Comparison of Multiple Systems for Laboratory Whole Room Fumigation

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Abstract

Fumigation of high-containment microbiology facilities is an international requirement and in the United Kingdom this process is still commonly undertaken using formaldehyde vaporization. Formaldehyde usage is simple and inexpensive, but concerns exist over its toxicity and carcinogenicity. Alternative fumigants exist, although independent, parallel comparison of these substances is limited. This study determined the level of biocidal efficacy achievable with formaldehyde and compared this with other commonly used fumigants. Three different hydrogen peroxide-based fumigation systems were evaluated (two vapor and one dry-mist methods), along with true gas systems employing ozone and chlorine dioxide. A range of challenge microorganisms was used at different room locations to assess the efficacy, usability, and safety of the fumigation equipment. These microorganisms included Geobacillus stearothermophilus, Clostridium difficile, Mycobacterium fortuitum*, and Vaccinia virus. Only chlorine dioxide and formaldehyde fumigants gave consistently high levels of antimicrobial efficacy across all bacterial challenge tests (typically greater than a 5-log reduction). All systems performed similarly against Vaccinia virus, but variable results were noted for* Geobacillus, C. difficile, *and* M. fortuitum *for the hydrogen peroxide- and ozone-based systems. The study also revealed inconsistencies in system reliability and reproducibility, with all fumigant systems aborting mid-cycle on at least one occasion. In contrast, formaldehyde fumigation was confirmed as extremely reliable, largely because of its simplicity (liquid plus hot plate). All the fumigants tested have UK workplace exposure limits of 2 ppm or less, yet residual fumigant was detected for the formaldehyde and hydrogen peroxide systems following cycle completion, even after room aeration.*

Introduction

A number of characteristics have been proposed for the ideal fumigant, and these deserve consideration when planning gaseous fumigation work (Joslyn, 2001). High on the list of desirable qualities is product safety, with the ideal fumigant being effective in its application but non-toxic to the user. The reality is that most gaseous fumigants are potentially harmful to people and animals and require containment to remain both effective and safe during use. At best, they may constitute a serious respiratory and mucosal irritant; while at worst; they may be highly toxic, even at low exposure levels. Joslyn (2001) describes the ideal fumigant as one that should leave no residues or should be capable of rapid removal to safe levels following fumigation. For a number of fumigant products this remains a challenge, and one that was considered within this study, alongside the important issue of efficacy.

Formaldehyde vapor was of particular interest in view of its historical significance and continued use. The earliest reports of its use as a fumigant date back to the 1880s (Lach, 1990), and it has remained the chemical of choice for laboratory fumigation for decades (Dreyfus, 1914). Formaldehyde is typically delivered by heating formalin (35%-40%) with an appropriate amount of water in a thermostatically controlled unit (Jones, 1995). As such, this study sought to draw a comparison between formaldehyde and other alternative products that might also be used for fumigation within the laboratory setting. The most commonly used of these, or at least the chemical receiving most attention in the literature, is hydrogen peroxide. This is available in vapor and dry-mist forms and has been evaluated in healthcare, laboratory, security, and food sector environments, often using the vaporized form. Hydrogen peroxide vapor can be delivered with different levels of associated humidity, depending on the brand of equipment used (French et al., 2004; Hall et al., 2007; Kahnert et al., 2005; Krause et al., 2001, McDonnell et al., 2002; Rudnick et al., 2009). A more recently developed approach, using a dry mist of hydrogen peroxide, is also available and uses a lower source hydrogen peroxide concentration (typically 5%) with silver cations (Andersen et al., 2006; Bartels et al., 2008; Grare et al., 2008; Shapey et al., 2008). In addition, both chlorine dioxide gas and ozone gas have been used effectively in fumigation applications (Pan et al., 1992; Rastogi et al., 2009; Sy et al., 2005; Wilson et al., 2005). Although other fumigants do exist, the above products were chosen for this study because they have received commercial attention as a result of their reported antimicrobial qualities and, for some, their alleged greater safety compared to formaldehyde. Some of these technologies have benefited from intelligent and strong marketing initiatives by their manufacturers, which have raised their profile above that of some other available systems.

All of these described fumigants have been available for room and vehicular use for a relatively short time, compared with the many decades that formaldehyde

has been in use. Despite its historical use, formaldehyde is now restricted to facilities that can be completely sealed and have some degree of ventilation control in place to minimize the risk of human exposure. This is because formaldehyde is known to be a human sensitizer and carcinogen and can leave undesirable residues if its vapor is poorly delivered or not evacuated from the treated area within a defined period of treatment (Cheney & Collins, 1995; Nelson et al., 1986).

Due to its toxicity, formaldehyde has a workplace exposure limit (WEL) of 2 ppm for both short- and long-term exposure (Health and Safety Executive [UK], 2006 & 2007). Decisions at the European Union (EU) level, under the Biocidal Products Directive (Directive 98/8/EC, 1998), may eventually lead to restrictions on its use, though it is unclear whether this will affect laboratory fumigation. Because formaldehyde remains relatively stable compared with some other available fumigants, its persistence presents both benefits and added risks. Despite the risks, formaldehyde remains an effective and easy-to-use fumigant, and available data suggest it is difficult to match in terms of broad antimicrobial efficacy.

Few independent data exist comparing the efficacy of multiple fumigation systems in the controlled laboratory setting, although some machine comparison work has been undertaken recently with viral challenges (Pottage et al., 2009). The aim of the current study was to investigate the alternatives to formaldehyde now available and to assess their efficacy within a contained microbiological facility, using bacterial and viral challenges. High levels of fumigant delivery were possible because the room could be properly sealed and ventilated. A controlled environment such as this presents an opportunity for fair comparison among the various fumigants, while applying consistent room conditions and microbial challenges. This information was required by Great Britain's Health and Safety Executive (HSE) to inform and advise inspection activity in this area and to enable HSE to provide accurate advice for its duty holders. With this in mind, the related experimental aims of this work were:

- To initially confirm the levels of biocidal efficacy achievable with formaldehyde and to use these data points for comparison testing of other fumigants
- To evaluate alternative methods of fumigation and the systems used to deliver them, using a range of challenge microorganisms located at various room positions
- To assess equipment usability and related safety measures in use for these test fumigation systems when evaluated in the controlled air chamber (CAC) of Great Britain's Health and Safety Laboratory (HSL) and in a Containment Level 3 (CL3) facility with scaled-up conditions
- To report the findings, with appropriate interpretation, to HSE for its use in advice or guidance provisions for end-users of fumigation.

Materials and Methods

Working with Chosen Bacterial Strains and Culture

This work involved preparation of seeded steel discs, using high-titre microbial challenges, which then required their placement, recovery, and cultivation. In view of the amount of sample-handling required and the need to deploy these discs within a chamber environment, HSL followed principles recommended by HSE in The Control of Substances Hazardous to Health (COSHH) Regulations (2002) to control and so minimize any contamination risk to those performing experimental work. There is a primary duty under COSHH to prevent exposure of laboratory staff to biological agents during planned research (HSE-ACDP, 2005), by either avoiding their use or substituting with a safer alternative. For some types of laboratory work, such as diagnostic work, this may not be possible. However, it can be achieved for other types of work, such as planned experimental testing. With this in mind, the following cultures and methods were used for fumigation testing:

Clostridium difficile. NCTC 11209, a widely used reference strain, was used as a surrogate for epidemic *C. difficile*. Cultures of *C. difficile* were grown anaerobically in cooked meat broth (Oxoid Ltd., Cambridge, England) using a shaking incubator at 37°C for 48 hours. Oxoid-cooked meat broth is designed to promote anaerobic growth conditions in a sealed flask without the need for additional anaerobic controls. However, as an additional precaution, all culture flasks were placed in anaerobic jars containing anaerobic gas packs (Anaerogen, Oxoid Ltd.). Liquid culture was agitated to ensure a uniform suspension before decanting 50 ml of the suspension. This was centrifuged at 3,000 rpm for 10 minutes, and the pellet was then resuspended in 5 ml fresh-cooked meat broth to concentrate the bacteria. The combination of using meat-broth culture and drying the seeded residues onto discs effectively induced sporulation. Spore concentrations were confirmed by staining the dried suspensions with malachite green. This process showed between 85% and 90% spore formation for each independent experiment (data not shown). Fifty microliters of *C. difficile* stock typically contained approximately 10^6 to 10^7 cells. This volume was seeded onto sterile stainless steel discs in quadruplet (triplicate replicas for exposure to fumigant, plus a single comparative positive process control for each room location). Seeded discs were dried for 1.5 hours prior to fumigation and then positioned in predetermined chamber locations prior to the start of the test fumigation.

