Chemical Engineering Communications

ISSN: 0098-6445 (Print) 1563-5201 (Online) Journal homepage: https://www.tandfonline.com/loi/gcec20

An Investigation into the Inactivation Kinetics of Hydrogen Peroxide Vapor Against *Clostridium difficile* Endospores

D. J. Malik, C. M. Shaw, G. Shama, M. R. J. Clokie & C. D. Rielly

To cite this article: D. J. Malik, C. M. Shaw, G. Shama, M. R. J. Clokie & C. D. Rielly (2016) An Investigation into the Inactivation Kinetics of Hydrogen Peroxide Vapor Against *Clostridium difficile* Endospores, Chemical Engineering Communications, 203:12, 1615-1624, DOI: 10.1080/00986445.2016.1223058

To link to this article: https://doi.org/10.1080/00986445.2016.1223058

Published online: 27 Oct 2016.

Chemical Engineering Communications, 203:1615–1624, 2016

ISSN: 0098-6445 print/1563-5201 online
DOI: 10.1080/00986445.2016.1223058

Taylor & Francis
Taylor & Francis Group

An Investigation into the Inactivation Kinetics of Hydrogen Peroxide Vapor Against *Clostridium difficile* Endospores

D. J. MALIK[1], C. M. SHAW[1], G. SHAMA[1], M. R. J. CLOKIE[2], and C. D. RIELLY[1]

[1]*Department of Chemical Engineering, Loughborough University, Loughborough, Leicester, LE11 3TU, UK*
[2]*Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, LE1 9HN, UK*

C. difficile spores are resistant to routine cleaning agents and are able to survive on inanimate surfaces for long periods of time. There is increasing evidence of the importance of the clinical environment as a reservoir for pathogenic agents and as a potential source of healthcare-associated infections (HCAIs). In this context, to reduce the risk of cross-transmission, terminal disinfection of hospital wards and isolation rooms using hydrogen peroxide vapor (HPV) is attracting attention. Spores of *C. difficile* (ribotype 027) were exposed to constant concentrations of HPV ranging between 11 and 92 mg m^{-3} (ppm) for a range of exposure times in a specially designed chamber. The inactivation data thus obtained was fitted using the modified Chick–Watson inactivation model to obtain decimal reduction values (*D* values). *D* values ranged from 23 to 1.3 min at HPV concentrations of 11 and 92 ppm, respectively. We present a simple mathematical model based on the inactivation kinetic data obtained here to estimate the efficacy of commercial HPV processes used in healthcare environmental decontamination. *C. difficile* spores showed linear inactivation kinetics at steady HPV concentrations ranging between 10 and 90 ppm. The data obtained here was used to provide estimates of the inactivation efficacy of commercial HPV process cycles, which employ unsteady HPV concentrations during the decontamination process.

Keywords: *Clostridium difficile*; Decimal reduction values; Decontamination; Healthcare-associated infections; Hydrogen peroxide vapor; Inactivation kinetics

Introduction

Clostridium difficile is a Gram-positive, spore-forming obligately anaerobic bacterium. The consequences of colonization by *C. difficile* can vary in severity from asymptomatic carriage, diarrhea, pseudomembranous colitis to death (Kuijper et al., 2006). *C. difficile* remains the principal cause of nosocomial diarrhea in the developed world, and its impact has been considerable. In the United States, for example, it has been estimated that there are approximately 500,000 cases of *C. difficile*-associated disease (CDAD) per year, claiming some 20,000 lives (Rupnik et al., 2009). A conservative estimate of the cost to the US healthcare system for CDAD management has been put at $3.2 billion per year (O'Brien et al., 2007).

Spores of *C. difficile* are able to survive in the environment for as long as 5 months, and possibly longer (Kim et al., 1981), and remain highly infective. This is undoubtedly an important factor in the apparent ease with which this organism can be transmitted. He et al. (2012) recently analyzed the global spread of a fluoroquinolone-resistant variant of *C. difficile*, and concluded that the ease and rapidity with which this variant was transmitted internationally highlighted what they referred to as the 'interconnectedness of the global healthcare system.'

Use of sporicides, e.g., hypochlorite for environmental cleaning and disinfection, has been recommended and shown to be effective at inactivating *C. difficile* spores *in vitro.* Mayfield et al. (2000) presented evidence of a significant decline in CDAD in bone marrow transplant patients from 8.6 cases to 3.3 cases per 1000 patient-days in a before-and-after intervention study using hypochlorite disinfectant solution in an area of high endemicity in an acute care hospital. However, the need for long exposure times and failure to maintain sufficiently high concentrations of biocide have been cited as the cause of ineffective environmental decontamination that is prevalent in hospital wards (Maillard, 2011). In addition to this, the inappropriate use of disinfectants may also lead to an increase in sporulation (Fawley et al., 2007).

