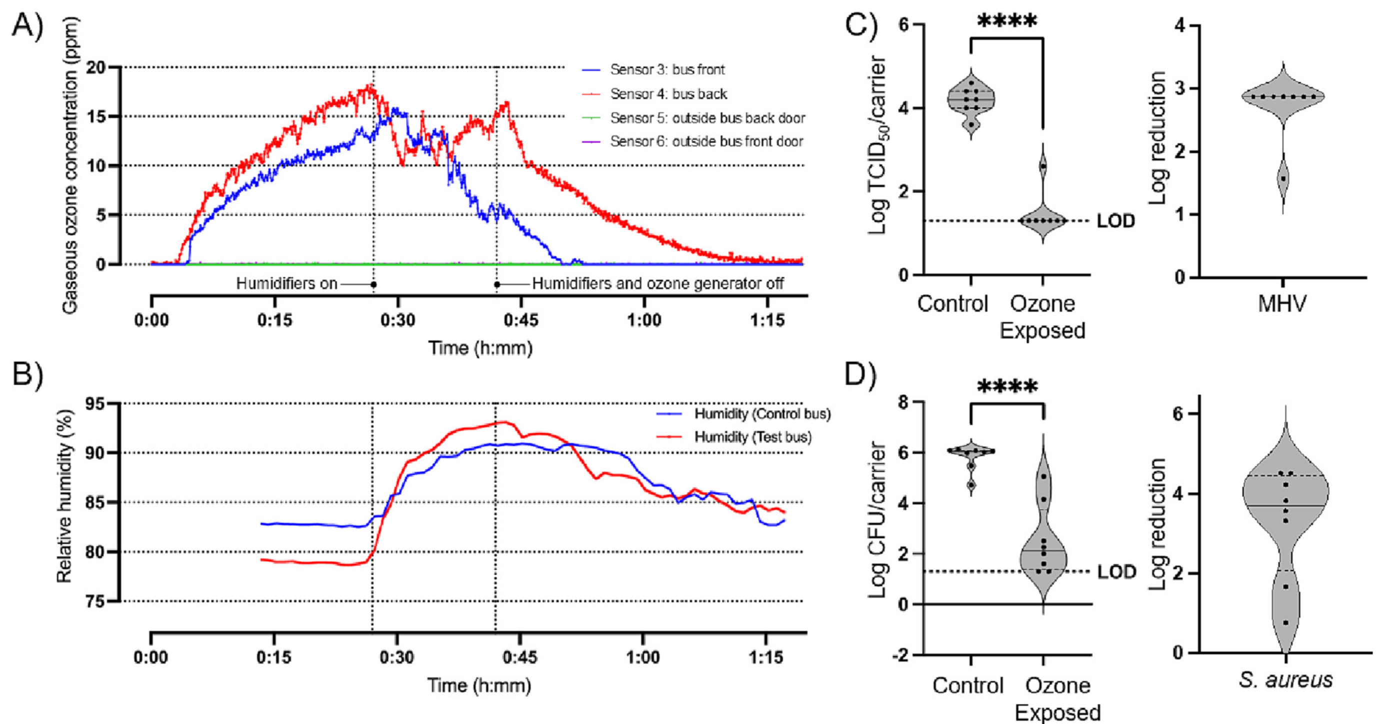

Fig. 4. Phase 2 efficacy test results with increased humidity. Measurements of A) ozone concentration and B) humidity during the decontamination cycle. Recovery of C) MHV and D) *S. aureus* from control and ozone exposed carriers, and their respective log reduction (Median (Solid line) with upper and lower quartiles (dotted lines), *t*-test **** $p < 0.0001$).

The mean recovery of MHV from control carriers was 4.95-log TCID50/carrier, while the mean recovery of MHV from ozone exposed carriers was 1.3-log TCID50/carrier, accounting for a log reduction of 3.65-log ± 0.06-log SEM (95 % CI – 3.51- to 3.78-log reduction) (Fig. 5B).

