Chapter

Hybrid Hydrogen Peroxide for Viral Disinfection

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Abstract

Decontamination is often necessary in facilities with sensitive spaces where pathogen elimination is critical. Historically, high concentration vaporized hydrogen peroxide technologies have been applied in these areas for pathogen disinfection. While effective, these high concentration solutions come with inherent risks to human health and safety. Alternatively, one recent innovation is a hybrid hydrogen peroxide system which combines a 7% hydrogen peroxide solution with a calibrated fogging device that delivers a mixture of vaporous and micro aerosolized particles, significantly lowering the risk of exposure to high-concentration hazardous chemicals. Studies performed with this technology demonstrate high level pathogen decontamination across a variety of tested pathogens and substrates. This chapter will cover a brief history of hydrogen peroxide technologies and their application processes; examine the correlations between viral inactivation, viral disinfection, and biological indicators for validation; demonstrate the necessity of dwell time for optimal efficacy; discuss the effects of viral disinfectant use on laboratory surfaces; and examine various studies, including virologic work performed in Biosafety Level 3 facilities and good laboratory practice (GLP) data performed by EPA-approved laboratories. This chapter will provide readers a deeper understanding of essential components and considerations when implementing hydrogen peroxide systems for viral decontamination.

Keywords: hydrogen peroxide, disinfection, high-level disinfection, decontamination, sterilization, vapor hydrogen peroxide, chlorine dioxide

1. Introduction

Decontamination is a fundamental requirement for research facilities where pathogen elimination is critical, and laboratory facility managers routinely employ various methods of fumigation or fogging disinfection in the never-ending battle against contamination. Historically, technologies such as chlorine dioxide and formaldehyde gas systems have been applied in these areas for pathogen disinfection. Likewise, high concentration vaporized hydrogen peroxide has also been relied on to achieve similar outcomes. A large percentage of these methods follow a familiar pattern of solution injection, dwell (contact time), evacuation, and validation; however, not every system delivers the same functionality or efficacy. Differences in formula and design influence personnel hours, material compatibility, and risk management.

While effective, these high concentration solutions come with inherent risks to health and safety. A recent innovation significantly lowers the risk of exposure to high-concentration chemicals— an HHP™ system which combines a 7% hydrogen peroxide solution with a calibrated fogging device to deliver a mixture of gaseous and micro aerosolized particles. Studies performed with this technology demonstrate high level pathogen disinfection across a variety of tested viruses, bacteria, and substrates. This chapter will provide readers with a deeper understanding of essential components and considerations when implementing systems for viral decontamination. This chapter introduces the latest evolution in hydrogen peroxide disinfection of viral pathogens to address these challenges: an HHP system using patented Pulse™ technology.

1.1 Addressing the need for disinfectants

A dichotomy of virology work is the need for both viral presence within the confines of research and the equally consistent need to establish pathogen-free research spaces. Throughout the world, contagious disease through viral contamination is an ever-present concern, and SARS-CoV-2 has brought the need to decontaminate to the forefront of virtually every industry. Scientific industries performing research, manufacturing pharmaceuticals, or providing healthcare services, all employ protocols for the disinfection of their environments in order for safe, successful, timely work to take place. These industries depend upon disinfection chemicals, and perhaps just as importantly the chemical delivery systems, that ensure the integrity of their work, personnel safety, and efficient transition from one research project or product type to the next.

1.2 Classification of antimicrobial effectiveness

Today, a number of distinct categories are used to classify and understand disinfection methods. Disinfection chemicals are tested with established protocols and classified according to their relative success at eliminating specific pathogens. The *Biosafety in Microbiological and Biomedical Laboratorie*s (BMBL) 6th edition makes a distinction between the inactivation of pathogens (rendering them non-viable) and the destruction of pathogens and their infectious particles (decontamination) [1]. This distinction is highly relevant to industries where establishing a sterile surface can be a critical determinant of success or failure [2]. The United States Environmental Protection Agency (EPA) classifies disinfectants by their ability to inactivate certain challenging pathogens, such as *Clostridioides difficile* (*C. diff*) and *Bacillus anthracis* (Anthrax), which delineates if the disinfectant is classified a sterilant, decontaminant, or sporicide [3] (**Box 1**). This delineation is based on the Spaulding classification, the microbiological hierarchy model standard, which classifies pathogens based on their environmental hardiness and relative resistance to disinfection [4, 5]. In this hierarchy, small non-enveloped viruses are considered moderately resistant, whereas spores are most resistant to disinfection methods. Beginning in 2016, the EPA developed its Emerging Viral Pathogen category to fast-track products proven against bacterial spores for use against newly appearing viral threats [5–8]. Beyond this classification testing, commercially available spore-based biological indicators can be used with certain solutions as an ongoing measurement and verification of sterilization results [2].

Biosafety in Microbiological and Biomedical Laboratories (BMBL) Definitions [1]	
Decontamination	The use of physical and/or chemical means to remove, inactivate, or destroy microbial pathogens (e.g., bloodborne or aerosolized) on a surface or item to the point where they are no longer capable of transmitting infectious particles and the item or surface is rendered safe to handle: however, this definition has been broadened by infection control specialists to include all pathogens and physical spaces (e.g., patient rooms, laboratories, buildings).
Disinfectant	A substance, or mixture of substances, that destroys or irreversibly inactivates bacteria, fungi, and viruses, but not necessarily bacterial spores or prions, in the inanimate environment.
Disinfection	A process that destroys pathogens and other microorganisms, except prions, by physical or chemical means.
High-Level Disinfection	A lethaI process utilizing a sterilant under less than sterilizing conditions (e.g., 10–30 min contact time instead of 6–10 h needed for sterilization). The process kills all forms of microbiaI life except for large numbers of bacterial spores.
lnactivation	A procedure to render a pathogen non-viable, viral nucleic acid sequences non-infectious, or a toxin non-toxic whiIe retaining characteristic(s) of interest for future use. Methods targeting tropism may be host-specific.
Sterilization	A physical or chemical process that kills or inactivates all microbial life forms including highly resistant bacterial spores.
Sterilant	A substance or mixture of substances that destroys or eliminates all forms of microbial life in the inanimate environment including all forms of vegetative bacteria, bacterial spores, fungi, fungal spores, and viruses.
Validation	Establishment of the performance characteristics of a method and provision of objective evidence that the performance requirements for a specified intended use are fulfilled.
Classification Definitions	
Aerosol	Particulate matter, solid or liquid, larger than a molecule but small enough to remain suspended in the atmosphere [9].
Gas	A substance or matter in a state in which it will expand freely to fill the whole of a container, having no fixed shape (unlike a solid) and no fixed volume (unlike a liquid) [10].
Hybrid H_2O_2	A mixture of gaseous and micro aerosolized substance which remain suspended in the air to fill the whole container [11]
Vapor	A substance diffused or suspended in the air, especially one normally Iiquid or solid [12].

Box 1.
Definitions*. Definitions relating to achieving and evaluating levels of antimicrobial effectiveness on environmental surfaces [1]. Definitions of substance phase or classification [9–12]. Depending on device design, the chemical being dispersed throughout the treatment space may be delivered in a variety of forms, phases, or states of matter. These definitions are provided for the sake of our understanding the differences in technologies and delivery methods described within this chapter.*

1.3 The evolution of disinfection systems

One growing understanding is that the application method of a disinfectant plays a critical role in the success of the disinfection results. While some of the most common spray and wipe surface disinfectants have been in use for decades, there are challenges to their application which can result in inconsistent or ineffective

results. Adequate distribution and required contact time are difficult to achieve on a consistent basis by hand application methods, especially in large spaces with high ceilings and complex surface profiles. These accessibility issues and failures may result in inconsistent and incomplete elimination of surface contamination [13]. To address inherent inconsistencies in manual disinfection and to provide alternative methods of delivery, various technologies have been applied. Those technologies include fumigation with formaldehyde, chlorine dioxide gas, fogging of hydrogen peroxide as vapor, silver hydrogen peroxide systems, and hybrid hydrogen peroxide systems. Their gaseous and vaporous form allows access to, and contact with, surfaces that spray and wipe methods alone often cannot access. Automated systems have taken these chemicals with known disinfectant action and paired them with dispersion devices, aiming to deliver an appropriate contact time and maximize surface exposure. These systems automate much of the disinfection process, helping to remove human error and mitigate safety concerns from contact with potentially caustic chemicals. In particular, H_2O_2-based systems have become a front-runner among automated high-level disinfection technologies due to H_2O_2's effectiveness, material compatibilities, lack of chemical residues, and increased safety over other technologies such as formaldehyde or chlorine dioxide gas [14–18]. When applied in multiple life science environments, H_2O_2 fogging is well documented to have efficacy against numerous viral pathogens and has seen a rise in use in environments where thorough efficacy and decontamination of a room and its contents are needed [19–22].