Mycobacterium fortuitum. NCTC 10394, a fast-growing, non-tuberculous *Mycobacterium* species, was used as a safe surrogate of *Mycobacterium tuberculosis*. *M. fortuitum* has the added advantage of growing as quantifiable colonies on standard agar plates, unlike other members of this genus, which require growth on

slopes. *M. fortuitum* was grown aerobically in Middlebrook 7H9 broth containing 10% Middlebrook ADC enrichment broth and 1% Tween and was maintained on Middlebrook 7H10 agar plates (Becton Dickinson, Franklin Lakes, NJ) at 37ºC for 4 days. To prepare a sample for fumigation experiments, 10 ml was removed from a 4-day-old broth culture of *M. fortuitum* and pipetted into a universal tube containing two glass beads. The sample was vortexed for 1 minute to disperse any bacteria and minimize cell clumping, and the resulting suspension was used to seed steel discs in 50-µl aliquots, as for *C. difficile*. The 50-µl aliquots of the *M. fortuitum* stock typically contained approximately 10^6 to 10^7 cells.

Commercially prepared spore discs of *Geobacillus stearothermophilus* 7953, at a concentration of approximately 10^6 spores/disc (ATI Atlas, West Sussex, UK), were used as an additional control and point of comparison. Recovered *G. stearothermophilus* was grown on Tryptone Soya Agar (TSA-Oxoid, Cambridge, England) at 55ºC overnight. This bacterium has been used extensively for the evaluation of disinfection and sterilization methods and was included here as a recognized standard within the industry. The steel disc preparation method was used for this organism, rather than the cellulose strip process, because the steel discs are designed for fumigation assessment.

Virus and Cell Culture

Vaccinia virus was chosen as a surrogate for *Variola virus*, the causative agent of smallpox. The Vaccinia virus (a vaccine strain that was previously adapted for growth on Vero E6 cells from the UK's National Collection of Pathogenic Viruses) was obtained from the European Collection of Cell Cultures (ECACC). The Vero E6 cells were also obtained from ECACC and cultured as directed by the supplier. High-titre stocks of virus were grown on cultured Vero E6 cells as previously described by Cann (1999) with the exception that virus was incubated for 3 days and staining was performed using a second overlay of agarose-containing neutral red (Knipe et al., 2001).

The titre of virus used for seeding stainless steel discs was measured using a standard plaque assay as described by Cann (1999) and Knipe et al. (2001). Six-well plates were seeded with $5x10^6$ Vero E6 cells per well grown in 3 ml of Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 units), and streptomycin (100 µg), GlutaMAX™ (2 mM), nonessential amino acids (0.1 mM), referred to hereafter as complete EMEM. Cells were incubated at 37ºC with 5% CO_2 overnight. The medium was removed from the wells, and the cells were washed with phosphate buffered saline (PBS) followed by infection medium (complete EMEM medium with 2.5% FCS instead of 10%). Dilution series of viral stocks were prepared using infection medium as the diluent. Infection medium was removed from the six-well plates and 1-ml aliquots of the virus samples were transferred to the wells. Plates were gently rotated for 30 seconds to ensure even viral adhesion, then incubated for 1 hour at 37ºC, 5% CO_2. The cells were then washed with PBS before adding 2 ml of overlay medium (1:1 ratio of complete EMEM and pre-warmed 3% LMP agarose, prepared in distilled water). Once the overlay had set (20 minutes at room temperature), plates were incubated at 37ºC, 5% CO_2 for 72 hours. Plates were stained with neutral red using a second agarose overlay of 2 ml as described above, with the addition of 3% neutral red. The numbers of plaque-forming units (pfu)/ml were then calculated by taking an average of the numbers of plaques counted from the highest dilutions showing between 1 and 50 plaques. The average number of plaques identified was then multiplied by the dilution factor. A 50-µl aliquot of a 10^7 (pfu)/ml Vaccinia virus culture was used for final challenge preparations.

Preparation of Samples for Fumigation

Stainless steel discs (surgical grade, 15 mm diameter) were prepared in the workshop at HSL. These were cleaned and pre-sterilized by autoclaving prior to use as a seeding surface. For each test location and for process controls, discs were prepared in a Petri dish in a biological safety cabinet and a 50-µl aliquot of each bacterial or viral culture was pipetted onto the surface of the discs. For the bacterial samples, an aliquot of liquid culture containing approximately 1×10^6 colony forming units (cfu)/ml was prepared on triplicate test discs, with additional (unexposed) controls prepared from the same stock suspension. For the virus samples, an aliquot of working viral stock containing approximately 2×10^5 (pfu)/ml was used. Test and control discs were prepared in the same manner as the bacterial discs. The discs were then left to air-dry for approximately 1.5 hours or until visibly dry.

Simulated, small-volume spills were set up in flat-bottom, multi-well plates. An aliquot of 750 µl of culture (using the same source culture as prepared for dried-down samples) was placed in 1 well of a 6-well plate, covering the bottom of the well. For bacterial samples, triplicate wells were set up, and for viral samples, a single well was set up. A single well of each sample was set up in a separate 6-well plate as a (unexposed) control.

Setup of Fumigation Experiment

Controlled Air Chamber (CAC)

The HSL's CAC had an internal volume of 35 m^3 and was set up as a mock laboratory for this series of fumigation experiments by adding basic laboratory furniture and equipment (Figure 1). Wherever possible, the fumigation machines were placed in one designated position within the chamber. Dried discs were placed on the open bench top, on the floor under a cupboard, and inside a

Figure 1

Typical set-up of exposure chamber to resemble a simple "mock" laboratory—bench top centrifuge, laboratory chair, twin laboratory cupboards, Formica-coated work top, safety vinyl floor section, heated shaker, and porous material (cardboard box) all present.

partially open centrifuge. The simulated spill plate was placed at floor level, under the front edge of a cupboard unit. Initial experiments showed that some fumigants were able to penetrate even double-sealed control samples that were intended to remain unexposed to the fumigant. In view of this, unexposed process controls of dried discs and simulated spills were prepared in an identical fashion to exposed samples, but were then kept in a separate laboratory of comparable temperature and relative humidity (RH), avoiding any exposure to the fumigant. Prior to the start of each experiment, the CAC room conditions were set to a room temperature of 23ºC and RH of 40%, to give a consistent starting point for the different experiments.

Containment Level 3 Laboratory (CL3)

Further fumigation experiments were carried out within HSL's CL3 laboratory that had an internal volume of 105 m^3. In this laboratory, the location of test samples was chosen to be as consistent as possible with those used for earlier CAC experiments; dried discs were placed on the open bench top, in a partially open centrifuge, on the floor under the front edge of a cupboard, and inside a Class I biological safety cabinet (Figure 2). As for the CAC-based experiments, the simulated spill was placed at floor level, under the front edge of a cupboard. Dried control discs and a simulated spill were placed in a separate laboratory of equivalent temperature and RH and, therefore, were not exposed to any fumigant. It was not possible to control the starting temperature or relative humidity in the CL3, and ambient conditions prevailed.

For all fumigation experiments in the CAC or the CL3, the air supply to the room was isolated before commencing fumigation. Following fumigation, the room was vented to remove any residual fumigant. Prior to re-entry, levels of fumigant were checked using a PortaSens II hand-held monitor containing the appropriate calibrated fumigant sensor (GemLog Controls Limited, Pulborough, UK). Personnel did not re-enter the room until the level of fumigant was below the WEL.

Processing Samples Following Fumigation

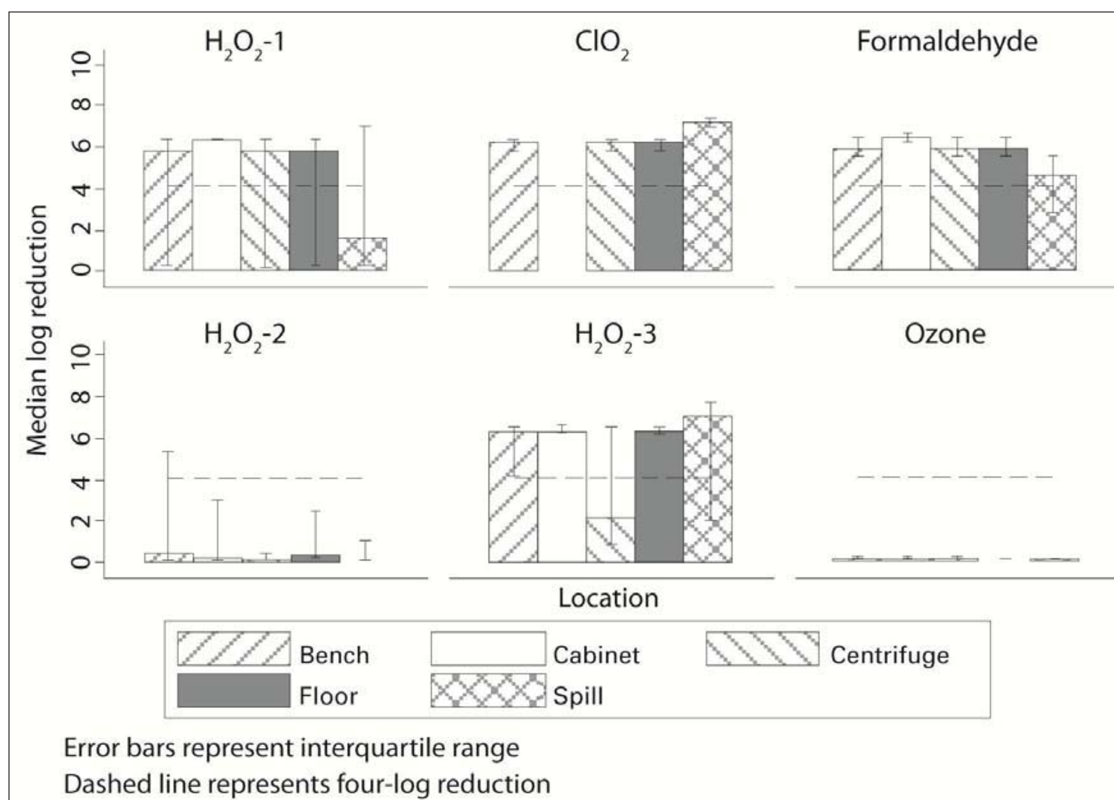
Bacterial Recovery from Steel Discs

Following exposure to the fumigant, each disc was placed in a tube containing two 5-mm sterile glass beads and 10 ml of PBS; the mixture was vigorously agitated for 1 minute by vortexing. The tubes were left at room temperature for 30 minutes and then vortexed again to ensure efficient rehydration and removal of the dried bacterial sample from the disc.