The mean recovery of *S. aureus* from the control carriers was 6.03-log CFU/carrier while the recovery from ozone-exposed carriers was 1.3-log CFU/carrier, demonstrating a log reduction of 4.73-log ± 0.06-log SEM (95 % CI – 4.58- to 4.88-log reduction) (Fig. 5C). These results demonstrated that prolonged exposure to gaseous ozone resulted in improved disinfection efficacy.

4. Discussion

Although antimicrobial properties of gaseous ozone have been well-reported in literature in laboratory settings (Table S1), there have been limited studies in field settings. To our knowledge, this is the first field trial done in a bus setting testing the disinfection efficacy of gaseous ozone against a virus and a bacterium in parallel. Furthermore, we have adapted the standard *BS EN 17272:2020* to a public bus setting, and this protocol can be replicated and used in future studies. This study reports the first field trial assessing the disinfection efficacy of gaseous ozone in a real-world public bus setting. At an optimal gaseous ozone regime of 18 ppm with high humidity (>90 %), decontamination efficacies of >3-log reductions were achieved for both murine hepatitis virus and *S. aureus*. Our findings highlight the utility of gaseous ozone disinfection of environmental surfaces in enclosed spaces, though parameters had to be adjusted to achieve disinfection efficacy.

To fit the operational requirements of a public transport operator, the initial phase of our study investigated the disinfection efficacy of gaseous ozone (15 ppm concentration) with a disinfection cycle of a shorter duration (approximately 15 min ozone generation time). The resulting log reduction observed in this setup was poor, with 0.96-log for MHV and 0.61-log for *S. aureus*.

To increase disinfection efficacy, we next explored the relationship between ozone exposure time and disinfection efficacy. This was supported by findings by Yano et al. reporting efficacious results using very low ozone concentrations of 1 ppm and 6 ppm, for 60 and 55 min respectively; revealing the strong correlation between ozone disinfection efficacy and incubation time (Yano et al., 2020). Grignani et al. (Grignani et al., 2021b) did a comprehensive literature review on the effectiveness of ozone as disinfectant and concluded that low concentrations of ozone for longer exposure times can have similar results to shorter exposures at higher concentrations. In one study using influenza virus that used ozone concentrations similar to what was used in our bus trial, H1N1 virus was exposed to gaseous ozone at a concentration of 10 ppm for 3.5 h or 20 ppm for 2.5 h; and reported a > 4-log reduction in H1N1 titers at 10 ppm, and > 5-log reduction at 20 ppm (Tanaka et al., 2009). Therefore, in our follow-up trial in phase 2 of our study, we prolonged the exposure of the challenge organisms to gaseous ozone to 2.5 h, and showed a disinfection efficacy of 3.65- to 4.73-log; a substantial improvement from the phase 1 result.

Varying durations for ozone disinfection cycles have been previously reported. Hudson et al. (Hudson et al., 2009) obtained the maximum antiviral efficacy against 12 different viruses at a concentration of 25 ppm of ozone for 15 min. For human coronavirus 229E, a 3-log reduction in virus titers on contaminated face masks was reported at a gaseous ozone concentration of 120 ppm, for as short as 1 min post exposure (Lee et al., 2021). However, it must be noted that unlike our study which explored the environmental decontamination of enclosed spaces, the test setting was for decontamination of specific items (i.e. masks) which were placed in close proximity (1 cm) from the ozone source. Hence, the high ppm and close distance of the generator would not be feasible for the purposes of environmental decontamination of enclosed spaces.