1.4 Mechanism of action of hydrogen peroxide

Anyone who has skinned their knee and poured hydrogen peroxide on the wound to stave off infection is familiar with the use of H_2O_2 as an antiseptic and anti-bacterial agent. Indeed, hydrogen peroxide is produced naturally in the body, acting as a beacon triggering the accumulation of white blood cells of the immune response [23]. Hydrogen Peroxide was first discovered in 1818 by Louis Jacque Theénard, who described it as 'eau oxygénéé or water oxygen for its composition containing one more oxygen atom than water [24]. This single oxygen–oxygen or peroxide bond is naturally unstable and prone to decomposition with or without the presence of a catalyst [25]. During decomposition, the active oxygen atom cleaves off, releasing energy and resulting in water and oxygen molecules [26]. The oxidizing activity, resulting from the presence of the extra oxygen atom, is what makes hydrogen peroxide an effective disinfectant. It is the reactive formulation of hydrogen peroxide which causes destruction of pathogens by breaking apart structures, interrupting key functions, causing damage to DNA, and eliminating infectious particles.

2. Hybrid hydrogen peroxide via pulse technology

One of the biggest challenges to any disinfectant application is ensuring a thorough and consistent disinfectant exposure to contaminated surfaces for an effective contact time. To achieve success, fogging technologies must perform a complicated dance between the amount of chemical injected, temperature, humidity, dew point, and method, all of which can affect efficacy from one application to the next. To answer this need, CURIS System designed and patented the concept of replenishing any naturally decomposing solution and called it Pulse technology, simplifying the complicated balance of a successful disinfection. Combining a 7% hydrogen peroxide solution with a calibrated fogging device, this HHP system delivers hybrid

hydrogen peroxide, a mixture of gaseous and micro aerosol particles. While effective in a liquid solution, fogging with hydrogen peroxide in this hybrid form increases the availability of each H_2O_2 molecule, maximizing oxidation opportunities and leading to the destruction of pathogens on surfaces. Beyond just inactivating pathogens, this oxidation causes a physical destructive action of pathogen components, which further delineates this substance as a decontaminant as defined by the BMBL.

A fundamental distinction of this system is its ability to disperse a lower concentration of 7% hydrogen peroxide at calibrated intervals, maximizing contact time while using less H_2O_2 to achieve microbicidal efficacy. The HHP device operates by delivering the HHP mixture in a two-part process. First, it fills an enclosure with disinfecting fog to an optimal level for killing pathogens. Second, it maintains the fog at the optimal level without oversaturation by periodically injecting more solution into the space being treated, and thereby prolonging the active contact time of the H_2O_2 (**Figure 1**). This not only helps to keep surfaces dry, it also reduces sensitivity to variations in temperature and other factors. One might consider this similar to cruise control in a vehicle—the initial phase continuously revs the engine to get the vehicle up to speed, while the second phase uses the engine just enough to keep it at the cruising speed without exceeding the limit. In the case of disinfection, it means keeping the fog concentration at the optimum "kill" level to achieve efficacy in a relatively short time, yet without exceeding this optimum level to the point where the fog condenses on surfaces in the treatment area.

2.1 Chemical concentrations and safety implications

With a concentration of 7% H_2O_2, the solution, known as CURoxide™, is below the 8% hazard threshold [27, 28]. Being below the threshold means special shipping considerations are not required. Moreover, this enables safer handling for personnel than the 35–59% H_2O_2 solutions traditionally employed for fogging applications [18, 29–31]. Likewise, the 7% solution is safer for laboratory materials than the 28.1–52% concentration of corrosive industrial strength grade hydrogen peroxide [27, 32]. This material safety (compatibility) is perhaps most evident when

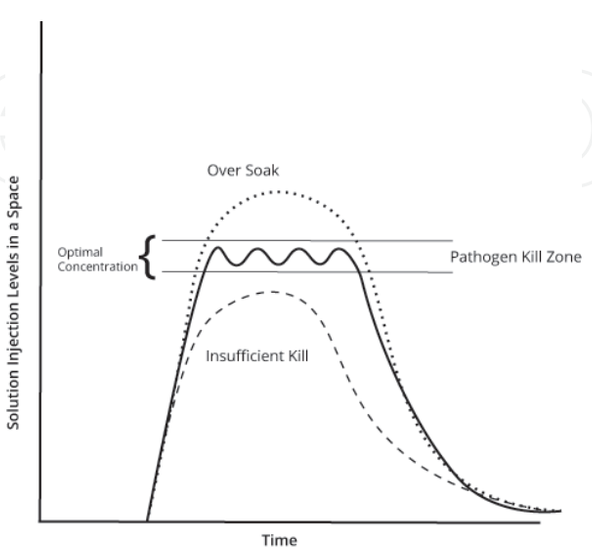

Figure 1.
***Pulse HHP Cycle**. Hydrogen peroxide released as a vapor or aerosol begins a natural decomposition into water and oxygen within 10 minutes. Most fogging delivery methods require longer contact time. Pulse technology periodically replenishes active hydrogen peroxide during the decontamination cycle, prolonging the effective contact time, and promoting an optimal pathogen kill zone.*

considering how the hydrogen peroxide concentration of a solution will evolve when the solution transitions through states of matter. Hydrogen peroxide is more resistant to leaving the liquid state and more likely to return to it than the water in the solution. When transitioning from vapor back into liquid, this can result in surface condensation at more than double the initial liquid concentration (**Figure 2**). At 7% H_2O_2, the HHP solution remains below the 45% known level of material incompatibility [33].

The levels of particle concentration used in typical high-level disinfection are of particular concern to facility managers. These concerns may be lessened by employing lower particle-producing products. Technologies utilizing formaldehyde, chlorine dioxide, and high concentration H_2O_2 operate at concentrations as high as 1,400 parts per million (ppm) [34–36]. By contrast, the HHP 7% solution has a lower operating concentration of approximately 138 ppm [37]. Traditional vaporized approaches require a concentration that is up to 10× higher than the lower 7% H_2O_2 concentration enables, which accordingly may result in a greater risk to personnel from leakage with typical high concentration systems [38]. This is particularly important because, according to the National Library of Medicine, "Inhalation of vapors from concentrated (greater than 10%) solutions may result in severe pulmonary irritation" [39]. This may be why there is a substantial safety concern among facility managers when it comes to typical fogging approaches, as these approaches utilize caustic chemicals at very high concentrations which are known to penetrate through gaps as small as a keyhole [38, 40].