The simulated spills were recovered from the wells and each transferred into a sterile tube. In some cases, particularly following overnight exposure or venting, the liquid in the spill wells had evaporated partially or completely. In these cases, the sample was reconstituted to

Figure 2

Observed median Log reduction by fumigation system and location for *C. difficile*.



NB. The ClO_2 system could not be used inside the CL3 facility so the ClO_2 graph above reflects data from the controlled air chamber only (i.e., no microbiology safety cabinet was in place).

its original 750-µl volume using PBS before transfer to a sterile tube.

The samples were serially diluted, 10-fold, and 100-µl aliquots of each dilution were plated onto duplicate agar plates; mycobacteria were recovered on Middlebrook 7H10 agar plates (Becton Dickinson), clostridia on Columbia blood agar, and *Geobacillus* on TSA. Following appropriate incubation, the plates were counted and the number of bacterial colonies recovered from each tile or spill well was calculated. Exposed samples were compared with unexposed control samples to determine the percentage kill and the $\log_{10}$ reduction.

Viral Samples

Virus Recovery from Steel Discs

Sterile swabs, moistened with sterile PBS, were used to gently wipe the viral load from each steel disc. The swab was immersed in a 2.5-ml cryovial containing 1 ml of cell culture medium and gently rotated to dislodge some of the virus from the swab into the medium. The nib of the swab was cut from the shaft, and the samples were refrigerated overnight to soak, releasing the maximum amount of virus into the medium. The swabs were then removed, taking care to remove as much medium as possible by pressing the swab against the side of the tube. Samples from simulated spill wells were recovered into cryovials. All samples were stored at -80ºC until they could be analyzed by plaque assay, as previously described.

Formaldehyde Fumigation

Formaldehyde fumigation was carried out using aqueous solutions of 37% formaldehyde (formalin) and additional water appropriate to the volume of the room, as described by others (Cheney & Collins, 1995; Lach, 1990). All runs were carried out using a theoretically calculated level of 600 ppm formaldehyde. Within the CAC and CL3 facilities, this equated to using 60 ml and 180 ml of formalin, respectively. The amount of formalin to added water was typically 1:20; this was higher than the 1:9 ratio used by some but was typical of the ratio used in our own facility at the time. This higher water component did not cause room condensation problems in either room facility. The formalin/water mixture was placed in an electrically operated wok, which effectively functioned like a hot plate, and was controlled from outside the room being decontaminated. The solution was

heated until all the liquid had evaporated. This typically required 40 and 60 minutes, respectively, for the CAC and CL3 facilities.

Other Fumigation Devices Evaluated in this Study

Fumigation devices were provided by five different companies for use in this project (see below). Each manufacturer was informed of the nature of the tests and the microorganisms being used in the study, and each advised on the most effective decontamination cycle for its machine, based on the room volumes. At least three fumigation runs for each system were performed in each facility. The exception to this was the chlorine dioxide system, which could not be used within the CL3 without a major door re-design. This additional cost was beyond the scope of the study, so this system was evaluated in the CAC only. If the results from an initial run demonstrated that the fumigation device had been less effective than expected, the supplier was given the opportunity to suggest changes to the cycle program prior to further work. These additional fumigation systems were as follows:

Hydrogen Peroxide—System 1 (H_2O_2-1)

This supplier has developed a machine that attempts to deposit an even layer of “micro-condensation” of hydrogen peroxide vapor over all surfaces. The manufacturer claims that this system generates an even spread of hydrogen peroxide vapor, due to a high-velocity gas distribution system that includes nozzles and fans. The system uses AnalR grade 30% hydrogen peroxide, and the decontamination cycle consists of four phases: conditioning; gassing; dwell; and aeration. Current uses of this technology include the healthcare sector, food manufacturers, and defense and life science laboratories.

The supplied system is not normally available for purchase and is usually operated by trained engineers as a decontamination service. This system consisted of a vapor generator with a gas distribution nozzle and fan, with approximate dimensions of 1.5 m high by 0.5 m wide by 0.5 m deep. The fumigant delivery system is used in conjunction with an aeration unit, an instrumentation module, and a computer module. Only one aeration unit was supplied, but the supplier would normally provide additional linked units, as necessary. For testing purposes here, a decision was made to use the existing room air purge to aid aeration, rather than to add more units to the room, which might possibly hinder experimental procedures. The first supplied machine tested at HSL had a laptop control unit, but this unit was reclaimed by the supplier following initial CAC testing, as the company needed it to fulfil commercial obligations. A second, identical unit was later provided that had new control software. The computer (laptop) was located outside the room being fumigated and was connected to the fumigant delivery system via computer cables.

Generally, an engineer would monitor the performance of the machine throughout the decontamination procedure, but for ease of use, a pre-programmed cycle was employed that had been prepared by the supplier for the type and volume of the rooms being fumigated. Although this supplier has other available biodecontamination units using this technology, this unit was chosen because it is still widely used by the company and by those who have purchased this technology in the past.

Hydrogen Peroxide—System 2 (H_2O_2-2)

This system tested by HSL uses a biocide containing 5% hydrogen peroxide and less than 50 ppm silver cations (supplied as silver nitrate). Fumigant delivery from this system is by “dry mist” technology, where a fine spray is generated from a single nozzle containing electrically charged droplets in the 8 μm to 12 μm range. The supplier claims this size range enables the disinfectant to be dispersed onto all surfaces during treatment. The system is pre-programmed to deliver a fumigation cycle based on the volume of the room. The machine is 1.1 m high by 0.5 m wide by 0.5 m deep. This was the smallest and lightest of all the specialist machines, though its three wheels (one front and two rear) meant that care was required when wheeling the device over distances or uneven surfaces. This technology is used in a number of National Health Service trusts across the UK and has been reported to have some effectiveness against a range of bacteria.

Hydrogen Peroxide—System 3 (H_2O_2-3)

This system maintains the vapor level below its condensation or dew point. The hydrogen peroxide vapor concentration in the room during fumigation is generally 0.1 mg/l to 3 mg/l with this system. This technology has been used in the healthcare sector, pharmaceutical and manufacturing industries, and life science and defense laboratories.

The supplied machine is not typically available for purchase, but a range of similar machines is available commercially. According to the manufacturer’s information, this machine was designed for decontamination of areas up to 85 m^3, though advice from the supplier’s engineer confirmed that it has been successfully used for larger room volumes during research work. The decontamination cycle consists of four phases: dehumidification; conditioning; sterilization; and aeration. Each phase of this cycle is programmed by the user and is dependent on the volume of the room.

The fumigation machine itself is 1 m high by 0.7 m wide by 0.4 m deep, and although heavy, it is highly manoeuvrable on its four wheels. The system uses 35% hydrogen peroxide provided by the supplier. A drying cartridge is required for operation of the fumigation machine, which must be dried on a customized cartridge

regenerator for 6 hours or overnight. Alternatively, single-use-only drying cartridges can be purchased from the supplier.

Ozone—System 4

The ozone delivery system used was designed to promote a reaction between ozone and water to generate biocidal-free radicals. This process, as described by the supplier, involves four stages: phase 1 is Ozone Debt Absorption, in which the latent contaminants in the room are addressed; phase 2 is the Build phase, in which ozone levels are raised by the generator; phase 3 is the Killing phase; and phase 4 is the Decay phase, in which residual ozone is converted to harmless by-products by a quenching agent.

The supplier has developed a number of ozone delivery systems and has only recently begun to market these commercially. This study was supplied with a fully functioning prototype machine, which was approximately 1 m high. It required a small oxygen cylinder and cans of quenching agent (formulation confidential). The system required the operator to input the ozone level and length of time required for the sterilization cycle; this information was optimized by the supplier prior to testing. Systems from this supplier are currently used in the food manufacturing industry, and its ozone technology is used widely in washer disinfectors.