Gaseous disinfectants possess inherent advantages over liquid biocides in terms of ease of dispersal for large-scale

Address correspondence to D. J. Malik, Department of Chemical Engineering, Loughborough University, Loughborough, Leics. LE11 3TU, UK. E-mail: d.j.malik@lboro.ac.uk

decontamination, e.g., in isolation rooms and multi-bed hospital wards. In this regard, there has been growing interest in the use of HPV as a so-called 'terminal decontamination' procedure (Passaretti et al., 2013). HPV treatment of hospital wards and isolation rooms necessitates the evacuation of patients and sealing off the area to be decontaminated. The HPV is generated by specialized equipment that is typically fully automated and releases a predetermined quantity of hydrogen peroxide into the atmosphere. There are a number of proprietary HPV systems currently on the market differing in the manner by which they generate HPV, in the concentration of the vapor produced and the way it is distributed in the space to be treated. Some systems use forced convection following release of the vapor/spray, which assists in the distribution of the vapor within the room, whereas others do not, relying solely on diffusion. There appears to be general agreement that the HPV decontamination method is effective, but criticism has been directed at the lengthy treatment cycle times (Vonberg et al., 2008; Davies et al., 2011; Maillard, 2011; Doan et al., 2012). Not only must the HPV be released and be allowed to act, but following the disinfection phase its concentration must fall to a safe level (typically 1 ppm) before healthcare personnel and patients can be readmitted.

The basis upon which release profiles and treatment times using HPV are determined by equipment manufacturers is far from clear. The intended targets of such decontamination procedures are the commonest causative agents of nosocomial infection outbreaks, namely *C. difficile*, norovirus, *S. aureus*, etc. However, for convenience estimation of cycle times is made by equipment manufacturers with reference to commercially available spore bioindicator (BI) strips (Otter and Yezli, 2012). These comprise either stamped metal discs or papers onto which spore suspensions of either *Bacillus subtilis*, or more commonly *Geobacillus stearothermophilus*, have been deposited. A number of studies on the efficacy of HPV generators have been conducted using commercial BIs, e.g., Fu et al. (2012) and Holmdahl et al. (2011). Moreover, published studies routinely fail to provide details of the spatial and temporal HPV concentrations achieved during decontamination. To cite one example, Barbut et al. (2012) recently reported on the activity of HPV against *C. difficile* spores using a commercial HPV system (Bioquell Clarus^{TM}) without any indication of the aerial HPV concentrations achieved and how these both varied over time as well as spatially within the single occupancy room (33–45 m^3 size). A full concentration-time record would be required to predict using validated mathematical models the extent of disinfection achieved at a particular location. This would need to be corroborated with associated microbiological sampling using suitable BIs positioned at the sampling points. Hard-to-reach surfaces, e.g., behind radiator grills, may not experience elevated concentrations of HPV, which may be achieved elsewhere during decontamination.

In order to enable decontamination processes to be founded on a rational basis, it is necessary to have inactivation data for specific microorganisms of interest obtained under controlled conditions. The most useful starting point for rigorously designing decontamination processes is to have access to survival curves obtained at constant sterilant concentrations from which corresponding decimal reduction times (D values) can be derived. In the work presented here, spores of *C. difficile* were deposited onto filter membranes and then placed in a specially constructed chamber where they were exposed to known steady HPV concentrations and spore survival curves were determined. The data obtained were fitted using a modified Chick–Watson inactivation model (Watson, 1908) from which the D values were obtained. Furthermore, a modeling approach based on the measured inactivation kinetics was employed to enable assessment of the decontamination efficacy of commercial HPV systems used in healthcare, where the concentration of HPV varies both spatially and temporally.

Materials and Methods

Hydrogen Peroxide Exposure Chamber

The environmental chamber comprised a hydrogen peroxide vapor (HPV) generation unit, an exhaust unit, and three exposure boxes connected in series (Figure 1). HPV was generated by metering hydrogen peroxide solution of the required strength on to a heated stage maintained at 130°C. The HPV was well-mixed with air and transported though the exposure boxes by means of a fan. Each of these boxes contained a sample support rack onto which membranes were placed at a height of 50 mm from the base of the box. The lids of the boxes were in position during operation, but could be rapidly removed by undoing the wing nuts by which they were secured, without affecting the samples in the two other boxes. The air–hydrogen peroxide mixture exiting the last of the chambers flowed into the exhaust unit, which housed a hydrogen peroxide sensor