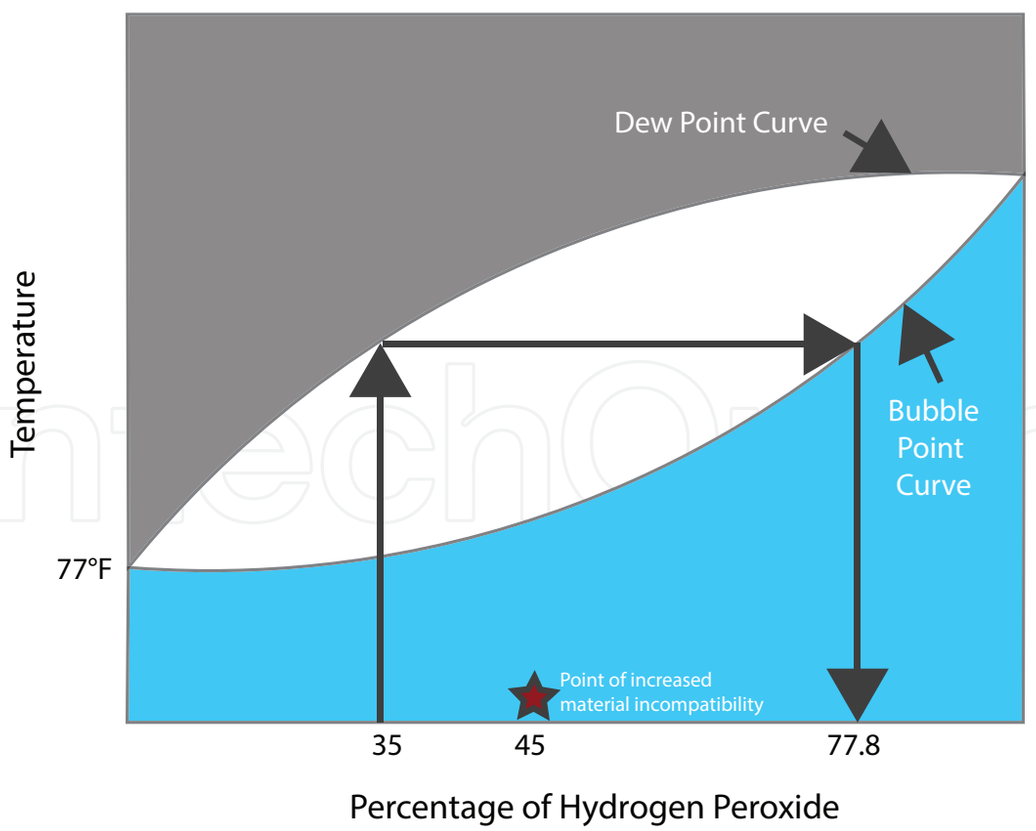

Figure 2.
Fluctuations in H_2O_2 Concentration. *Adapted from Hultman et al. [33] the concentration of hydrogen peroxide changes throughout different states of matter. When vapor condenses onto a surface the peroxide is more likely to enter the liquid state than the water vapor. This results in surface concentrations significantly higher than the original solution concentration. Concentrations exceeding 45% H_2O_2 are higher than the recommended maximum concentration for suitable interaction with other materials. In this manner a 35% solution that has been vaporized and condensed out on surfaces can reach concentrations of 77.8% H_2O_2 [33].*

2.2 HHP device description

Roughly the size of a small suitcase, the 36-pound (16 kg) HHP system fogs enclosures from an adjustable stainless-steel nozzle at the top of the unit. It can be wheeled or carried throughout a facility to disinfect a wide variety of spaces, large or small, and its Rotomold design provides durability for long-term use and sturdiness during transport. A push-button design allows users to input area dimensions through the device's manual digital interface, or users may operate the device remotely via a tablet for touchless disinfection from outside the treatment space. The system self-calculates the cubic footage of the space to be fogged to determine the amount of disinfectant needed, and an indicator light shows users when the appropriate amount of solution has been added to the reservoir. An electronically sequenced A/C electrical outlet provides optional connection for any desired additional equipment.

2.3 Smart technology

In a world where everything is documented to defend, reinforce, train, and track information, technologies with the ability to employ these methods are invaluable to present and future decontamination applications. The HHP system incorporates patented smart technology, allowing operation not only from a device interface but also remotely through its control app for phones and tablets (**Figure 3**). For larger spaces, multiple devices may simultaneously work together via wireless communication to combine their capacities to fill the larger volume without the added complications of cables. Whether used alone or in a network, the fogging device(s) self-calculates the dosage required for a space once dimensions are provided. For each disinfection cycle, a job report is wirelessly generated and saved into a secure data system, providing the facility with trackable records in support of risk management protocols. On-demand training, reference materials, and technical support are also available through this secure data storage system, which includes security codes, usernames, and password protection against unauthorized operation and modifications. These smart technology components give laboratory personnel the

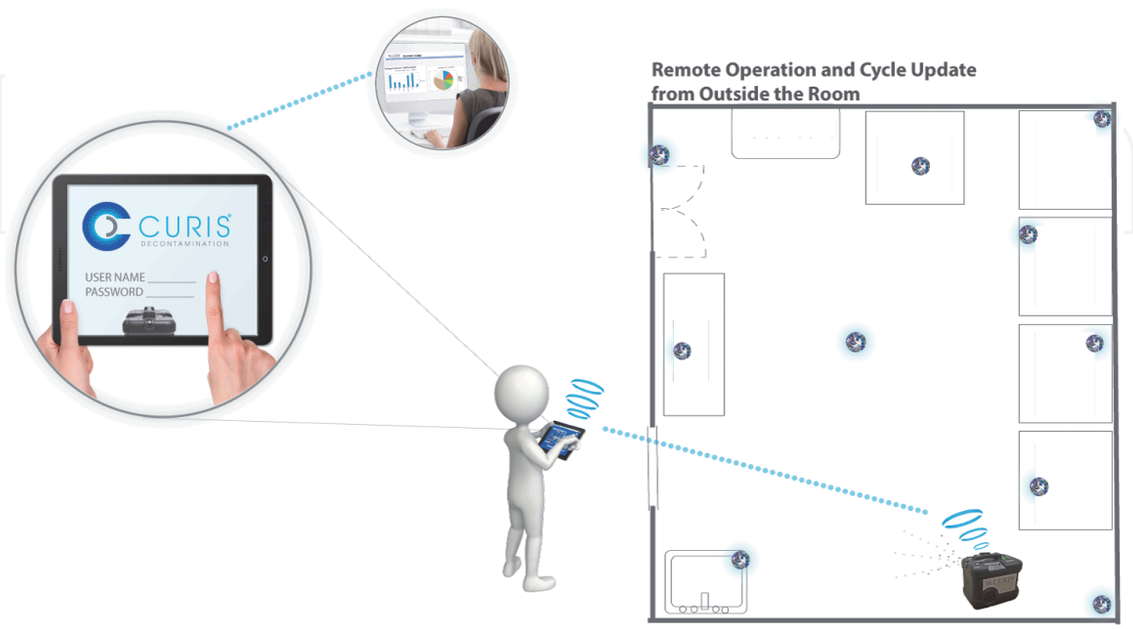

Figure 3.
Hybrid Hydrogen Peroxide (HHP) Smart Technology*. Wireless remote operation via tablet, with secure data management. The HHP device is operated from outside the enclosure. Once the treatment cycle is complete, the data are uploaded and recorded to a secure database for customer analytics and job reports.*

ability to remotely operate and monitor the system, lessening concerns affiliated with exposure to high concentrations of H_2O_2.

2.4 Versatility

2.4.1 Large enclosure decontamination

The HHP device offers the ability to decontaminate enclosed spaces as large as 14,000 ft^3 (396 m^3) by itself or wirelessly pair up to 25 devices together to treat spaces as large as 350,000 ft^3 (9,911 m^3) at once. Although the EPA approvals are for 3,682 ft^3 (103 m^3) due to the size limitation of the testing laboratory, efficacy of bacterial spores are documented in much larger spaces [41]. The small, compact design also reaches tall ceilings efficaciously, as noted in studies where 6-log_{10} reductions of *Geobacillus stearothermophilus* challenged indicators were proven at 21 ft. However, all treated spaces are to be validated with 6-log_{10} biological indicators for optimal application.