Chlorine Dioxide (ClO_2)—System 5

HSL was supplied with a commercially available chlorine dioxide unit designed for the decontamination of rooms and buildings. The system has a maximum volume delivery capacity of up to 800 m^3. The system requires a large supply gas cylinder, and gas is typically supplied from compressed gas cylinders containing a mixture of 2% chlorine gas in 98% nitrogen (inert carrier). This allows production of ClO_2 at point of use. The ClO_2 is passed in a controlled manner through catalyst cartridges containing a sodium chlorite mixture that results in a contained chemical reaction, producing pure ClO_2 gas. The gas is delivered to the target area via kynar tubing. The decontamination device must be situated outside the treated room. Gas is piped into the room via door or wall apertures that must be sealable. For this reason the equipment could not be tested in the CL3, as the scope of the project did not extend to modification of the CL3 door. A small humidifier and a desk fan were used within the treated area. This machine was the largest of the devices tested at nearly 2 m high by almost 1 m wide. Source gas bottles must be removed during transit.

The ClO_2 decontamination cycle consisted of five phases: Preconditioning, in which the relative humidity is raised and checks are made for leaks; Conditioning, in which the raised relative humidity is held; Charging, in which ClO_2 is generated and delivered; Controlled air, in which the required level of ClO_2 is maintained; and Aeration to finish. The user programs the cycle, which is dependent on the room volume. No aeration unit was supplied with the machine, although one could be fitted into the ventilation ducts of rooms that are frequently treated. The CAC room purge was used for fumigant removal on completion of the experiments. All lights had to be turned off in the room during decontamination, as ClO_2 is broken down in the presence of UV light.

Statistical Analysis

Specific questions were considered at the study outset, and both experimental design and statistical analyses were formulated to address these questions. The key outcome of interest from this study was the log reduction observed for microorganisms exposed to the fumigation process compared with unexposed controls. The data were not normally distributed; therefore, medians plus interquartile ranges were used to describe the data for each organism by fumigation system and location.

Mixed effects linear regression was used to compare fumigation systems and locations for each organism. Organism, fumigation system, and location of sample were entered as fixed/main effects, including all two- and three-way interactions (full three-factorial model). The ClO_2 system was used as the reference fumigation system, and the bench top was used as the reference location. The logarithm of the control concentration was also entered as a main effect to adjust for the different control concentrations observed. The experiment number was entered as a random effect to take into account possible clustering. This controls for factors that could affect all observations from an experiment, such as the settings of the fumigation system or the room in which the experiment was conducted. Jackknife estimation (a method of estimating the standard errors [and therefore confidence limits] that does not rely on the underlying distribution of the data) with clustering on experiment number was used to estimate standard errors and construct 95% confidence intervals. The Wald test was used to test the overall significance of variables (significance, $p \le 0.05$). Testing different components of the interaction terms (using the Wald test) provided a statistical test of interaction between fumigation system and location for each organism—that is, it tested whether the differences in log reduction between locations varied significantly from fumigation system to fumigation system. All analyses were conducted in Stata/SE 10 for Windows.

Results

Formaldehyde Level Used for Fumigations

Initial experiments were completed to assess the efficacy of formaldehyde as a fumigant, using *Geobacillus stearothermophilus* spores only. Experiments in the

HSL CAC facility initially employed a level of 1,400 ppm formaldehyde. This reflected the levels of formaldehyde used in our own CL3 safety procedure, for emergency fumigation, and is similar to the formaldehyde concentration described by others (Lach, 1990). However, following further literature searching, it was decided to reduce the level of formaldehyde delivery as far as possible, while still seeking to achieve a reliable biocidal effect, to provide a fairer comparison with other fumigation technologies. Based on this principle, a level of 600 ppm was found to be the threshold at which any breakthrough (survival) from 10^6 *G. stearothermophilus* spores was prevented (data not shown). This concentration of fumigant was therefore used as the threshold value for subsequent formaldehyde tests; with the caveat that repeated failure to achieve effective killing would result in a review of the amount of formaldehyde used. Subsequent data confirm the effectiveness of this chosen formaldehyde level, and these are presented below.

Comparison of the Efficacy of Different Fumigation Systems

At least three fumigation runs were completed within the CAC with each system. A similar number of runs were completed within the higher room volume of the CL3 laboratory, with the exception of the ClO_2 system, which could not be trialled in the CL3 for reasons previously described. The data presented represents overall data from two room locations, where this was available, or reflect the CAC data only for ClO_2. Because no biological safety cabinet was available in the CAC, cabinet data are not available for the ClO_2 system (Figures 2-4).

Overall Results by Room Location, Challenge Organism, and System Type

Efficacy trends are evident from data plots, allowing a comparison between what might be reasonably expected of any disinfection process, as indicated by a dashed line 4-log reduction, and what was actually achievable. Figures 2, 3, and 4 shows the observed median log reduction by fumigation system and location for *C. difficile*, *M. fortuitum*, and Vaccinia virus, respectively. Clear differences were observed in overall efficacies among fumigation systems, sample locations, and challenge organisms.

Figure 2 shows results against *C. difficile*, with formaldehyde, hydrogen peroxide vapor systems, and ClO_2 all demonstrating spore kill potential of up to 6- to 7-log for most room locations. Formaldehyde and ClO_2 provided the most consistent data against *C. difficile*, both for the dried residues and simulated spill. Exceptions were noted. For the simulated 750-µl spill, hydrogen peroxide delivered by system H_2O_2-1 appeared to be less effective (overall 1.5-log reduction). For the penetrative test of the partly open centrifuge, hydrogen peroxide delivered by system H_2O_2-3 appeared to be less effective than against dry residues (approximately 1.6-log reduction). Another important observation from these data concerns the variation in performances for individual systems among their identical repeat cycles. The interquartile ranges (Table 1) indicate that considerable variation was seen for hydrogen peroxide-based systems, compared with the more consistent results seen for formaldehyde and ClO_2; this is particularly visible in Figure 2, where the error bars actually represent the interquartile ranges. Ozone and dry mist hydrogen peroxide/silver systems were the least

Table 1

Number of observations—median log reduction plus interquartile range (in parentheses)—by fumigation system and organism.

System	Organism			
	Geobacillus[a]	*C. difficile*	*M. fortuitum*	Vaccinia
H_2O_2-1	7 5.69 (5.30 – 5.79)	31 5.73 (0.12 – 6.20)	31 2.28 (0.76 – 4.22)	31 3.05 (2.78 – 4.41)
ClO_2	3 5.04 (4.85 – 5.96)	11 6.12 (5.85 – 6.24)	11 7.35 (6.44 – 7.38)	12 3.97 (3.30 – 4.64)
Formaldehyde	6 2.90 (2.27 – 5.55)	36 5.73 (5.30 – 6.33)	36 6.35 (6.24 – 7.19)	17 2.43 (2.06 – 3.76)
H_2O_2-2	9 2.27 (0.85 – 5.17)	40 0.13 (0.00 – 1.24)	40 0.26 (0.05 – 0.79)	25 2.92 (2.35 – 3.88)
H_2O_2-3	7 5.43 (2.27 – 5.69)	37 6.20 (3.24 – 6.55)	37 2.93 (0.51 – 5.35)	37 2.96 (2.38 – 3.30)
Ozone	7 0.58 (0.52 – 3.03)	30 0.01 (0.00 – 0.07)	31 0.50 (0.08 – 0.71)	26 3.72 (1.37 – 4.18)

Data are number of observations, median values, and lower and upper quartiles in parenthesis.
[a]: *Geobacillus* placed only at one location (the bench).

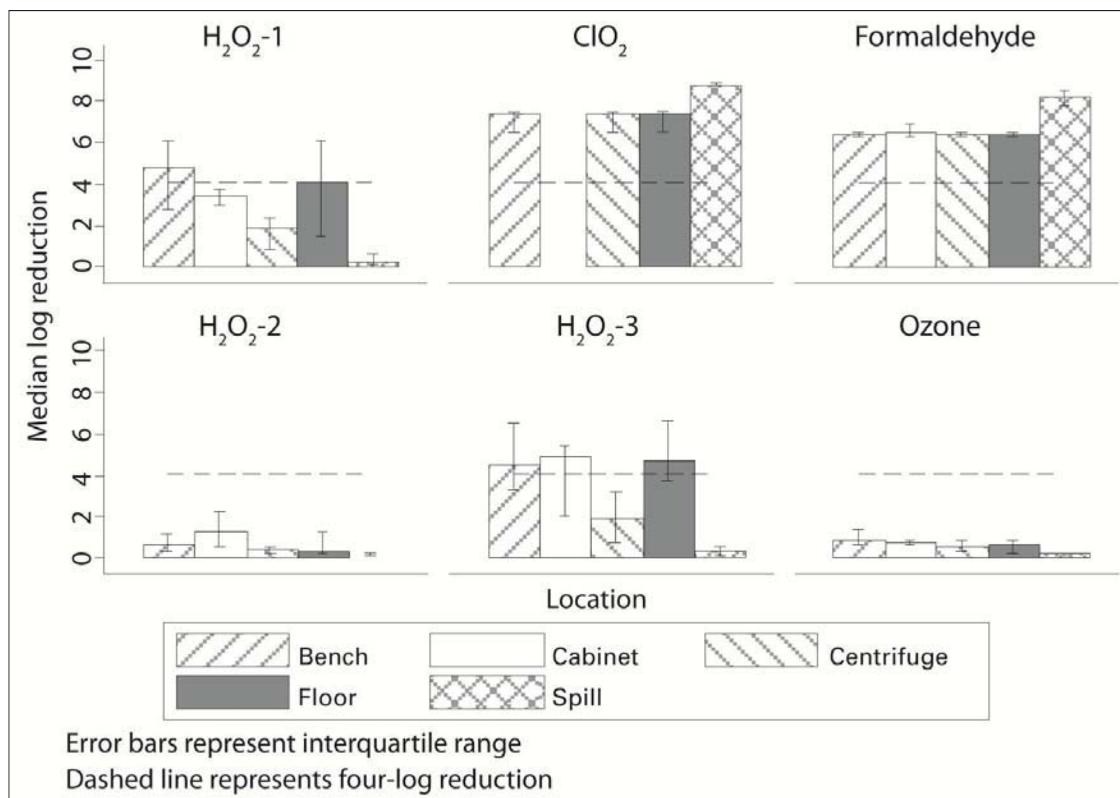
effective for *C. difficile*, with both achieving less than 0.5-log reductions overall when using the standard cycle settings provided by the suppliers (Table 1).