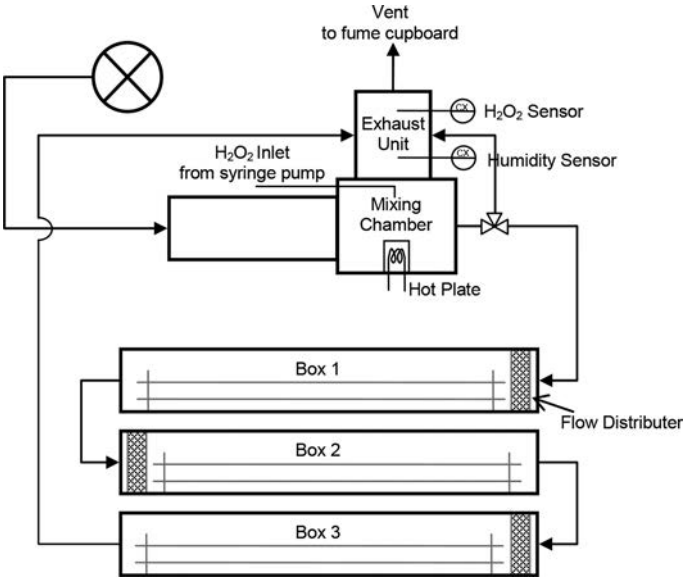

Fig. 1. Schematic diagram of the hydrogen peroxide exposure chamber used to investigate *C. difficile* inactivation kinetics.

and a combined humidity and temperature logger. By having three boxes in series, experiments at steady-state operation for different exposure times could be facilitated, e.g., by opening the 3rd box in series at the earliest exposure time point, followed by the 2nd box at the intermediate time point, and the 1st box at the latest time point.

The establishment of steady-state conditions within the environmental chamber took approximately 2 h. BIs were placed in the boxes only after steady state had been reached; the process of opening a box and placing the membranes in position did not cause undue perturbations of the hydrogen peroxide concentration in the exposure chamber, which returned to the desired steady-state value within minutes following closure, as revealed by the sensor.

Calibration of the Hydrogen Peroxide Sensor

Hydrogen peroxide electrochemical sensors (ATI GasSens A11/34 hydrogen peroxide sensor/transmitter, from ATI Limited, UK) were calibrated using a batch calibration bath (Figure 2). A known concentration of hydrogen peroxide was equilibrated with its vapor at a constant temperature of 25°C in a 1 L vessel containing 0.5 L of hydrogen peroxide solution. The liquid and vapor phases were agitated (at 1000 rpm) using a motor-driven stirrer. The calibration bath was allowed to equilibrate for at least 2 h. After this time the hydrogen peroxide sensor head was inserted into the head space. The sensor output was recorded over a period of 1 h using an EasyLog USB data logger (Lascar, UK). The concentration of the equilibrated liquid hydrogen peroxide was titrated against potassium permanganate using the method of Jeffery and Vogel (1989). Published vapor–liquid equilibrium data (Schumb et al., 1955) were used to calculate the equilibrium vapor concentration. If the read-out from the sensor was within 10% of the reading, the sensor was taken as calibrated and suitable for use. Otherwise, the span on the transmitter was adjusted until the sensor output matched the expected value. Any adjustment was followed by a repeat procedure (as outlined above) to check the sensor readout was within the desired range of accuracy (10% of the nominal value). All sensors were calibrated prior to usage and the calibration checked every week. Sensors were calibrated typically at 15 ppm and checked at 90 ppm to ensure sensor response was within the calibration range of usage (the recommended range of usage is around 10–100 ppm).

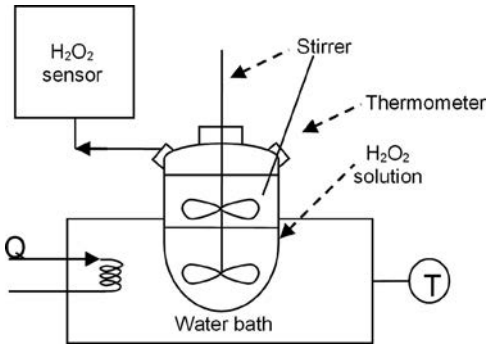

Fig. 2. Schematic diagram of the experimental hydrogen peroxide electrochemical sensor calibration arrangement.

Deposition of C. Difficile *Spores on Membranes*

A high-titer spore stock (10^{10} spores/mL) of *C. difficile* (isolated from a clinical fecal sample and PCR ribotyped as 027 using the method reported by Shan et al. (2012)) was prepared as described by Perez et al. (2011). The spore stock suspension was diluted in phosphate buffer saline (PBS, Oxoid Basingstoke, Hants, UK) to obtain the required spore concentrations to yield membrane BIs with spore surface loadings ranging from 10^2 to 10^7 per membrane. Spore suspension (1 mL) of the required dilution contained within a sterile hypodermic syringe was filtered through an Isopore™ (Millipore Ltd., Watford, UK) membrane filter of 13 mm diameter and of 0.22 µm pore size mounted in a membrane filter holder, the whole assembly having been previously sterilized by autoclaving. Following this, the filter holder was subjected to gentle vacuum filtration at 0.5 barg in order to remove any liquid adhering to the membrane. Membranes laden with uniformly deposited *C. difficile* spores were prepared immediately before experiments and were never stored.