2.4.2 Attachments

Since many life science facilities are made up of diversely sized spaces and needs, the next generation of Pulse technology device was developed. Retaining the core fogging unit's design, the new attachment model offers the ability to fog, hand spray, or port in, all from the same unit. This fogging model can disinfect large open spaces with a hand sprayer (with proper personal protective equipment). The device can also port into enclosed spaces, such as labs or mobile equipment, with extension nozzles, or it can connect to various enclosures found within laboratories.

2.4.3 Scalable decontamination

To enable decontamination of small enclosures, the HHP system pairs with a mobile cart designed to attach to biological safety cabinets, isolators, incubators, filters, and filter housings (**Figure 4a**) [42]. This modular pairing delivers low concentration H_2O_2 solution to the closed system environment, extracts vapor once decontamination has been achieved, and conditions the space to return it to its normal operating environment. No disassembly of lab equipment is required. The system achieves decontamination of the entire chamber, including filters, and contents. The rolling cart weighs approximately 50 pounds (22 kg) and includes a pullout tray to house the HHP fogging device. For scalable applications, the fogging device can fog a whole laboratory or be coupled to the mobile cart as needed for smaller enclosures.

2.4.4 Facility integration

The HHP system also enables integration with a laboratory or stand-alone chamber. This modular design allows for custom installation into facilities—including integrated nozzles and touchscreen operation—to provide decontamination to these essential spaces (**Figure 4b**). For facilities requiring unified operation of environmental or electronic controls, the HHP system works in tandem with smart integration technology to provide remote operation, automation, and mounted disinfection for one or more enclosed spaces at a time. Decontamination chamber or washer integration includes cycles of less than 120 minutes, including aeration. This chamber integration enables users to operate the entire chamber from one common point, the display screen. It is suitable for coupling with chambers from a variety of manufacturers.

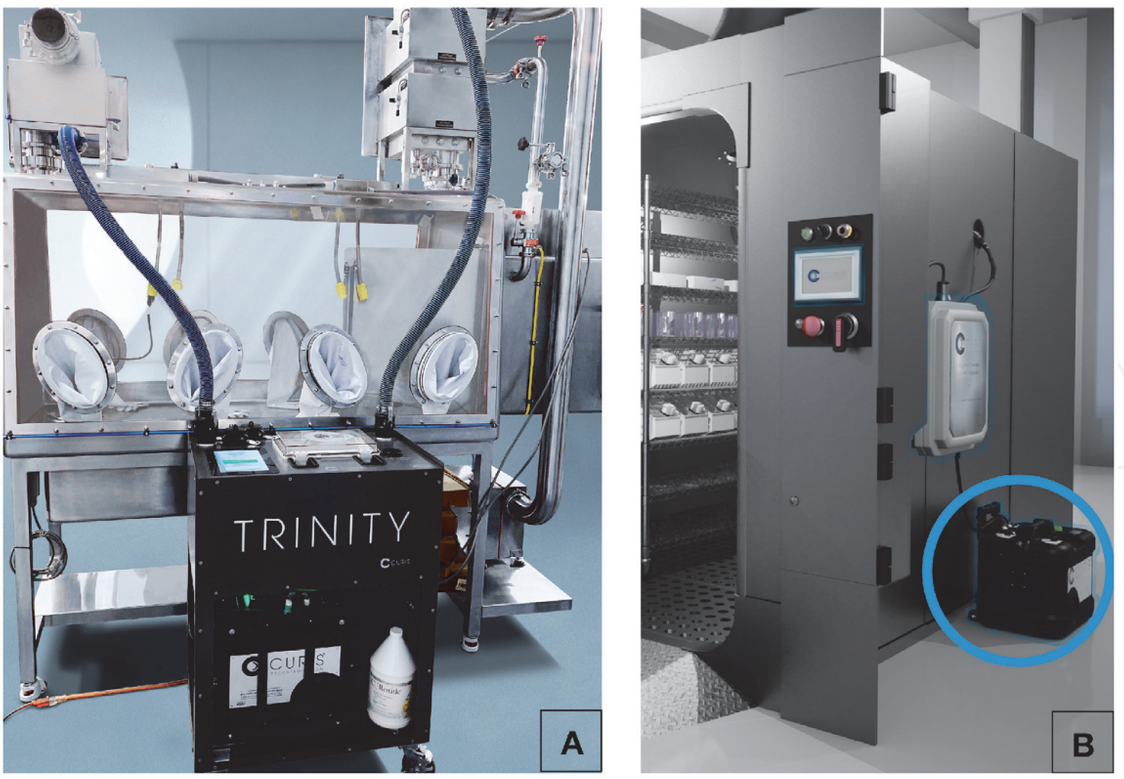

Figure 4.
Scalability and Integration*. A. Modular cart coupled with hybrid hydrogen peroxide (HHP) device, shown here decontaminating a glove box. B. HHP system integration for decontamination of a laboratory or chamber and its contents.*

2.4.5 HHP applications

During the 2020–2021 COVID-19 pandemic, the HHP system was approved by the EPA for use against SARS-CoV-2 through the Emerging Viral Pathogen designation due to its sporicidal efficacy [37]. As a result, the HHP system was used in many different environments as a tool for mitigating risk to personnel, research, and equipment. Healthcare facilities faced with shortages of personal protective equipment (PPE) employed the system to decontaminate and safely reuse PPE until the supply could be reestablished. Life science facilities incorporated the HHP system for decontaminating manufacturing spaces where vaccine work was taking place. The HHP system was also instrumental in multiple military applications, significantly aided by the portable design and accessible use. Some prior and ongoing uses include disinfection of manufacturing facilities with a need for sterilization, sterile processing facilities, drug manufacturing facilities, vivariums, laboratory contents, laboratories with interstitial spaces, laboratory filter housings, compounding pharmacies, surgical suites, healthcare patient rooms, ambulances, equipment for service providers, biological safety cabinets, isolator filters, and gnotobiotics.

3. HHP testing efficacy data

3.1 Introduction

Studies performed with Pulse technology demonstrate high-level pathogen disinfection across a variety of tested viruses, bacteria, and bacterial spores. The data presented here include a mixture of peer-reviewed studies, Good Laboratory Practice (GLP)-regulated testing, and real-world applications where disinfection can be

further complicated by condition-dependent factors such as biofilms, soil loads, and surface type (porous/non-porous), all of which can protect and harbor infectious pathogens [13, 43]. Across the body of this work, the target of high-level disinfection is not only to reduce the present contamination, but to reduce it sufficiently to prevent an infectious dose or the potential for colony regrowth. The work presented here demonstrates the HHP system's ability to decontaminate, destroying microbial pathogens. This complete decontamination is critical as any surviving pathogens have the potential to interfere with or invalidate research, contaminate sterile products, and cause health hazards.

3.2 Validating the HHP process

When targeting pathogens invisible to the eye, there must be some way to measure the efficacy of disinfection. Employing validation tools gives the ability to verify a disinfection process using living organisms and giving results rooted in science. Though several types of chemical and pH indicators exist, indicators of *Geobacillus stearothermophilus* bacterial spores (1×10^6 organisms) are used as the international standard for validation of sterilization by hydrogen peroxide [44, 45]. These 6-$\log_{10}$ indicators consist of a verified population of approximately 1 million bacterial spores. The evolutionary hardiness of bacterial spores has led to them being used as a standard of measurement for sterilization [2]. Inactivation of these difficult-to-penetrate spores also represents confirmation of efficacy in disinfecting lower-level pathogens, such as non-enveloped viruses, gram-negative and gram-positive bacteria, molds, yeasts, and enveloped viruses (**Figure 5)** [4, 5, 45]. Likewise, proving inactivation of these robust organisms predicts successful disinfection of more susceptible pathogens [7, 8].