In contrast, others had also demonstrated successful disinfection efficacy of mumps virus (>5-log reduction) using much lower ozone concentrations of 20–25 ppm, albeit with a longer contact time of 20 min (Pekovic and Kacimi, 2015). Of note, the researchers also intentionally increased the relative humidity of the test chamber to >90 %. Mazur-Panasiuk et al. (Mazur-Panasiuk et al., 2021) also demonstrated that increased humidity favored MHV disinfection efficacy. Similarly in the second phase of our trial, we explored using increased humidity, while maintaining a short

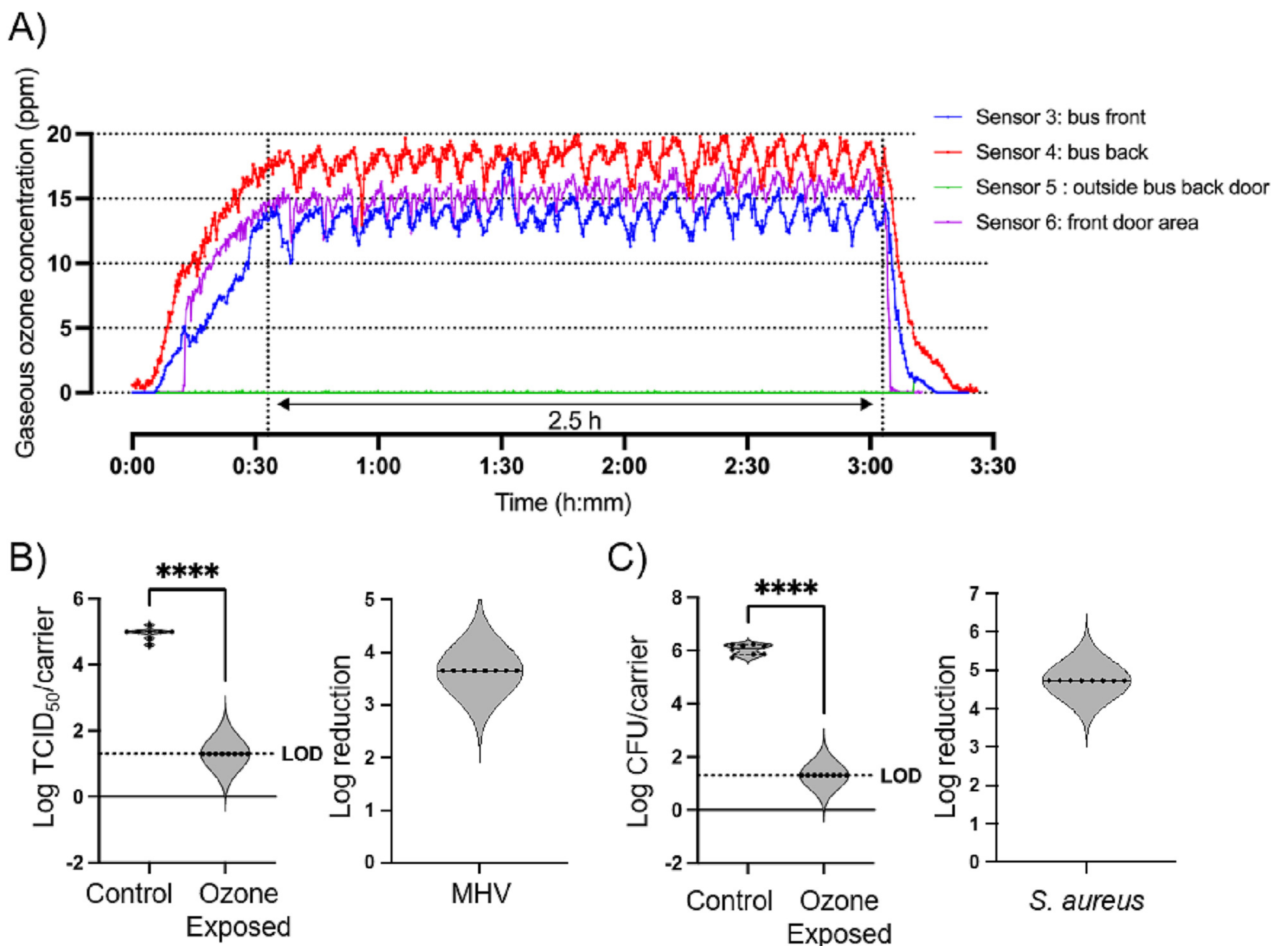

Fig. 5. Phase 2 efficacy test with increased incubation time. Measurements of A) ozone concentration during the decontamination cycle. Recovery of B) MHV and C) *S. aureus* from control and ozone exposed carriers, and their respective log reduction (Median (Solid line) with upper and lower quartiles (dotted lines), *t*-test **** $p < 0.0001$).

duration of ozone exposure. In agreement with literature, we showed that a low concentration gaseous ozone used in conjunction with an increased relative humidity resulted in improved disinfection efficacy of 2.71-log against MHV and 3.29-log against *S. aureus*.

5. Conclusion

In summary, we demonstrated that different durations of gaseous ozone exposure (both short and long) have shown to be efficacious. Of note, shorter durations of gaseous ozone required close proximity to ozone source and relied on achieving higher ppm which are not feasible in large spaces. We have demonstrated that for shorter durations of gaseous ozone exposure, a large space could be adequately decontaminated at lower gaseous ozone concentrations if relative humidity could be increased. Although technically a higher concentration could reduce disinfection time, our study protocol had a maximum ozone concentration of ~20 ppm, due to the use of in situ ozone generators. These generators produce ozone from oxygen in the air that becomes progressively concentrated with ozone, making difficult to increase even more the concentration. Furthermore, high ozone concentrations present a hazard in an operational setting by rising the safety risk to operators.

The occurrence, stability, and infectivity of SARS-CoV-2 varies depending on the surface materials where the droplets land. Although we only tested here one type of surface (stainless steel), the high virus survivability on stainless steel makes this the most stringent condition (van Doremalen et al., 2020). Although the interior of a bus is made of different types of material including porous surfaces, recent studies demonstrated that gaseous ozone is able to penetrate these surfaces effectively (Uppal et al., 2021; Wolfgruber et al., 2022).