Spore Recovery and Estimation of Spore Survival and Calculation of Decimal Reduction Times

Following exposure to HPV, the membranes were transferred to sterile Universal bottles containing 2 mL of reduced brain heart infusion broth (Oxoid) containing 0.2 mg bovine liver catalase (2000–5000 units/mg, Sigma Chemical Co., UK). Catalase was added in order to arrest the action of any hydrogen peroxide that may have adsorbed on to the membranes; Johnston et al. (2005) employed a similar approach. Membranes were incubated anaerobically at 37°C for 48 h in an anaerobic chamber (mini MACS, Don Whitley Scientific Ltd., UK) before assessing whether growth had occurred based on the observation of turbidity of the media. Exposing multiple membrane BIs (covering a range of spore loadings (10^2–10^7)) during the same experiment (fixed exposure time and concentration of HPV) allowed assessment of the log-inactivation (i.e., absence of growth), which was accurate to within ±1 log. Six replicates (n_i) at each spore loading and at each time point were used.

Hydrogen peroxide inactivation data for *C. difficile* spores were fitted using a modified Chick–Watson model. The model assumes a first-order dependence of the inactivation rate on the HPV concentration (C) and the number concentration of viable spores (N) as shown in Equation (1), which is the integrated form of the differential equation. k' is the inactivation rate coefficient and N_o the initial spore loading.

$$-\log\left(\frac{N}{N_o}\right) = k' \int Cdt \tag{1}$$

Average decimal reduction times were estimated using the method given in Lewis (1956). Briefly, given a starting

BI loading of N_0 viable spores per BI coupon, the inactivation rate is modeled as a first-order decay process (described above), which for a fixed steady peroxide concentration yields:

$$N = N_0 e^{-kt} \tag{2}$$

where N is the number of surviving spores at time t (in minutes). The decimal reduction time is related to the survival rate constant k (min^{-1}) by

$$D = \frac{\log_e 10}{k} = \frac{2.3026}{k} \tag{3}$$

N was estimated by a binomial count at each initial spore loading (10^2–10^7), i.e., by the numbers of sterile and viable BI coupons in groups exposed to a given peroxide concentration for a given exposure period. An estimate of N, $\hat{N}$, was calculated for a particular period by the following relationship:

$$\hat{N}_i = \log_e\left(\frac{n_i}{r_i}\right) \tag{4}$$

where n_i is the number of samples for the i'th period (six replicates) that were incubated and r_i is the number that were sterile. The validity of Equation (4) rests on the applicability of the Poisson probability distribution for events that occur with low frequencies, i.e., for a small proportion of spores that survive a period of hydrogen peroxide exposure. For a fixed steady hydrogen peroxide concentration and each given initial spore loading (10^2–10^7), at different exposure times, fractional survival values were obtained using Equation (5):

$$\frac{N}{N_0} = \frac{\hat{N}_i}{N_0} = \frac{\log_e\left(\frac{n_i}{r_i}\right)}{N_0} \tag{5}$$

Regression fitting of experimental data using the linear form of the modified Chick–Watson Equation (1) was undertaken using Datafit 9.0 software (Oakdale Engineering, USA). The coefficient of multiple determination (r^2) was chosen as a suitable indicator of the 'goodness of fit' to the experimental data.

Scanning Electron Micrographs (SEM)

The technique employed for fixing the spores to the membranes and their subsequent drying for SEM was based on that of Perdigao et al. (1995) and employed glutaraldehyde for the latter stage. Samples prepared by this method were then coated with a layer of gold-palladium and then imaged using a Hitachi scanning electron microscope (S3000H, Hitachi High-Tech, Tokyo, Japan) operated at 15 kV using a tungsten filament at a working distance of 25 mm.

Results

Deposition of the Spores onto the Membrane Surface

The method of filtering spores of *C. difficile* onto the isopore membrane filters resulted in an even surface distribution of the spores (Figure 3). Spores (typically ~1 μm in size) appear as a monolayer without aggregation on the surface of the filter medium (pore size ~0.2 μm). Under such conditions, all of the spores on the surface of the membrane would be equally exposed to the constant concentrations of HPV maintained in the environmental chamber.

HPV Sensor Calibration

Calibration data for the hydrogen peroxide electrochemical sensors showed good correspondence with the theoretical vapor–liquid equilibrium values reported by Schumb et al. (1955). Measurements were within 10% of the equilibrium value for calibrated sensors (Figure 4). Operation of the environmental chamber for periods in excess of 2 h showed that the concentration of hydrogen peroxide remained constant (95% confidence intervals are given in Table I). However, it was found that the sensors needed regular calibration. Previous studies do not mention this aspect of operation, and apparently made use of sensors supplied with the HPV generators. Therefore, the veracity of any absolute hydrogen peroxide concentrations reported in previous published work should be treated with caution.