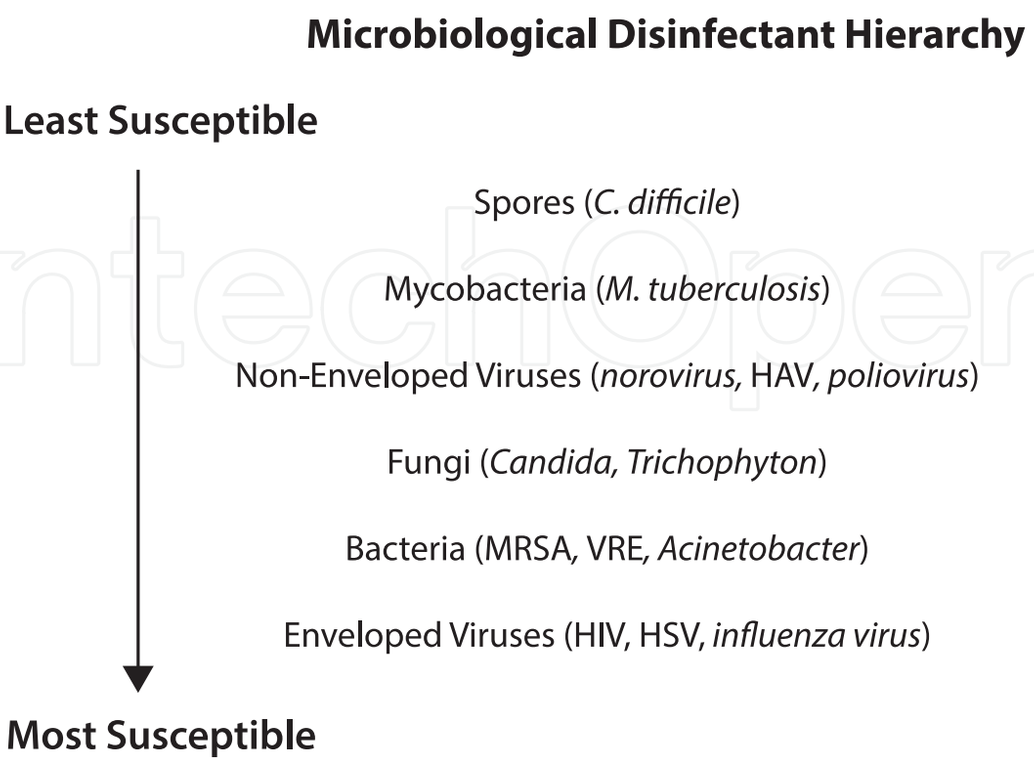

Figure 5.
Microbiological Disinfection Hierarchy*. Described in* chemical disinfection of medical and surgical materials, *EH Spaulding ranked the microbiological hierarchy of disinfectants, listing organisms from least susceptible to most susceptible, according to their vulnerability to disinfectants [4, 5, 45].*

Recognizing a disinfectant's ability to kill less susceptible pathogens as an indicator of broader effectiveness, the EPA offers a variety of specific designations a chemical or system can claim. In 2018, the HHP system was approved for sporicidal classification by the EPA for a 6-$\log_{10}$ reduction of *Clostridioides difficile* (*C. diff*) in a tripartite soil load [46]. The EPA's Emerging Viral Pathogens claim was additionally approved for the HHP system on the basis of this sporicidal data [37]. Granting of this classification may further support the validity that efficacy against bacterial spores will likely conclude efficacy against enveloped and non-enveloped viruses. Targeting a 6-$\log_{10}$ or greater reduction of bacterial spores for validation is a key component of achieving a successful high-level disinfection [47]. Achieving this 6-$\log_{10}$ sporicidal kill will enable confidence against more susceptible organisms, such as enveloped or non-enveloped viruses [5] which may exist in a soil load or biofilm, making them more difficult to inactivate [13, 43].

3.3 Viral efficacy data: norovirus

Norovirus, a single stranded non-enveloped virus of the Caliciviridae family, is a leading cause of acute gastroenteritis in humans. The most common genogroup GII is responsible for 95% of infections, which can have severe and even fatal outcomes in at-risk populations such as young children or the elderly. Norovirus, once present, can become a pervasive problem due to the environmental stability of the virus, low infectious dose, resistance to alcohol and chlorine-based disinfectants, and the potential for prolonged asymptomatic shedding of infected individuals. Norovirus is also used as a target organism for testing, as it is considered to be a non-enveloped virus with relatively low susceptibility to disinfectants [48].

In 2018, a 1,600-bed assisted living facility had a norovirus outbreak affecting 1/4 of the residents within a 2-week period with an average of 40 new cases a day, despite protective measures such as the quarantine of afflicted individuals. A biodecontamination company employing HHP technology was brought into the facility for outbreak response and control. HHP fogging was implemented as part of a 5-point process including continued quarantine and enhanced staff education. After a four-day implementation period, no new cases were reported, effectively ending the outbreak [49].

The HHP system was also tested under GLP conditions for efficacy against the norovirus testing surrogate feline calicivirus [20]. In this testing, 21 inoculated glass agar carrier plates were placed throughout the test room, ranging from floor level to 12 feet (3.6 m) in height, and exposed to the HHP fogging protocols. There was no recovered virus from the challenged plates for an overall reduction of 7.6 $\log_{10}$ (**Table 1**). Interestingly, efficacious results were also noted in GLP compliant testing when a carrier plate lid was accidentally left on during the HHP fogging cycle. This protocol deviation allowed for the observation that, even under these challenging conditions, the HHP fog migrated underneath the lid and achieved inactivation of viral particles [20].

The combination of these two studies demonstrates that the HHP system effectively disinfects complex spaces contaminated with norovirus or its surrogates in both laboratory and real-world conditions. Though the assisted living facility case study did not measure a numerical reduction of viral burden, the effective outbreak control of 100% reduction in new cases leads to the conclusion that norovirus was reduced to levels less than the infectious dose.

3.4 Viral efficacy data: within porous materials

In the spring/summer of 2020, the COVID-19 pandemic triggered a scarcity, and subsequent shortage of personal protective equipment (PPE) used by hospitals and

HHP Efficacy				
Pathogen [reference]	**Characteristics**	**Strain/ Source**	**Carrier Type**	**Results**
Bacillus subtilis [50]	Gram-positive, rod-shaped, endospore formation	19615	Dacron suture loop Porcelain Penicylinders (50% Tyvek/Tyvek)	75 of 77 carriers negative 5.2 $\log_{10}$ reduction (Penicylinder) 6.2 $\log_{10}$ reduction (suture)
Clostridium sporogenes [50]	Gram-positive, rod-shaped, endospore formation	3584	Dacron suture loop Porcelain Penicylinders (50% Tyvek/Tyvek)	73 of 74 carriers negative 6.1 $\log_{10}$ reduction (Penicylinder) 6.3 $\log_{10}$ reduction (suture)
Geobacillus stearothermophilus [41]	Gram-positive, rod-shaped, endospore formation	ATCC 7953	Tyvek/Tyvek stainless steel coupon	206 carriers negative 6.2 $\log_{10}$ reduction
Clostridioides difficile [46]	Gram-positive, rod-shaped, endospore formation	ATCC 43598	Stainless Steel Disk	90 carriers negative 6.6 $\log_{10}$ reduction
***Pseudomonas* phi6 (phi6)** [19]	Enveloped, icosahedral	phi 6	Porous N95 Mask	36 of 37 ≥ 6.0 $\log_{10}$ reduction*
Norovirus [49]	Non-enveloped, icosahedral	Unknown	Wild type	100% reduction of cases
Feline calicivirus (U.S. EPA-approved norovirus surrogate) [20]	Non-enveloped, icosahedral	Strain F-9, ATCC VF-782	Glass Petri Dish	40 of 40 plates ≥7.58 $\log_{10}$ reduction
Herpes simplex virus 1 (HSV-1) [19]	Enveloped, icosahedral	Strain F	Porous N95 Mask	64 of 65 ≥ 5 $\log_{10}$ reduction*
Coxsackievirus B3 (CVB3) [19]	Non-enveloped (naked), icosahedral	Strain B3	Porous N95 Mask	6o of 63 ≥ 4.3 $\log_{10}$ reduction*
SARS-CoV-2 [19]	Enveloped, no icosahedral capsid	Isolate USA-WA1/ 2020	Porous N95 Mask	48 of 48 reduced below LOD