As with most global cities, metropolitan buses are an important part of the Singapore public transport system. Approximately half the population of Singapore commute on them every day, and a safe, effective, and quality-controlled method for sanitizing the fleet of vehicles is called as we continue to live with COVID-19. Although the initial disinfection duration could not be achieved, using a longer disinfection cycle or higher humidity, our field trial demonstrated the utility and efficacy of gaseous ozone disinfection in public transport buses. Where bus operators could afford the time, gaseous ozone decontamination could be a less laborious, safe and efficacious solution for disinfection.

Our results agree with the literature on the importance of relative humidity, duration of exposure and concentration as important parameters for optimal efficacy of gaseous ozone as a disinfectant. Due to its wide antimicrobial range, ozone is considered one of the best biocides against microorganisms, and it can provide a valuable decontamination tool for the removal of pathogens in public transport and other larger indoor venues.

CRediT authorship contribution statement

Erica Sena Neves: Methodology, Investigation, Writing – original draft, Writing – review & editing, Data curation. **Cheng Teng Ng:** Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Han Bin Pek:** Investigation, Writing – review & editing. **Vanessa Shi Li Goh:** Investigation, Writing – review & editing. **Roslinda Mohamed:** Investigation, Writing – review & editing. **Sheereen Osman:**

Investigation, Writing – review & editing. **Yi Kai Ng:** Investigation, Writing – review & editing. **Sharain Abdul Kadir:** Investigation, Writing – review & editing. **Mohammad Nazeem:** Investigation, Writing – review & editing. **Alan She:** Methodology, Validation, Investigation, Resources, Writing – review & editing. **Glenndle Sim:** Investigation, Resources, Writing – review & editing, Funding acquisition. **Joel Aik:** Conceptualization, Investigation, Writing – review & editing, Funding acquisition. **Lee Ching Ng:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Sophie Octavia:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Supervision. **Zhanxiong Fang:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Supervision. **Judith Chui Ching Wong:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Yin Xiang Setoh:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

Aydogan, A., Gurol, M.D., 2006. Application of gaseous ozone for inactivation of Bacillus subtilis spores. J. Air Waste Manag. Assoc. 56 (2), 179–185.