Inactivation Kinetics

Figure 5 shows the survival curves along with the regression model fits (solid lines) for the exposure of *C. difficile* spores at hydrogen peroxide concentrations of 11, 25, 51, and

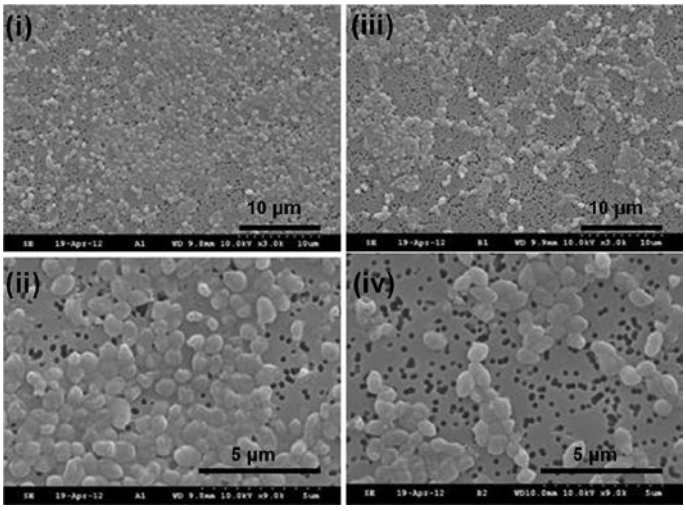

Fig. 3. SEM images of *C. difficile* spores deposited onto a membrane: (i) top left 10^8 spores/filter, bar scale 10 μm, (ii) bottom left 10^8 spores/filter, bar scale 5 μm, (iii) top right 10^7 spores/filter, bar scale 10 μm, (iv) bottom right 10^7 spores/filter, bar scale 5 μm.

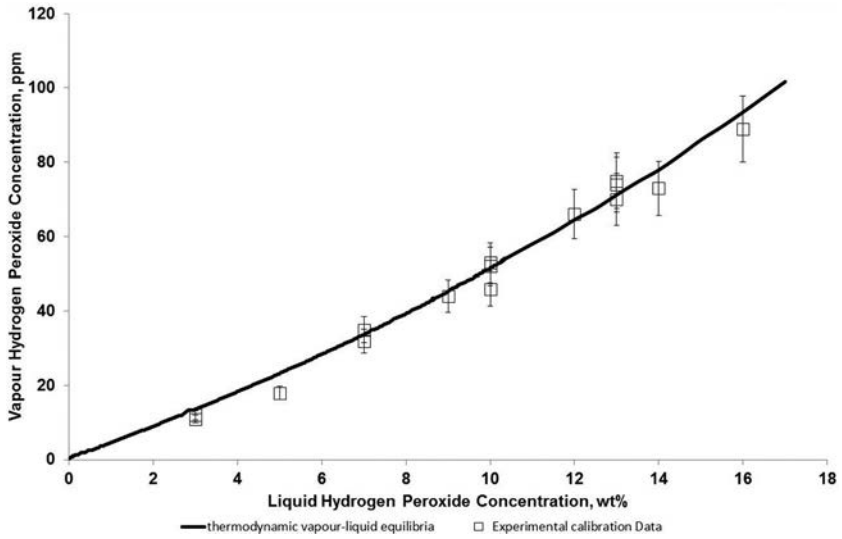

Fig. 4. Hydrogen peroxide sensor calibration plot showing the experimental data and the theoretical thermodynamic vapor–liquid equilibrium curve (the error bars represent one standard deviation at the measured concentration).

Table I. *C. difficile* inactivation kinetics (10–90 ppm) modeling parameters, decimal reduction values

HPV (ppm)	95% CI (ppm)	T °C	RH %	Modified Chick–Watson		
				k, $min^{-1}\,ppm^{-1}$	D, min	r^2
11	11.1–11.2	25 ± 2	46	0.0043	23.3	0.983
25	24.5–24.6	25 ± 2	47	0.0066	6.1	0.965
51	50.9–51.1	25 ± 2	51	0.0090	2.2	0.943
92	92.3–92.5	25 ± 2	62	0.0085	1.3	0.890

92 ppm for times up to 2 h. The results obtained by fitting the modified Chick–Watson inactivation model are shown in Table I. The table shows the fitting parameters for the model, the goodness of fit (r^2), and an estimate of the decimal reduction value (D). The D values ranged from 23 min at 11 ppm to 78 s at 92 ppm.