Table 1.
Efficacy*. Summary table of data presented within this chapter demonstrating efficacy of the HHP system against a range of pathogens and substrates. Sporicidal results show inactivated (negative) carriers by log reduction, viral results show either log reduction or limit of detection (LOD) where applicable. * indicates where* $\log_{10}$ *reduction is the starting log titer and the LOD = log titer.*

other healthcare facilities. In an attempt to find ways to mitigate this emergency, researchers at Pennsylvania State University (Penn State) employed HHP to disinfect expired N95 respirators to assess the applicability of the HHP system for this use. Respirators were tested both for any physical degradation effects of the treatment on the respirator material and for efficacy of disinfection of respirator components via inoculation with three viral pathogens and one bacteriophage. Viral work performed at the Eva J Pell Biosafety Level 3 laboratory at Penn State used viruses of different characteristics, as well as a bacteriophage, to represent the range of physical characteristics of pathogens to which healthcare workers may be exposed (**Table 1**) [19]. Three viruses: herpes simplex virus (HSV-1; enveloped

virus; family Herpesviridae), coxsackievirus (CVB3; non-enveloped virus; family Picornaviridae), and SARS-CoV-2 (isolate USA-WA1/2020; enveloped virus; family Coronaviridae), as well as pseudomonas bacteriophage (phi6; enveloped), were chosen for testing (**Figure 6**). The inside, outside, and strap materials of the respirators were used as inoculation sites. While the majority of these surfaces are made up of porous materials, at least one type of respirator had an outer layer of hydrophobic material which caused the inoculation droplet to dry into a 'coffee ring' pattern on the respirator. This testing of porous materials is significant because it presents a more difficult challenge to disinfection than non-porous surfaces, since the materials which absorb the pathogen may also provide a degree of protection, at least temporarily [51]. Disinfectant efficacy testing is commonly done on non-porous surfaces, which does not reflect the difficulty and variables that porous surfaces present.

Testing performed at Penn State also included the use of biological indicators as validation of the protocol for a successful HHP cycle. For each HHP cycle, 6 to 12 biological indicators (*Geobacillus stearothermophilus* ATCC® 7953) with a mean spore count 2.4×10^5 on stainless steel carriers encased in Tyvek®/Glassine pouches were placed throughout the room. In the total of 14 disinfection cycles, only 2 of 138 indicators returned positive for spore growth. These included preliminary cycles, which were intended to establish optimal cycle parameters [19].

3.5 Viral efficacy indicated through bacterial spore validation

The EPA and the Centers for Disease Control and Prevention (CDC) recognize that certain microorganisms can be ranked with respect to their tolerance to chemical disinfectants [7]. As a result, efficacy against less susceptible bacterial spores can be extrapolated to indicate efficacy against more susceptible microorganisms, including enveloped and non-enveloped viruses [4, 5, 52].

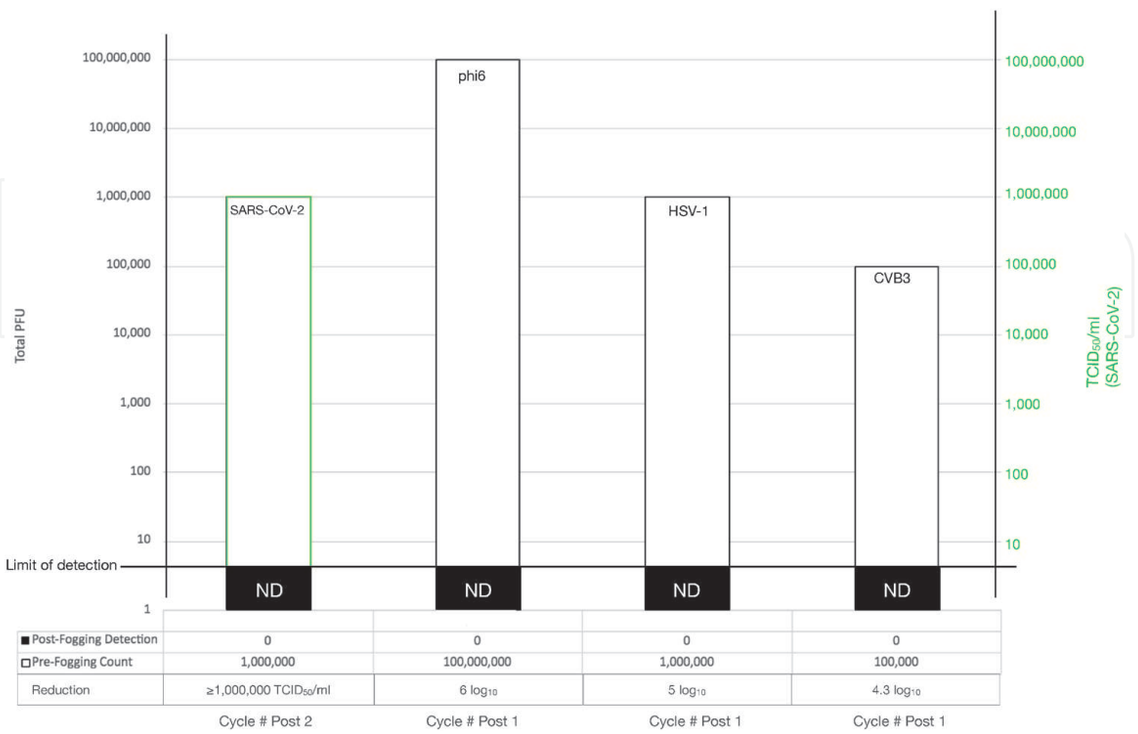

Figure 6.
Viral Reductions Post Hybrid Hydrogen Peroxide (HHP) Fogging. *Data table demonstrating the efficacy of HHP fogging for reducing tested viruses and bacteriophage to below the limit of detection–not detected (ND)–measured as either plaque-forming units (PFU) or median tissue culture infectious dose ($TCID_{50}$) [19].*

3.5.1 Bacterial spore efficacy data: necropsy, laboratories, and interstitial spaces

To assess efficacy within various Biosafety Level 3 Agricultural (BSL-3Ag) environments, Kansas State University challenged the HHP system within their Biosecurity Research Institute, a BSL-3Ag facility. Testing was performed in three laboratories representing a range of sizes: 2,281 ft^3 (65 m^3), 4,668 ft^3 (132 m^3), and 44,212 ft^3 (1,252 m^3). Each of the two smaller laboratories were tested over a series of three disinfection cycles with biological indicators of *Geobacillus stearothermophilus* (6.2 log_{10} spores) encased in Tyvek/Tyvek and placed throughout the laboratories, in laboratory equipment such as biological safety cabinets, and in the overhead interstitial space (drop ceiling). Testing in these laboratories resulted in a greater than 6-log_{10} reduction of all 252 challenged indicators, including those placed in the difficult to access interstitial space.

Within the largest space tested, the 44,212 ft^3 (1,252 m^3) necropsy laboratory, four HHP devices were used for the disinfection cycle. The smart technology of the HHP system automated the connection of multiple Pulse fogging devices for a synchronized, custom-calibrated, HHP cycle. A total of 206 biological indicators were tested over two HHP cycles in locations throughout the laboratory, including at the 21-ft (6.4 m) ceiling height, soft-sided anteroom, walk-in cooler, and change rooms. All 206 challenged indicators were negative for spore growth, demonstrating a greater than 6-log_{10} reduction of *G. stearothermophilus*. This BSL-3Ag testing provides real-life results within the targeted environment for the HHP system. The smart controls and automation allowed this testing to be performed in house by the laboratory personnel [41].