Challenging the systems with *M. fortuitum* again showed that the most consistently efficacious results were for formaldehyde and ClO_2 (6- to 7-log reductions; Figure 3; Table 1). The overall performance of both hydrogen peroxide vapor systems (H_2O_2-1 and H_2O_2-3) was more variable against this bacterium, with reductions typically on the order of 5-log, at best (Table 1). For both these hydrogen peroxide systems, the penetrative centrifuge test resulted in a reduced efficacy of only about 1.7-log. Mycobacterial spill test results were particularly poor for these hydrogen peroxide vapor systems, with each producing less than 0.5-log reduction against *M. fortuitum* in 750 µl of challenge broth. The dry mist hydrogen peroxide/silver system and ozone fumigation again gave a low level of efficacy against this bacterium, with less than 1-log reduction overall, for all room locations (Figure 3). Individual data for the dry mist hydrogen peroxide/silver system indicated potential reductions of up to 2-log, though this was not typical (Table 1).

Observed results for Vaccinia virus challenges are given in Figure 4. Seeding levels in the order of 10^6 pfu of Vaccinia virus were typically dried onto tiles. The air-drying process (approximately 1.5 hours) caused a 2- to 3-log reduction in viable virus in the absence of any fumigant exposure, despite the protective presence of viral infection medium (data not shown). This meant that the remaining viable challenge for each test was reduced to levels of approximately 10^3 to 10^4 pfu, depending on the extent of viability loss. So, although Figure 4 indicates log reductions in the range 10^2 to 10^4 for Vaccinia virus, this actually equated to an elimination of all remaining virus in most cases. While differences in viability loss post-drying were noted among fumigation cycles (a minimum of three cycles were undertaken for each fumigation system and lab setting), viable virus reduction occurring on individual samples within individual experiments was consistent. Replicate samples were used for each location within each experiment, with the exception of simulated spills, which maintained a single sample per experiment. For these low-volume liquid challenges, the viability of virus was retained more effectively, leading to 5- to 6-log reductions. This equated to elimination of all viable virus for most fumigation systems. The exception was the ozone system, which appeared to have the least penetrative ability with the liquid challenge, achieving less than a 2-log reduction against the Vaccinia virus spill (Figure 4).

Figure 3

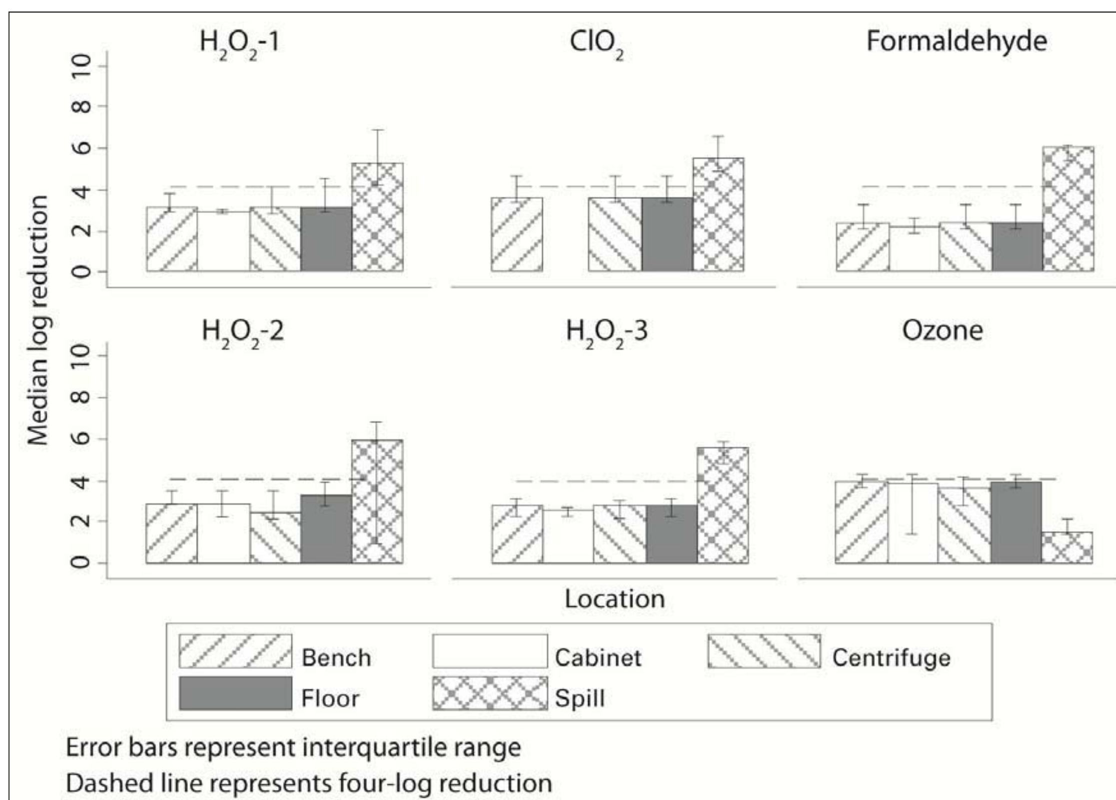
Observed median Log reduction by fumigation system and location for *M. fortuitum*.



NB. The ClO_2 system could not be used inside the CL3 facility so the ClO_2 graph above reflects data from the controlled air chamber only (i.e., no microbiology safety cabinet was in place).

Figure 4

Observed median Log reduction by fumigation system and location for Vaccinia.



NB. The ClO_2 system could not be used inside the CL3 facility so the ClO_2 graph above reflects data from the controlled air chamber only (i.e., no microbiology safety cabinet was in place).

Efficacy Results Related to Individual Challenges and Their Room Locations

A good insight into each system's maximum efficacy can be provided by assessing log reduction for samples located on the open bench (i.e., in one of the most exposed sites within the treated room) and comparing these data to less exposed room locations. Table 2 presents the differences in log reduction through mixed effects linear regression. There were statistically significant differences among fumigation systems for *G. stearothermophilus*, *C. difficile*, and *M. fortuitum* for samples taken at the open bench (all Wald test p-value $<< 0.0001$). For *G. stearothermophilus*, the formaldehyde, H_2O_2-2, and ozone systems all had statistically significantly lower log reductions than the ClO_2 system. For *C. difficile*, the H_2O_2-2 and ozone systems had statistically significantly lower log reductions than the ClO_2 system. For *M. fortuitum*, the H_2O_2-1, H_2O_2-2, H_2O_2-3, and ozone systems all had statistically significantly lower log reductions than the ClO_2 system. There were no statistically significant differences among systems for samples of Vaccinia virus located on the bench, with all achieving similar efficacy (Wald test p-value = 0.262).

Next, differences in log reduction by fumigation system and location were analyzed for each organism. Results for *G. stearothermophilus* are not reported since this was placed only on the bench.

Table 3 shows differences in log reduction for *C. difficile* estimated using mixed effects linear regression. For *C. difficile*, differences in log reduction due to location differed among fumigation systems (overall p-value for this interaction, < 0.0001). There were no statistically significant differences among locations for the H_2O_2-1, ClO_2, and H_2O_2-2 systems (all Wald test p-values > 0.05). However, in the remaining three systems, there were statistically significant differences in log reduction among locations (p-values ≤ 0.05). For the formaldehyde system, a spill had a lower log reduction than the bench; for the H_2O_2-3 system, the centrifuge location gave a lower log reduction than the bench; and for ozone, a spill had a lower log reduction than the bench.

Table 4 shows differences in log reduction for *M. fortuitum* estimated using mixed effects linear regression. For *M. fortuitum*, differences in log reduction due to location differed among fumigation systems (overall p-value for this interaction, p-value < 0.0001). There were

Table 2

Difference in Log reduction by organism and fumigation system for samples taken at the bench, estimated using mixed effects linear regression.