Blanco, A., Ojembarrena, F.D.B., Clavo, B., Negro, C., 2021. Ozone potential to fight against SAR-COV-2 pandemic: facts and research needs. Environ. Sci. Pollut. Res. 28 (13), 16517–16531.

Boegel, S.J., Gabriel, M., Sasges, M., Petri, B., D'Agostino, M.R., Zhang, A., Ang, J.C., Miller, M.S., Meunier, S.M., Aucoin, M.G., 2021. Robust evaluation of ultraviolet-C sensitivity for SARS-CoV-2 and surrogate coronaviruses. Microbiol. Spectr. 9 (2), e0053721.

Brié, A., Boudaud, N., Mssihid, A., Loutreul, J., Bertrand, I., Gantzer, C., 2018. Inactivation of murine norovirus and hepatitis A virus on fresh raspberries by gaseous ozone treatment. Food Microbiol. 70, 1–6.

Brodowska, A.J., Nowak, A., Śmigielski, K., 2018. Ozone in the food industry: principles of ozone treatment, mechanisms of action, and applications: an overview. Crit. Rev. Food Sci. Nutr. 58 (13), 2176–2201.

Castaño, N., Cordts, S.C., Kurosu Jalil, M., Zhang, K.S., Koppaka, S., Bick, A.D., Paul, R., Tang, S.K.Y., 2021. Fomite transmission, physicochemical origin of virus-surface interactions, and disinfection strategies for enveloped viruses with applications to SARS-CoV-2. ACS Omega 6 (10), 6509–6527.

CDC, .. Science Brief: SARS-CoV-2 and Surface (Fomite) Transmission for Indoor Community Environments https://www.cdc.gov/coronavirus/2019-ncov/more/science-and-research/surface-transmission.html (accessed 12/01/2022).

Cheng, P., Luo, K., Xiao, S., Yang, H., Hang, J., Ou, C., Cowling, B.J., Yen, H.L., Hui, D.S., Hu, S., Li, Y., 2022. Predominant airborne transmission and insignificant fomite transmission of SARS-CoV-2 in a two-bus COVID-19 outbreak originating from the same pre-symptomatic index case. J. Hazard. Mater. 425, 128051.

Cristiano, L., 2020. Could ozone be an effective disinfection measure against the novel coronavirus (SARS-CoV-2)? J. Prev. Med. Hyg. 61 (3), E301–E303.

van Doremalen, N., Bushmaker, T., Morris, D.H., Holbrook, M.G., Gamble, A., Williamson, B.N., Tamin, A., Harcourt, J.L., Thornburg, N.J., Gerber, S.I., Lloyd-Smith, J.O., de Wit, E., Munster, V.J., 2020. Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1. N. Engl. J. Med. 382 (16), 1564–1567.

Dubuis, M.E., Dumont-Leblond, N., Laliberté, C., Veillette, M., Turgeon, N., Jean, J., Duchaine, C., 2020. Ozone efficacy for the control of airborne viruses: bacteriophage and norovirus models. PLoS One 15 (4), e0231164.

Goh, T., .. 9 COVID-19 cases in Blk 111 Tampines St 11, residents of 58 households and their visitors offered testing https://www.straitstimes.com/singapore/health/coronavirus-9-confirmed-covid-19-cases-detected-in-blk-111-tampines-st-11-residents (accessed 1st March 2023).