3.5.2 Bacterial spore efficacy data: sterilization study on porous surfaces

The BMBL (6th edition) defines sterilization as; "a physical or chemical process that kills or inactivates all microbial life forms including highly resistant bacterial spores." The importance of sterilization is well understood in life science, pharmaceutical, and healthcare industries. Through the process of sterilization, researchers and physicians alike establish the basis for reliable and safe protocols and procedures. Standards for fogging sterilization testing are developed by the Association of Official Analytical Chemists (AOAC International), a globally recognized, third party not-for-profit, that provides education and facilitates the development of test methods and standards.

The HHP system was challenged with the Fogging Devices Sterilant Test (OCSPP 810.2100) for efficacy against *B. subtilis* (strain 19615) spores, an opportunistic pathogen, which is tolerant of ultraviolet light and high temperatures, and *Clostridium sporogenes* (strain 3584) spores, a strain of *Clostridium botulinum*. These two spores are designated for this test due to their enhanced survivability compared to other spore types. Two carrier formations were used for both spore types, porcelain Penicylinders and Dacron™ suture loops. Each carrier type was saturated with the substrate, distributing spores throughout these materials. Half of each type of carrier was placed inside Tyvek/Tyvek pouches, with the remaining carriers placed in glass petri dishes. Carriers with these bacterial spores were placed throughout the 9′11″ × 14′6″ × 12′9″ (1,833 ft^3 / 51 m^3) testing room. A total of 151 carriers were tested, with only three carriers being found positive for spore growth, all on porcelain Penicyliner carriers enclosed in Tyvek/Tyvek pouches (1 *B. subtilis*, 2 *C. sporogenes*) [50]. This testing method is designed to challenge a fogging system's penetration and subsequent disinfection of spores within these porous carriers. These results demonstrated the HHP system's ability to penetrate through two forms of porous surfaces to inactivate the resistant spores.

3.5.3 Bacterial spore efficacy data: sporicidal study in a tripartite soil load

Clostridioides difficile is a bacterium responsible for causing almost half a million infections in the United States alone each year, with fatal outcomes for 1 in 11 people over the age of 65 within one month of infection [53]. *Clostridioides difficile (C. diff)* is considered one of the most epidemiologically important pathogens, as its environmental persistence, antibiotic resistance, and low infectious dose have led to this bacterium plaguing hospitals and long-term care facilities alike [54]. Precisely due to the hardiness of this bacterium in spore form, *C. diff* has become a standard against which to measure disinfectant efficacy and forms the basis of the EPA's Emerging Viral Pathogen efficacy and approval [8]. With the understanding that pathogens in the environment do not exist in a vacuum, but rather are more likely to be found within a soil load consisting of physiological fluids such as blood, purulent material, or feces, the EPA updated testing requirements for sporicidal classification to challenge not only against hardy *C. diff* spores, but to test such spores within three protective materials (tripartite load; bovine serum albumin, yeast extract, mucin). In 2018, the HHP system was awarded sporicidal classification in the EPA's most stringent *C. diff* test; elimination of *C. diff* spores in a tripartite soil load. A total of 63 carrier plates over three testing lots were exposed to the HHP cycle, resulting in the inactivation of all 63 carriers and an average $\log_{10}$ reduction of 6.6 for this difficult to kill bacterial spore. This testing confirmed the HHP system's ability for high-level disinfection with sporicidal classification [46].

4. Comparison to existing technologies

4.1 Fumigated formaldehyde devices

Formaldehyde is a naturally occurring compound consisting of hydrogen, oxygen, and carbon which is used as a disinfectant in both its liquid and gaseous states [55]. Used as a laboratory fumigant since the late 19th century, formaldehyde has remained in use due to its efficacy and low cost [56, 57]. For use as a disinfectant, formalin, the aqueous form of formaldehyde, is heated into a vapor producing formaldehyde gas [58]. When encountering microbes, this gas causes a cross-linking of molecules leading to protein clumping and loss of structure [59]. While an effective sterilant, formaldehyde must be handled with extreme care as exposure can cause asthma-like respiratory problems, cancer, or even be fatal to humans [55]. In gaseous form, formaldehyde is used at 8,000–10,000 ppm concentration and leaves behind a residue which must be removed through manual cleaning [56, 60]. Due to the potential health hazards and the required labor-intensive clean-up of residue, formaldehyde use is declining in favor of less hazardous and faster solutions. Indeed, the European Union lists formaldehyde as a substance of very high concern and has issued regulation calling for the progressive substitution when suitable alternatives have been identified [61]. While generally compatible with laboratory materials, formaldehyde can be absorbed into porous materials such as HEPA filters, off-gassing slowly and extending the time needed for safe re-entry [56, 62]. Formaldehyde production equipment ranges from as small as an electric fry pan requiring timers or externally controlled circuits to larger automated devices roughly the size of a household refrigerator and weighing approximately 396 pounds (180 kg) [63].

4.2 Chlorine dioxide devices

Chlorine dioxide (ClO_2) is a synthetic, green-colored gas that gives off a bleach-like odor. Despite the familiar scent, chlorine dioxide gas is toxic and must be

carefully contained when employed as a fumigant [64]. Consisting of unstable chlorine (Cl_2) and oxygen molecules (O_2), ClO_2 disassociates when heated into chloride (Cl-), chlorite (ClO-) and chlorate ions (ClO_3-). Some formulations can leave residues of sodium chlorite or inert salts, such as sodium chloride, on surfaces [65]. The disinfection cycle for ClO_2 commonly consists of five steps: pre-conditioning, conditioning, charge (gas injection), exposure (contact time), and aeration [66]. The cycle is humidity-dependent, requiring a dosage increase of approximately 500 ppm for each 10% change in humidity, leading to an operating concentration range of 600–1550 ppm [66]. Similar to formaldehyde, ClO_2 can be absorbed into porous surfaces and thus take longer to aerate than non-porous materials [65]. One consideration for system use is material compatibility with laboratory equipment. Some device manufacturers recommend that the ClO_2-generating equipment remain outside the space being disinfected to prevent repeated exposure [34]. Instable in solution, chlorine dioxide must be mixed on-site by laboratory personnel. The effectiveness of ClO_2 in penetrating treated spaces may also cause concern for personnel safety, as it can migrate out of seemingly enclosed spaces [38, 40]. As a result, facilities employing ClO_2 systems must carefully monitor the disinfection cycle to ensure safety [64]. Roughly the size of an office bookcase and weighing approximately 230 pounds (104 kg), one system can treat up to 70,000 ft^3 (2,000 m^3) which may maximize the treatment space per device compared to other systems. ClO_2 can also be dispensed from smaller devices which fit into a biological safety cabinet to treat that equipment [67, 68].

4.3 High concentration H_2O_2 vapor

High concentration H_2O_2 devices are roughly the size of a medium file cabinet, wheeled around facilities on four castors and can be very heavy, weighing up to 500 pounds (227 kg). They are operated via touchscreen displays and the range of treatment area is between 8,800 to 20,000 ft^3 (249 to 566 m^3), depending on the device. One system can connect up to 10 devices via ethernet cables linking one device to another and enabling the treatment of larger spaces. Validation of these vaporous systems is determined using chemical and biological indicators, often *G. stearothermophilus* (1×10^6) an international standard for determining success in sterilization procedures [44]. These systems may not offer hand-spray or port-in capabilities; however, they can integrate into various chambers or rooms.

High concentration vaporous H_2O_2 systems traditionally employ a 35–59% H_2O_2 liquid solution, heated to a vaporous state [29]. These chemicals must be handled with care, since human contact with the liquid or vapor can be harmful and has been known to result in second- and third-degree burns [29–31]. Once heated, these chemicals are delivered to the treatment space, where vapor concentrations can reach peak levels of up to 1,400 ppm H_2O_2 [36], often necessitating precise operating conditions and continuous monitoring of the treatment cycle by the operator(s). A myriad of sensors precisely measures peak concentrations and these aid in delivering a specific combination of conditions to result in efficacy. These systems can be highly complex, accompanied by user manuals nearing a hundred pages of instructions. The four-part fogging process—dehumidification, conditioning, decontamination, and aeration—may require a technician to be present during the entire cycle of several hours [34, 69]. One reason for this vigilant monitoring may be to respond quickly should the system over or under deliver the high concentrations of H_2O_2 required. Another reason for persistent oversight may be a valid fear of escaped H_2O_2 vapor, which could migrate out of the treated space at high concentrations and affect personnel [38, 40].