	Geobacillus			***C. difficile***		
	Diff.	**(95% CI)**	**Wald Test**	**Diff.**	**(95% CI)**	**Wald Test**
Fumigation system			p<<0.0001**			p<<0.0001**
ClO_2	(ref)			(ref)		
H_2O_2-1	0.13	(-0.22, 0.48)		-2.31	(-4.68, 0.07)	
Formaldehyde	-2.06	(-3.70, -0.42)*		-0.08	(-0.28, 0.12)	
H_2O_2-2	-2.93	(-4.61, -1.24)**		-3.49	(-5.33, -1.64)**	
H_2O_2-3	-0.85	(-2.25, 0.56)		-0.86	(-2.12, 0.40)	
Ozone	-3.48	(-4.64, -2.33)**		-6.00	(-6.35, -5.64)**	
	M. fortuitum			**Vaccinia**		
	Diff.	**(95% CI)**	**Wald Test**	**Diff.**	**(95% CI)**	**Wald Test**
Fumigation system			p<<0.0001**			p=0.2616
ClO_2	(ref)			(ref)		
H_2O_2-1	-2.47	(-4.05, -0.90)**		-0.38	(-0.81, 0.05)	
Formaldehyde	-0.21	(-0.52, 0.11)		-0.38	(-0.96, 0.20)	
H_2O_2-2	-5.86	(-6.59, -5.13)**		-0.57	(-1.19, 0.05)	
H_2O_2-3	-2.05	(-3.61, -0.48)*		-0.39	(-0.84, 0.07)	
Ozone	-5.32	(-6.17, -4.47)**		-0.05	(-0.53, 0.42)	

Diff. = Estimated difference in Log reduction using mixed effects linear regression adjusted for the control concentration.
ref: reference category
CI: Confidence interval estimated using jackknife standard errors.
* significant at $p \leq 0.05$
** significant at $p \leq 0.01$

no statistically significant differences among locations for the ClO_2 and formaldehyde systems (both Wald test p-values > 0.05). There were statistically significant differences in the log reduction among locations in the remaining systems (p-values ≤ 0.05). For the H_2O_2-1 system, the cabinet, centrifuge, and spills all had lower log reduction than the bench; for both the H_2O_2-2 and H_2O_2-3 systems, the centrifuge and spills had a lower log reduction than the bench; and for the ozone system, the cabinet and spills had a lower log reduction than the bench.

Table 5 shows differences in log reduction for Vaccinia virus estimated using mixed effects linear regression. For Vaccinia virus, differences in log reduction due to location differed among fumigation systems and location (p-value for this interaction, p-value ≤ 0.01). There were no statistically significant differences among locations for the H_2O_2-1, ClO_2, formaldehyde, and H_2O_2-3 systems (all Wald test p-values > 0.05). For the H_2O_2-2 system, the centrifuge had a statistically significantly lower log reduction than the bench (p-value ≤ 0.05), and for the ozone system, spills had a statistically significantly lower log reduction than the bench (p-value ≤ 0.05).

Discussion

This study evaluated available alternatives to formaldehyde for the fumigation of CL3 and CL4 laboratories. The work involved an investigation of system efficacy and usability in a laboratory setting. Other factors also need to be evaluated when considering alternative fumigants for such facilities, including reproducibility and reliability of system performance. The safety of the system, how easy it is to use, its cost, and time taken to fumigate are also important to the end-user and are considered below.

Table 3

Difference in Log reduction by fumigation system and sample location for *C. difficile*, estimated using mixed effects linear regression.

	H_2O_2-1			ClO_2			Formaldehyde		
	Diff.	(95% CI)	Wald Test	Diff.	(95% CI)	Wald Test	Diff.	(95% CI)	Wald Test
Location			p=0.2125			p=0.0615			p=0.0353*
Bench	(ref)			(ref)			(ref)		
Cabinet	1.67	(-0.03, 3.37)		N/A			0.17	(-0.07, 0.42)	
Centrifuge	-0.15	(-0.42, 0.13)		0.00	(-0.00, 0.00)		-0.40	(-1.24, 0.45)	
Floor	-0.05	(-0.14, 0.05)		0.00	(-0.00, 0.00)		0.00	(-0.00, 0.00)	
Spills	-0.91	(-2.15, 0.32)		0.26	(-0.04, 0.56)		-2.21	(-4.20, -0.22)*	
	H_2O_2-2			**H_2O_2-3**			**Ozone**		
	Diff.	(95% CI)	Wald Test	Diff.	(95% CI)	Wald Test	Diff.	(95% CI)	Wald Test
Location			p=0.1170			p=0.0068**			p<<0.0001**
Bench	(ref)			(ref)			(ref)		
Cabinet	-0.45	(-2.40, 1.51)		0.89	(-0.27, 2.04)		-0.12	(-0.56, 0.32)	
Centrifuge	-1.50	(-2.97, -0.02)*		-2.23	(-4.03, -0.43)*		0.02	(-0.01, 0.05)	
Floor	-0.65	(-1.59, 0.28)		0.90	(-0.36, 2.15)		-0.02	(-0.08, 0.03)	
Spills	-1.61	(-3.11, -0.11)*		-0.99	(-3.43, 1.46)		-0.72	(-1.18, -0.25)**	

The p-value for the overall fumigation system by location interaction was < 0.0001. The Wald tests show whether there were any statistically significant differences in Log reduction between locations for each of the fumigation systems.
Diff. = Estimated difference in Log reduction using mixed effects linear regression adjusted for the control concentration.
ref: reference category
CI: Confidence interval estimated using jackknife standard errors.
N/A: not applicable
* significant at p≤0.05
** significant at p≤0.01

Efficacy

A priority when evaluating alternative fumigants to formaldehyde is the efficacy of other available systems, i.e., how effective is a particular fumigant at significantly reducing or eliminating microorganisms? Within the healthcare environment *at least* a 4-log microbial reduction is required before a disinfection process is regarded as effective. However, reduction on that scale may be somewhat less than needed for the laboratory environment, where higher levels of efficacy may be necessary if high titres of pathogens are in use. In this situation, there is a requirement for an effective emergency fumigation procedure to eradicate these high-level contaminants should that become necessary.

Formaldehyde was shown to be an effective fumigant, giving up to 6-log reductions for all organisms tested. This included efficacy for the simulated spill, though it was marginally less effective than ClO_2 when used against *C. difficile*. The 600-ppm level chosen for comparison with alternative systems did not deliver the maximum efficacy achievable for formaldehyde, and some laboratories may be using 1,200 ppm to 1,400 ppm for their whole-room treatments. However, the higher levels of formaldehyde used by some may exceed the necessary dose required and the findings here indicate that many laboratories could almost certainly reduce their overall use of formaldehyde. This lower-level formaldehyde demonstrated efficacy and consistency across the full range of experimental challenges.

The ClO_2 system demonstrated efficacy against all challenge organisms and was therefore used as the reference category for statistical analysis. Its performance

Table 4

Difference in Log reduction by fumigation system and sample location for *M. fortuitum*, estimated using mixed effects linear regression.

	H_2O_2-1			ClO_2			Formaldehyde		
	Diff.	(95% CI)	Wald Test	Diff.	(95% CI)	Wald Test	Diff.	(95% CI)	Wald Test
Location			p<<0.0001**			p=0.0640			p=0.1348
Bench	(ref)			(ref)			(ref)		
Cabinet	-2.01	(-3.21, -0.82)**		N/A			0.06	(-0.13, 0.25)	
Centrifuge	-2.74	(-3.81, -1.67)**		0.00	(-0.00, 0.00)		0.00	(-0.00, 0.00)	
Floor	-0.45	(-1.05, 0.15)		0.00	(-0.00, 0.00)		0.00	(-0.00, 0.00)	
Spills	-4.94	(-6.33, -3.56)**		0.44	(-0.03, 0.91)		0.53	(-0.02, 1.08)*	
	H_2O_2-2			H_2O_2-3			Ozone		
	Diff.	(95% CI)	Wald Test	Diff.	(95% CI)	Wald Test	Diff.	(95% CI)	Wald Test
Location			p<<0.0001**			p<<0.0001**			p<<0.0001**
Bench	(ref)			(ref)			(ref)		
Cabinet	0.90	(-0.10, 1.90)		-0.64	(-2.79, 1.52)		-0.64	(-1.19, -0.09)	
Centrifuge	-0.46	(-0.88, -0.04)*		-2.60	(-3.94, -1.26)**		-0.52	(-1.34, 0.30)	
Floor	-0.01	(-0.35, 0.35)		0.06	(-1.72, 1.84)		-0.48	(-0.98, 0.02)	
Spills	-1.25	(-1.92, -0.58)**		-5.26	(-6.75, -3.76)**		-1.67	(-2.56, -0.79)**	

The p-value for the overall fumigation system by location interaction was < 0.0001. The Wald tests show whether there were any statistically significant differences in Log reduction among locations for each of the fumigation systems.
Diff. = Estimated difference in Log reduction using mixed effects linear regression adjusted for the control concentration.
ref: reference category
CI: Confidence interval estimated using jackknife standard errors.
N/A: not applicable
* significant at $p \leq 0.05$
** significant at $p \leq 0.01$

against *C. difficile* and *M. fortuitum* was especially notable, with 6- to 7-log reductions overall. The ClO_2 method was capable of killing these bacteria in a simulated spill as well as in dry residue form. Although good levels of efficacy were observed for hydrogen peroxide vapor systems, these exhibited greater variation in performance across location and sample type and did not consistently achieve the same efficacy levels as the ClO_2 system.