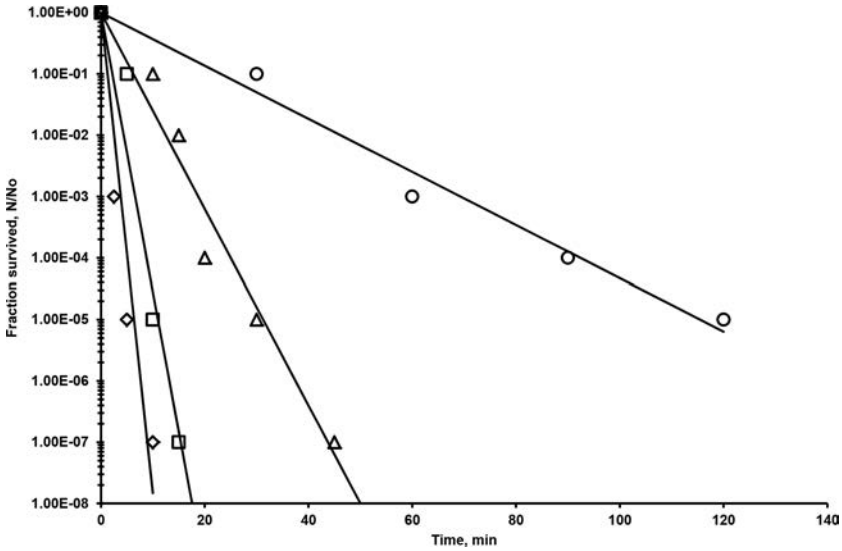

Fig. 5. Inactivation data for *C. difficile* spores (solid lines are fits using a modified Chick–Watson inactivation model), circles (11 ppm), triangles (25 ppm), squares (51 ppm), diamonds (92 ppm).

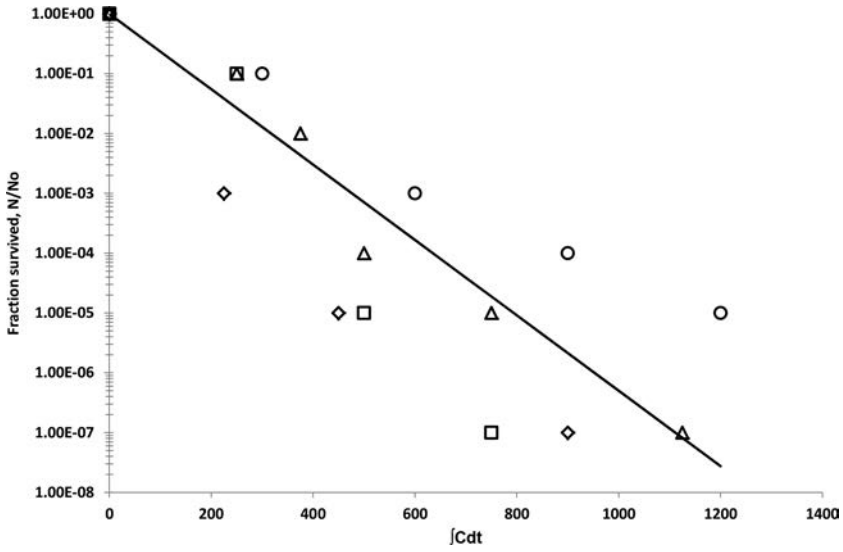

Fig. 6. Rescaling the inactivation data using $\int$Cdt as the rescaling parameter (regression equation $-\log(N/No) = 0.0063 \int Cdt$, $r^2 = 0.622$); circles (11 ppm), triangles (25 ppm), squares (51 ppm), diamonds (92 ppm).

Analysis of Inactivation with Varying HPV Concentrations

Commercial HPV generators used for the decontamination of healthcare facilities typically release a fixed quantity of hydrogen peroxide into an enclosed space. A consequence of this is that the environmental concentration peaks and then rapidly drops to low values as mixing and decomposition of hydrogen peroxide occur. In other words, the HPV concentration does not remain constant over time but rises, reaches a peak value, and then declines. Surprisingly few previously published microbial inactivation studies with hydrogen peroxide have provided full details of the concentration of the oxidant over the time course of their experiments. One way of addressing the inactivation efficacy of HPV due to the temporal variation of the HPV concentration is to investigate the relationship between the *C. difficile* inactivation using $\int Cdt$ as the independent variable. The inactivation data obtained here was fitted using this relationship and is shown in Figure 6. Reasonable agreement with the Chick–Watson relationship was obtained. At low HPV concentrations (~10 ppm), the regression line overestimates the potential inactivation of *C. difficile* spores, whereas at high concentrations the model provides a more conservative estimate.

Discussion

In previous studies, commercially available BIs comprising spores of either *B. subtilis* or *G. stearothermophilus* spores were typically used to assess the efficacy of HPV disinfection cycles (Andersen et al., 2010; Pottage et al., 2012). These BIs are typically produced by depositing (usually by pipetting) the spore suspension onto thin metal coupons or paper strips and then allowing them to dry before packaging them in a gas-permeable envelope. This is the procedure also followed by researchers who prepare their own spore indicators (Barbut et al., 2012). The status of the deposited spores on these BIs remains unknown as no images of the spores on the surfaces have previously been provided. The SEM images of *C. difficile* spore-laden membranes reported in the present study provide clarity regarding the uniform deposition of the spores achieved.