4.4 Hydrogen peroxide silver ion devices

Chemical solutions, even within the range of H_2O_2 technologies, differ not only in concentration, but also in their formulation. Some available H_2O_2 solutions contain additional active ingredients, such as the heavy metal silver nitrate [70]. Although silver has a long history of use in wound care, it is also known to cause a permanent retention of silver once in the body [71]. Silver ions are one of the most toxic known forms of heavy metal [70]. Accidental ingestion of these invisible silver residues can cause problems for the microbiome of the human digestive system, since these metals lack the ability to differentiate beneficial bacteria from pathogenic bacteria [72]. Silver persists not only in the body, but also in the environment, where it remains toxic and can be lethal to organisms [70]. As a result of a growing understanding of these unintended negative consequences, the use of silver for disinfection is regulated by the European Union (BPR, Regulation (EU) 528/2012) which states that "It may unnecessarily expose humans, animals and the environment to biocidal active substance, generate health and/or environmental risks and impacts, and may also contribute to the development of resistance to biocides leading to other health and/or environmental issues" [73]. Likewise the EPA acknowledges the potential health hazards related to exposure to silver, and has issued cautionary documents to this effect [74]. Due to the high level of potential exposure during residue cleanup, and the resulting inhalation or dermal absorption of this heavy metal, proper protocols and control should be always employed [74]. Devices for aerosolizing H_2O_2 with silver vary in size from toolbox-sized fixed systems in mobile transportation to large, stand-alone portable systems. Some of these systems spray in a mist, while others use a more wet delivery method which may impede the generation of floating aerosols [75].

5. Key considerations when choosing a disinfection approach

There are several key elements to consider when deciding on a decontamination system. An ideal anti-microbial disinfectant should have the following characteristics: (1) is destructive to the greatest variety of pathogens, including bacterial spores, bacteria, viruses, molds, and fungi; (2) minimizes risks to personnel; (3) is non-corrosive and compatible with materials under normal application conditions; (4) is easy to implement; (5) imparts no harmful residue to the laboratory space or equipment; and (6) provides affordable decontamination. When comparing various disinfection systems, consider the most pertinent aspects below:

5.1 Highest efficacy

First and foremost, it is important for the system to not only be efficacious against more susceptible organisms, but efficacious against less susceptible organisms to the degree necessary to confidently implement the system as a regular component of the research cycle. Commensurate with the definitions of disinfection and decontamination [1], disinfection inactivates pathogens, while decontamination goes to the further degree of inactivating and denaturing them. In industries where pathogen-free environments form the foundational block for successful research, only decontamination will suffice. A detail-conscious manager should not only look for a decontaminant but select one which can demonstrate proof of efficacy with both porous and non-porous surfaces, most accurately representing the array found within life science sectors. Further supporting efficacy, laboratories should be able to validate their chosen system using biological indicators in adherence to international

standards [44]. In support of risk management, the system should enable validation of sterilization through a 6-$\log_{10}$ sporicidal reduction that can be tracked and recorded [2]. With only the most efficacious systems under consideration, facility managers should evaluate each system's impact on personnel safety, ideal laboratory operation, equipment material compatibility, and integrity of research.

5.2 Safety

Even more important than the safety of materials is the safety of personnel, which should be a top priority when implementing a decontamination system. Safety should be considered from the perspective of normal operation as well as in the event of an accidental exposure. Under normal conditions, devices which can be operated remotely create a layer of isolation between the decontamination system and the human operator, allowing for implementation without direct contact for personnel. In the unlikely event of an accidental exposure, higher concentration solutions may come with risks for exposure to high-consequence chemicals either from contact or inhalation [39]. Choosing a product with lower operating concentrations may likewise decrease the potential for risks associated with accidental exposure caused by unintended fog leakage [38, 40]. As with most gaseous systems, the Occupational Safety and Health Administration (OSHA) has defined a minimum reoccupation level, Permissible Exposure Limit (PEL), which must be considered: ClO_2 = 0.1 ppm; H_2O_2 = 1 ppm; and formaldehyde = 0.75 ppm. Technologies employing lower operational ppm may reach reoccupation levels more quickly due to a lower peak threshold [15, 16, 76].

5.3 Consequences of repetitive use

Decontamination within facilities is a recurring need, so both the physical devices as well as the chemicals or solutions used in them should be reviewed for the consequences of regular use. Devices with instructions requiring the operating machinery to remain outside of the room being disinfected may call into question the safety of exposed laboratory equipment within this space [34]. Likewise, systems with operating concentrations that can condense at levels beyond known material compatibility, such as 45% hydrogen peroxide, may also damage laboratory equipment [33].

5.4 Ease of use

Decision makers should critically examine the number of parts necessary for implementing a system. Multiple components may appear to create value but instead may only introduce complication and risks. Hosing laying on the floor add contamination risk in two ways: (1) hoses may impede a complete disinfection of any surfaces they touch and (2) those same hoses may contribute to cross contamination as they are moved throughout the facility. Additionally, a system with many components also comes with many opportunities to misplace or damage a critical element, potentially disrupting scheduled disinfection cycles. Quality and durability of the equipment is paramount as well.

While not strictly required, the degree of support available also contributes to the ease of use of a system. Whether creating new protocols, training personnel, or troubleshooting unique challenges, ensuring there is a commitment from the vendor to provide support can mean the difference between a quick phone call or time spent deciphering a 100-page manual.

5.5 Residues

Besides providing ease of use, the optimal disinfectant will also be free of byproducts which can leave precipitates or residues behind on the treated surfaces, or damage those surfaces [56, 65, 73, 74]. Additives such as metals are often marketed as beneficial catalysts, yet any benefit imparted can be overshadowed by what is left behind. Any disinfection system should benefit the facility by controlling contaminants, rather than introducing them to sensitive laboratory environments. It is essential for the integrity of research that no residual components be left in a space perceived sterile which can interfere with, invalidate, or otherwise impact the scientific work taking place.

5.6 Costs

As cost-cutting measures within laboratory spaces continue to be important, one way to save money is to choose a system that can readily be operated in-house by personnel who feel safe doing so. Outsourcing can be associated with significantly higher costs. Systems that are safer, scalable, trackable, easy to use, and modular can be employed for more than one application, resulting in even more cost savings.

6. Conclusion

When striving to meet strict viral disinfection requirements yet achieve balance with ease of use, timeliness, and safety requirements, facility managers should assess the disinfection needs of individual laboratory environments and the facility as a whole. Ideal disinfection systems should include technologies that have the ability to achieve validated decontamination with the lowest risk to equipment and personnel. We believe that the Hybrid Hydrogen Peroxide system introduced and discussed in detail here merits consideration as a versatile tool for viral disinfection. Pulse technology provides an unexpected efficacy with a 7% H_2O_2 solution equaling the best commercially available high-concentration H_2O_2 systems. The simplicity of one portable device with optional accessories and integration capabilities offers intriguing possibilities for reaching and decontaminating viral pathogens that may be found in the myriad of spaces within laboratory environments. Although conceived with sterilization efficacy in mind, its simplicity of use and safer operation enabled widespread adoption into multiple markets such as education and the military, with applicators ranging from entry level technicians to experienced personnel. As research continues to venture into unknown territories, awareness of potential viral threats has increased as well. Current adoption into the life sciences field is robust and underscores the value which can be added through implementing a targeted yet versatile system for facility decontamination. This chapter provides encouragement that innovations in disinfection technology, such as the HHP system, continue to keep pace with these viral threats with fact-based, science-driven results.

Notes/thanks/other declarations

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