Overall, formaldehyde, ClO_2, H_2O_2-1, and H_2O_2-3 systems were effective for *C. difficile*, but efficacy observed with the H_2O_2-2 and ozone systems gave significantly lower log reductions. With *M. fortuitum*, H_2O_2-1, H_2O_2-2, H_2O_2-3, and ozone systems provided significantly lower log reductions than ClO_2, with formaldehyde not being statistically significantly different to ClO_2.

For Vaccinia virus, all fumigation systems providing 3- to 4-log reductions in viable virus and no statistically significant differences in efficacy were observed among the different systems. Most viruses begin to lose viability once removed from their host cell culture, and the viral challenges used here (10^7 (pfu)/ml, ~ 10^6 (pfu)/disc) meant that typical levels presented for fumigation treatment were in the order of 10^3-10^4 (pfu)/disc. The observed 3- to 4-log reduction, therefore, represented close to complete eradication of remaining virus.

Some of the differences in efficacy among organisms may be due to the physical properties of the microorganisms, such as the presentation of *C. difficile* and *G. stearothermophilus* as spores, making them more resistant to killing by external agents. The target organism

Table 5

Difference in Log reduction by fumigation system and sample location for Vaccinia, estimated using mixed effects linear regression.

	H_2O_2-1			ClO_2			Formaldehyde		
	Diff.	(95% CI)	Wald Test	Diff.	(95% CI)	Wald Test	Diff.	(95% CI)	Wald Test
Location			p=0.0628			p=0.0592			p=0.1350
Bench	(ref)			(ref)			(ref)		
Cabinet	-0.59	(-1.18, 0.01)		N/A			-0.14	(-0.52, 0.23)	
Centrifuge	-0.04	(-0.33, 0.25)		0.00	(-0.00, 0.00)		0.00	(-0.00, 0.00)	
Floor	0.20	(-0.18, 0.58)		0.00	(-0.00, 0.00)		0.00	(-0.00, 0.00)	
Spills	0.40	(-0.88, 1.69)		0.53	(-0.02, 1.08)		1.03	(-0.08, 2.15)	
	H_2O_2-2			H_2O_2-3			Ozone		
	Diff.	(95% CI)	Wald Test	Diff.	(95% CI)	Wald Test	Diff.	(95% CI)	Wald Test
Location			p=0.0062**			p=0.0508			p=0.0010**
Bench	(ref)			(ref)			(ref)		
Cabinet	-0.01	(-0.66, 0.64)		-0.26	(-0.5, 0.001)		-0.24	(-0.80, 0.33)	
Centrifuge	-0.43	(-0.80, -0.06)*		-0.18	(-0.28, 0.02)		-0.31	(-0.85, 0.24)	
Floor	0.17	(-0.34, 0.68)		-0.11	(-0.22, 0.01)		-0.00	(-0.00, 0.00)	
Spills	-0.68	(-3.45, 2.09)		0.90	(0.11, 1.70)*		-3.55	(-6.44, -0.65)*	

The p-value for the overall fumigation system by location interaction was < 0.01. The Wald tests show whether there were any statistically significant differences in Log reduction among locations for each of the fumigation systems.
Diff. = Estimated difference in Log reduction using mixed effects linear regression adjusted for the control concentration.
ref: reference category
CI: Confidence interval estimated using jackknife standard errors.
N/A: not applicable
* significant at p≤0.05
** significant at p≤0.01

is an important consideration, since differences in efficacy are apparent for the various microorganisms and the systems used to kill them. This finding underlines the need for validation of a particular system within the area to be treated, using the microorganism(s) likely to be handled in that area or using effective surrogate challenges. Similarly, the evaluation of a particular type of fumigation system within the treated area will ensure that the chosen system can effectively deliver the desired treatment under the conditions found there.

There was some observed variation among locations for penetration of the fumigant, with locations such as the cabinet, centrifuge, and spills located under a cupboard often giving lower log reductions than more exposed locations, such as the bench top. These differences were particularly evident for *C. difficile*, where penetration of the centrifuge sample was limited for the H_2O_2-3 system (less than 2-log) when compared to other locations for this same machine (about 6-log). This same system performed well in challenging spill tests with *C. difficile* (6- to 7-log reduction). Conversely, the H_2O_2-1 system performed well against dried *C. difficile* in the centrifuge location and in other locations (6-log typical), but less effectively in the spill test (less than 2-log). These data, observed for a single bacterial species, underline the variability that may be observed for similar fumigants delivered in different ways. Penetrative ability against recesses and liquids, therefore, remains a challenge for fumigation technologies. The partially open centrifuge presented such a challenge and the data indicate that some fumigants penetrated less effectively here when compared to open-bench locations. This is

likely to be due to the physical qualities of the fumigant and its resulting concentration within different parts of the treated room.

These observations underline the need for end-user optimization of the chosen system, based on individual laboratory requirements. These data also showed that antimicrobial efficacy of some systems, including the H_2O_2-1, H_2O_2-2, H_2O_2-3, and ozone systems were reduced for liquid spills compared with dry residues on the open bench. This may have been due to a localized dilution effect at the point of contact and corresponding difficulty in fumigant penetration of the liquid. However, this would require further investigation to confirm.

Reproducibility

For CL3 and CL4 fumigations, there is a need for confidence that the chosen system will give reproducible reductions in viable microorganisms every time it is used. Formaldehyde and ClO_2 gave consistently good results in all experiments in this study. The simplicity of formaldehyde delivery may have contributed to its reproducible efficacy, and it demonstrated the ability to kill microorganisms of all types. The ClO_2 system gave similarly consistent results for all microbial challenges and conditions under which it was used.

Some systems demonstrated less reproducibility and reliability than formaldehyde and ClO_2. In particular, a marked difference was shown between the performances of two machines of the same type (H_2O_2-1); the first machine HSL tested had to be returned at the supplier's request and was replaced soon after by an identical model. The replacement system performed better than the first and it was thought that a software control upgrade might have improved the performance of the second system. With the H_2O_2-2, H_2O_2-3, and ozone systems, more variation was observed among experiments than would be acceptable for routine, laboratory-based fumigation procedures.

Reliability

Formaldehyde fumigation is an inherently reliable approach and uses a simple method of delivery (a hotplate approach). All the other systems tested in this study involved more complex machines and various reliability problems were encountered with each system. All alternative systems aborted on at least one occasion during use. If any of these alternative technologies is to be used in laboratories, they will require improved reliability.

The greatest number of problems was encountered with the H_2O_2-2 system, where narrow-bore tubing delivering fumigant to the nozzle became pinched between control valves after standing unused for longer than a week, limiting further use. Such weaknesses would need to be overcome for laboratories that do not perform regular weekly fumigations.

The H_2O_2-3 system was extremely reliable in tests performed in the CAC. However, in the larger CL3 lab, problems were encountered with the performance of the system's desiccant cartridge. This cartridge contains a silicone-based desiccant that aids moisture removal at the end of the fumigation cycle. This needs to be dried (re-charged) between uses and problems were experienced in completely re-drying the cartridge. This may have related to the larger room size of the CL3 facility, although the supplier indicated that the system could handle the room volumes used for the tests.

With the H_2O_2-1 system, machine problems arose in relation to fumigant delivery and fan rotation, with both faults requiring supplier intervention.

The ozone system aborted on several occasions because the required ozone level in the room was not being reached. A problem was identified with the ozone sensors on the machine and required supplier intervention to rectify.

The ClO_2 machine experienced several aborted runs when it failed to reach its target humidity level within the room. The supplier had to visit HSL on only one occasion to remedy this.

Safety

Formaldehyde is a toxic chemical and has been classified as a Group 1 human carcinogen. For this reason, a choice of alternative, effective fumigation technologies is desirable. However, none of the fumigants evaluated here are harmless, and all have workplace exposure limits (WELs).

Hydrogen Peroxide

Three of the systems tested use hydrogen peroxide-based fumigant, which is an irritant and can be corrosive. Airborne residues of the fumigant must be checked prior to room re-entry to avoid inhalation and eye exposure. The need to open a bottle of concentrated H_2O_2 (25% to 30% $H_2O_{2(aq)}$) did arise during testing and this increased potential exposure to this substance, which can burn skin and damage clothing. Suitable personal protection must therefore be worn. The H_2O_2-2 system used a lower 5% H_2O_2 source from a sealed cartridge; these were sometimes difficult to install, although no spills occurred. Unwanted condensation of H_2O_2 vapor (e.g., below cold windows or walls) poses a risk of damaging the fabric of the room and equipment upon repeated fumigations. However, serious condensation effects with H_2O_2 were not observed during or after testing in the current study.