Bayliss et al. (2012) compared the filtration and drop deposition of *Bacillus* spp. spore suspensions and found that the latter method resulted in highly uneven spore distributions with extensive spore stacking at the periphery. Wiencek et al. (1990) found that spores produced by *Bacillus* spp. were generally more hydrophobic than those of the genus *Clostridium*. Hydrophobic spores may concentrate on the contact line during drying, thereby resulting in stacked structures at the periphery. Destrez (2012) has also commented that drop deposition is likely to result in clumps of cells. The difficulty posed by stacks of spores is that those at the surface of the stack protect those beneath the surface, and moreover the spore stack itself constitutes a diffusional resistance to HPV. This effect was demonstrated by Yu et al. (2006) for *B. subtilis* spores undergoing treatment with an atmospheric gas plasma. Although not identical to the case described here, the gaseous species produced by the gas plasma included hydrogen peroxide. Uneven spore distribution may explain potential variation among replicate BIs observed during disinfection studies, e.g., Fu et al. (2012). The method of filtering spores used in the present study ensured that an even surface distribution of *C. difficile* was achieved and therefore the HPV inactivation kinetics were not affected by diffusional effects arising from spore stacking.

Comparison of the *D* values obtained for spores of *B. subtilis* obtained by Malik et al. (2013) using similar experimental conditions to those for *C. difficile* obtained in the current work is shown in Table II. In each case the spores were exposed in the form of monolayers deposited

Table II. Comparison of decimal reduction values (D) for *B. subtilis* and *C. difficile*

HPV concentration (ppm)	Decimal reduction values (D) min	
	Bacillus subtilis[a]	*Clostridium difficile*
11	2085	23.1
25	628	6.3
51	221	2.2
92	93	1.3

[a]Data from Malik et al. (2013); reproduced with permission from Elsevier under the terms of the Creative Commons Attribution License.

on the same membrane material, which makes the comparison particularly valid. The D values for *B. subtilis* are two orders of magnitude greater than those of *C. difficile*. Rogers et al. (2005) compared the susceptibility of *Bacillus subtilis* and *Geobacillus stearothermophilus* spores deposited on a variety of indoor surface materials and exposed to HPV. *G. stearothermophilus* spores (routinely used as biological indicators to determine the efficacy of HPV decontamination cycles) were reported to be slightly more resistant to inactivation by HPV compared to *Bacillus subtilis*. The results were consistent with those reported previously by Klapes and Vesley (1990). Pottage et al. (2012) have challenged the use of *G. stearothermophilus* spore BI for decontamination studies as being inappropriate for use in healthcare settings (due to their greater HPV susceptibility), and shown *S. aureus* to be more resilient. *S. aureus* produces a protective biofilm and additionally possesses a powerful catalase, which provides an efficient pathway for the decomposition of hydrogen peroxide, potentially making this organism more resistant to HPV. Fu et al. (2012) compared the efficacy of two commercial HPV decontamination systems and reported good correspondence between commercial *G. stearothermophilus* BIs (loading of $\sim 10^6$ cfu) and in-house-prepared meticillin-resistant *Staphylococcus aureus* (MRSA) (loading of $\sim 10^6$ cfu) test discs. Ali et al. (2016) reported the persistence of catalase-producing MRSA (at a loading of $\sim 10^6$ cfu) and *C. difficile* spores (at a loading of $\sim 10^5$ cfu) used as BIs following commercial HPV decontamination cycles for whole-room aerial disinfection of single isolation rooms. Typical aerial concentrations of hydrogen peroxide were not reported in their study. Maillard (2011) claimed that there is a widespread view that spores of *C. difficile* are more susceptible than those of *B. subtilis* and that this could not be traced to 'robust scientific evidence' and that the persistence of this view was 'not accurate' and could provide a false sense of security. The data of Table II provide compelling evidence of the greater susceptibility of *C. difficile* spores to HPV in comparison with *B. subtilis* spores.

The convenience of commercially available BIs is incontestable as Otter and Yezli (2012) have pointed out. HPV decontamination cycles optimized based upon inactivation results obtained with *Bacillus spp*. are likely to greatly overestimate the HPV concentrations and/or exposure time required for the disinfection of surfaces contaminated with *C. difficile* spores. In healthcare settings where there is a *C. difficile* outbreak or a risk to the patient of *C. difficile* acquisition from the environment, HPV decontamination cycles optimized for *C. difficile* eradication may permit shorter cycle times. This may be possible due to the need for less-severe decontamination conditions, i.e., use of lower peroxide vapor concentrations and shorter exposure durations in comparison with cycles optimized for the inactivation of *G. stearothermophilus* BIs. However, it should be pointed out that there is evidence in the published literature of the reduced efficacy of HPV against *C. difficile* spores when the latter are suspended in a menstruum designed to simulate more accurately the physical state by which *C. difficile* exists in healthcare environments (Ali et al., 2016). Further work is therefore needed to assess the level of protection against HPV afforded to *C. difficile* spores associated with biological fluid and fecal material.