Grignani, E., Mansi, A., Cabella, R., Castellano, P., Tirabasso, A., Sisto, R., Spagnoli, M., Fabrizi, G., Frigerio, F., Tranfo, G., 2021. Safe and effective use of ozone as air and surface disinfectant in the conjuncture of COVID-19. Gases 19–. [Online].

Grignani, E., Mansi, A., Cabella, R., Castellano, P., Tirabasso, A., Sisto, R., Spagnoli, M., Fabrizi, G., Frigerio, F., Tranfo, G., 2021. Safe and effective use of ozone as air and surface disinfectant in the conjuncture of COVID-19. Gases 1, 19–32.

Hamzavi, I.H., Lyons, A.B., Kohli, I., Narla, S., Parks-Miller, A., Gelfand, J.M., Lim, H.W., Ozog, D.M., 2020. Ultraviolet germicidal irradiation: possible method for respirator disinfection to facilitate reuse during the COVID-19 pandemic. J. Am. Acad. Dermatol. 82 (6), 1511–1512.

Huang, H.L., Lee, M.G., Tai, J.H., 2012. Controlling indoor bioaerosols using a hybrid system of ozone and catalysts. Aerosol Air Qual. Res. 12 (1), 73–82.

Hudson, J.B., Sharma, M., Petric, M., 2007. Inactivation of norovirus by ozone gas in conditions relevant to healthcare. J. Hosp. Infect. 66 (1), 40–45.

Hudson, J.B., Sharma, M., Vimalanathan, S., 2009. Development of a practical method for using ozone gas as a virus decontaminating agent. Ozone Sci. Eng. 31 (3), 216–223.

Kampf, G., Voss, A., Scheithauer, S., 2020. Inactivation of coronaviruses by heat. J. Hosp. Infect. 105 (2), 348–349.

Knobling, B., Franke, G., Klupp, E.M., Belmar Campos, C., Knobloch, J.K., 2021. Evaluation of the effectiveness of two automated room decontamination devices under real-life conditions. Front. Public Health 9, 618263.

Körner, R.W., Majjouti, M., Alcazar, M.A.A., Mahabir, E., 2020. Of mice and men: the coronavirus MHV and mouse models as a translational approach to understand SARS-CoV-2. Viruses 12 (8).

Kumar, A., Sagdeo, A., Sagdeo, P.R., 2021. Possibility of using ultraviolet radiation for disinfecting the novel COVID-19. Photodiagn. Photodyn. Ther. 34, 102234.

Lee, J., Bong, C., Bae, P.K., Abafogi, A.T., Baek, S.H., Shin, Y.B., Bak, M.S., Park, S., 2021. Fast and easy disinfection of coronavirus-contaminated face masks using ozone gas produced by a dielectric barrier discharge plasma generator. Environ.Sci.Technol.Lett. 8 (4), 339–344.

Manjunath, S.N., Sakar, M., Katapadi, M., Geetha Balakrishna, R., 2021. Recent case studies on the use of ozone to combat coronavirus: problems and perspectives. Environ. Technol. Innov. 21, 101313.

Mazur-Panasiuk, N., Botwina, P., Kutaj, A., Woszczyna, D., Pyrc, K., 2021. Ozone treatment is insufficient to inactivate SARS-CoV-2 surrogate under field conditions. Antioxidants 10 (9) (Basel).

Megahed, A., Aldridge, B., Lowe, J., 2018. The microbial killing capacity of aqueous and gaseous ozone on different surfaces contaminated with dairy cattle manure. PLoS One 13 (5), e0196555.

Mohamed, Z., Barbara, R., 2021. Inactivation of microbes by ozone in the food industry: a review. Afr. J. Food Sci. 15 (3), 113–120.

Moreno, T., Pintó, R.M., Bosch, A., Moreno, N., Alastuey, A., Minguillón, M.C., Anfruns-Estrada, E., Guix, S., Fuentes, C., Buonanno, G., Stabile, L., 2021. Tracing surface and airborne SARS-CoV-2 RNA inside public buses and subway trains. Environ. Int. 147, 106326.