Ozone

The ozone system used an oxygen cylinder (industrial grade O_2) to generate ozone, and this required supplier training to install and change, as well as an appropriate location to store spare bottles. This sys-

tem also used an aerosol quenching agent, which is generated at the end of ozone treatments and reacts with the ozone to assist its removal. This process generates by-products, including acetic acid residue, which have an obvious odor. The supplier reported that these residues are non-toxic, as demonstrated by independent testing. Odor levels were reduced during testing by improved quencher control, though some level of aeration was required after ozone quenching to assist in odor removal prior to room re-occupation.

Chlorine Dioxide

The ClO_2 system requires a supply of stabilized chlorine gas, provided by twin gas cylinders containing 2% chlorine gas in nitrogen under pressure (2,000 PSIG); gas data provided by supplier. As chlorine is a strong oxidizing agent and irritating to skin and mucosal membranes, a safety procedure, including personal protective equipment (PPE) or some type of gas-containment system, must be in place prior to use. Like other powerful oxidizing agents, ClO_2 may cause progressive damage to room surfaces and equipment that is not impervious to fumigation. The above information is well described within the training provided with the ClO_2 system.

During tests in the CL3 laboratory, all machines had to be operated from within the room, since we could not allow cables or pipes to extrude from the room via door seals. This would have compromised the ability to seal the CL3 laboratory. Unfortunately, the ClO_2 system supplier could not sanction the use of its machine within the CL3, so ClO_2 test data are not available for this location. Technical solutions allow other suppliers to provide control of their systems from outside the treated area, while their machines remain within the treated room. These include delays in start function on the machine or wireless operation of the system from outside the room. Although such systems allow for easier remote control and may provide useful information via remote screens during fumigation, they remain dependent on the ability to fully expose the machine to its own fumigant. This was not a preferred option for the ClO_2 system at the time of testing and was the only real limitation to this machine's use.

Fumigant Monitoring Prior to Room Re-entry

This study revealed, without exception, that levels of fumigant were above the WEL at the end of fumigation cycles and required further air purging prior to room re-entry. This would not typically pose a risk of exposure in a containment laboratory, as it is always possible to use the room air-handling system to clear fumigant, but it is something users should be aware of, particularly those planning to use such systems in other settings (e.g., healthcare). A recommendation is that all systems be sold with a device for monitoring fumigant levels at the end of a cycle. Examples of portable monitors include the Portasens II (GemLog Controls Limited) and Dräeger hand-held systems.

Ease of Use

The vaporization of formaldehyde is a simple process. All the other systems involve more complex machines that require initial training to use safely and effectively. During this study, some of the machines required re-programming, and the H_2O_2-2 machine was the easiest to use, despite the increased complexity when modified cycles were used. The H_2O_2-3 system was the most difficult to program and its operation found to be non-intuitive. The ozone and ClO_2 machines had clear touch-screens, taking the user logically through the programming procedure. Although the H_2O_2-1 machine has multiple units to connect, it would typically be operated by trained operators from the supplier, and the company markets other systems that may be easier to operate but were not evaluated here.

Door modification would be essential for the ClO_2 unit, though it might not be desirable to have such a large machine located immediately outside a CL3 main door (e.g., in a lobby area). Some of the other machines are not normally operated from within the room being fumigated but were able to be operated effectively once installed there by the supplier. For example, the ozone machine is typically positioned outside the room, mainly to preserve the integrity of its ozone sensors, with ozone being piped into the room. Similarly, the H_2O_2-1 machine normally has a computer outside the room (connected via PC cables) for its operation, although the machine itself can reside within the room. For some of these mobile machines, the manufacturers need to consider their use in sealed rooms and to adapt them accordingly to make their use as straight-forward as possible within these higher-risk areas. In addition, the authors did have some problems with a machine setting off a particulate sensor fire alarm (dry mist system H_2O_2-2). This may be a problem for other users who switch from formaldehyde to alternative fumigants, since this problem was not associated with formaldehyde vapor alone.

For formaldehyde fumigation, a hot plate is in place for use in an emergency. Each of the alternative technologies involves a large machine, which would need to be stored between uses and maneuver into place for fumigation. This could be difficult in an emergency situation. In the case of newly built laboratories, some of these new technologies could be integrated within the fabric of the room, which should make treatment delivery easier. Alternatively, mobile machines could be located within facilities such as CL3 laboratories, but they would need to have the system operated remotely from outside the laboratory area. Such remote operation should, for safety reasons, include the ability to cancel and restart cycles in the event of machine failure.

Cost

While cost was not a primary consideration for this study, it is likely to influence user choice of alternative fumigation technologies. Formaldehyde remains a cost-effective method for fumigation, involving only the cost of purchasing formalin and a hot plate (probably less than £100 [$160 USD] for up to 1 year's treatment, including the cost of a simple hot plate [e.g., a wok]). All the alternative systems tested involve a large initial outlay of thousands of pounds (£15K to £50K [$25,000-$80,000 USD] at the time of testing) to purchase the equipment, as well as servicing and repair costs. The consumables required for these systems vary in price but tend to be higher where there is the requirement for a custom-made product (e.g., the need to purchase dedicated cartridges to operate the H_2O_2-2 system, or the recommended H_2O_2-3 active product sold for that system). In other cases, the consumables are relatively inexpensive; for example, the H_2O_2-1 supplier recommended the purchase of good quality, 30% hydrogen peroxide from any chemical supplier. Industrial oxygen supplies for the ozone system were approximately £16 ($16 USD) per 10 kg bottle, and this was generally enough for three fumigation cycles of the CAC facility, though only one complete cycle could be reliably completed for the larger-volume CL3. Piped oxygen, if available, would probably be less expensive but also less versatile if the system needed to be used elsewhere.

Length of Time to Fumigate

Formaldehyde fumigation takes at least 6 hours with formalin/water first heated and vaporized, left to dwell in the room for a minimum of 4 hours, and then removed by a process of room venting. This is routinely done overnight in most laboratories for convenience, with final purging of the room using mechanical ventilation. All of the alternative technologies evaluated here are considerably faster for fumigation and often also have a defined fumigant removal step, although this was frequently found to be less effective than stated by the supplier. All CL3 and CL4 rooms have a ventilation facility for purging the air, and by using this to speed up removal of the fumigant, most systems allow re-entry to the room within 3 to 4 hours after the start of fumigation. The duration of room treatment may be an important consideration for laboratories that carry out regular (e.g., weekly) fumigations, but less of a priority for laboratories that carry out whole room fumigations only in emergency situations or prior to major maintenance work.

Geobacillus stearothermophilus as a Fumigation Test Organism

Geobacillus stearothermophilus is often used as a test organism to validate fumigation, and this role has evolved historically from its extensive use for the testing of steam sterilizers. In this study, variable levels of log reduction were observed for different microorganisms, and some were more difficult to eliminate than *G. stearothermophilus,* despite its use here in spore form. This underlines the need for system validation within the room to be fumigated, using **relevant** challenge organisms appropriate for that work area. Based on the findings here, it is concluded that a fumigation system can only be used with confidence when its efficacy is confirmed in this way.

Final Recommendations

This study has highlighted that differences in performance exist among the various fumigation systems, and that efficacy against different microorganisms can vary for one machine. The following recommendations are made from this study:

- Validation is important. Supportive data should be requested on the efficacy of the fumigation system, with particular attention to the type of microbial challenges likely to be faced by the end-user. Ideally, individual laboratories need to find appropriate surrogates (test challenges) if considering replacing formaldehyde with an alternative fumigation system, since not all fumigants are as widely effective as formaldehyde;
- When considering major equipment purchases, a full working demonstration of the chosen system prior to purchase or hire is recommended. Certain systems will be better suited to some room environments than others;
- Consider the logistics and ease-of-use of fumigation equipment as well as outright efficacy. Look at more than one available system, if possible, to allow comparison.
- Equipment purchases should not be made in isolation; include the views of appropriate partners (e.g., scientific staff, occupational health and safety advisors, risk management staff).
- When considering the cost of new fumigation equipment, ask for information on service provision as well as outright purchase (if options exist). Ensure that consumable costs will be acceptable as a long-term commitment.
- Health and safety are of paramount importance when using fumigation equipment, and comprehensive information and advice should be available prior to delivery and use of the system. This should include:
    - Effective, onsite training if a system is to be operated by the purchaser's own staff and not by the supplier
    - Handling of chemicals in line with Control of Substances Hazardous to Health regulations for transportation, handling, and storage
    - A risk assessment, identifying who might be at risk from fumigant exposure and how any risk can be mitigated
    - Checks for residual levels of fumigant after use of the machine, usually performed with an appropriate hand-held monitoring device

Finally, manufacturers of all the machines tested in this study need to address the issue of poor reliability. This is especially important for emergency fumigations,

where reliability would be paramount. It would also be helpful if manufacturers investigated the increased use of remote control of their systems for use within sealed rooms.

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- *Autoclave Safety: The Proper Use of an Autoclave to Decontaminate Biohazardous Waste* is available on You Tube. This is a Laboratory Safety Project produced by The National Biosafety and Biocontainment Training Program, The National Institutes of Health, and Dartmouth College. Versions are available in English, German, Dutch, and Arabic.