NEA, .. List of household disinfectants and self-disinfecting surface coating products against COVID-19 virus https://www.nea.gov.sg/our-services/public-cleanliness/environmental-cleaning-guidelines/guidelines/list-of-household-products-and-active-ingredients-for-disinfection-of-covid-19 (accessed 15 March).

Pekovic, D.D., Kacimi, H., 2015. Efficacy of ozone gas against mumps virus under experimental environment conditions. EC Microbiol. 1, 184–189.

Pung, R., Chiew, C.J., Young, B.E., Chin, S., Chen, M.I., Clapham, H.E., Cook, A.R., Maurer-Stroh, S., Toh, M.P., Poh, C., Low, M., 2020. Investigation of three clusters of COVID-19 in Singapore: implications for surveillance and response measures. Lancet 395 (10229), 1039–1046.

Schwartz, A., Stiegel, M., Greeson, N., Vogel, A., Thomann, W., Brown, M., Sempowski, G.D., Alderman, T.S., Condreay, J.P., Burch, J., Wolfe, C., 2020. Decontamination and reuse of N95 respirators with hydrogen peroxide vapor to address worldwide personal protective equipment shortages during the SARS-CoV-2 (COVID-19) pandemic. Appl.Biosaf. 25 (2), 67–70.

Selma, M.V., Ibáñez, A.M., Cantwell, M., Suslow, T., 2008. Reduction by gaseous ozone of salmonella and microbial flora associated with fresh-cut cantaloupe. Food Microbiol. 25 (4), 558–565.

Sharma, M., Hudson, J.B., 2008. Ozone gas is an effective and practical antibacterial agent. Am. J. Infect. Control 36 (8), 559–563.

Tanaka, H., Sakurai, M., Ishii, K., Matsuzawa, Y., 2009. Inactivation of influenza virus by ozone gas. IHI Engineering Review. 42, pp. 108–111.
Tseng, C., Li, C., 2008. Inactivation of surface viruses by gaseous ozone. J. Environ. Health 70 (10), 56–62.
Uppal, T., Khazaieli, A., Snijders, A.M., Verma, S.C., 2021. Inactivation of human coronavirus by FATHHOME's dry sanitizer device: rapid and eco-friendly ozone-based disinfection of SARS-CoV-2. Pathogens 10 (3), 339.
Wang, J., Shen, J., Ye, D., Yan, X., Zhang, Y., Yang, W., Li, X., Zhang, L., Pan, L., 2020. Disinfection technology of hospital wastes and wastewater: suggestions for disinfection strategy during coronavirus disease 2019 (COVID-19) pandemic in China. Environ. Pollut. 262, 114665.
WHO, .. WHO Coronavirus (COVID-19) Dashboard https://covid19.who.int/ (accessed 15 March).
Wolfgruber, S., Loibner, M., Puff, M., Melischnig, A., Zatloukal, K., 2022. SARS-CoV2 neutralizing activity of ozone on porous and non-porous materials. New Biotechnol. 66, 36–45.
Xie, C., Zhao, H., Li, K., Zhang, Z., Lu, X., Peng, H., Wang, D., Chen, J., Zhang, X., Wu, D., Gu, Y., 2020. The evidence of indirect transmission of SARS-CoV-2 reported in Guangzhou, China. BMC Public Health 20, 1–9.
Yano, H., Nakano, R., Suzuki, Y., Nakano, A., Kasahara, K., Hosoi, H., 2020. Inactivation of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by gaseous ozone treatment. J. Hosp. Infect. 106 (4), 837–838.
Zucker, I., Lester, Y., Alter, J., Werbner, M., Yecheskel, Y., Gal-Tanamy, M., Dessau, M., 2021. Pseudoviruses for the assessment of coronavirus disinfection by ozone. Environ. Chem. Lett. 1–7.