Employing exposure times of less than 10 min, but at concentrations much higher than those used here (a maximum hydrogen peroxide concentration of 355 ppm), Johnston et al. (2005) obtained linear inactivation kinetics ($\log_{10}$ survivors vs. time) for *C. botulinum* spores. Whereas in a study of inactivation of a variety of nosocomial bacteria, Otter and French (2009) also obtained linear inactivation kinetics showing a 3-log reduction in 10 min and a 6-log reduction in 20 min for *C. difficile* spores (i.e., an averaged D value of ~3.3 min) upon exposure to HPV using a Bioquell Clarus^{TM} system (using in-house BIs). The peak HPV concentration for the Bioquell Clarus^{TM} system is greater than 100 ppm but is not held constant (Barbut et al., 2013). The temporal variation of HPV concentration during process cycles has not previously received much attention in the published literature. Rutala and Weber (2011) reviewed previous published work on room decontamination using a number of commercially available gaseous hydrogen peroxide decontamination systems. They implied that the uniform distribution of hydrogen peroxide is achieved by commercial gaseous hydrogen peroxide systems via automated dispersal systems. This assumption may not necessarily be valid as discussed below and has implications for the achieved efficacy of HPV decontamination.

The D values at low HPV concentrations (~10 ppm) in Table I are of the order of 25 min. Concentrations of this order persist over significant time periods following release of the gaseous hydrogen peroxide using commercial systems as reported by Fu et al. (2012). Fu et al. (2012) compared the decontamination efficacy of two commercial hydrogen peroxide systems: (i) Clarus R^{TM} (Bioquell) and (ii) SR2 Sterinis (Advanced Sterilization Products (ASPs)). They published hydrogen peroxide concentration– time curves for their work on the inactivation of *G. stearothermophilus* and *C. difficile* spores. The SR2 system releases an aerosol of hydrogen peroxide, whereas the Clarus R^{TM} system flash evaporates hydrogen peroxide solution to generate a high-concentration vapor. Fu et al. (2012) provided concentration–time data for typical 120 min decontamination process cycles for each of the two commercial systems; these

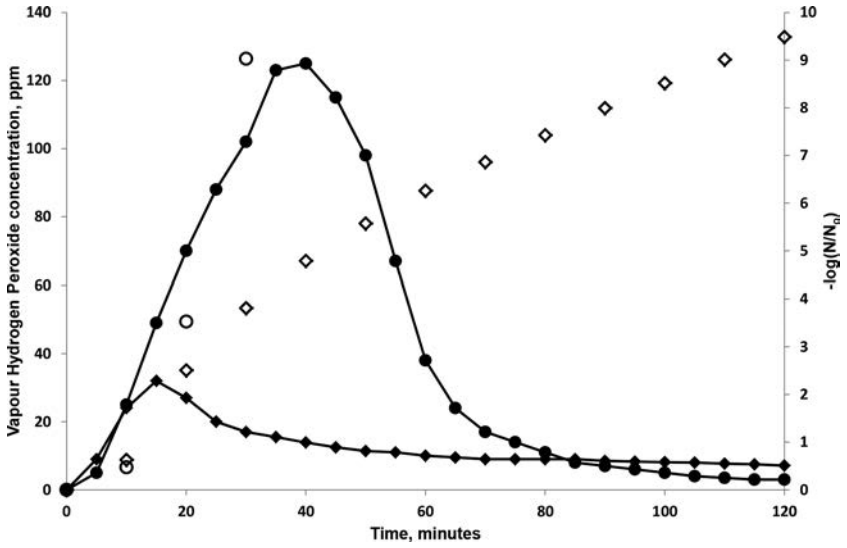

Fig. 7. Temporal hydrogen peroxide vapor concentration (ASP, solid diamonds; Bioquell, solid circles) for two commercial disinfection processes (data replotted from Fu et al. 2012; reproduced with permission from Elsevier under the terms of the Creative Commons Attribution License). Open diamonds (ASP process) and open circles (Bioquell process) represent calculated log reduction values estimated by integrating the concentration versus time data up to each time point and using the regression equation from Figure 6.

data have been replotted in Figure 7. The data of Fu et al. (2012) showed the concentration of hydrogen peroxide peaking at 30 ppm (~15 min) for the ASP Sterinis cycle and at 125 ppm (~40 min) for the Bioquell cycle and naturally decaying to around 10 ppm after 60 min (ASP). The Bioquell process employs catalytically enhanced decomposition of hydrogen peroxide (reaching ~10 ppm after 80 min) due to the relatively high peak concentration achieved during decontamination. The concentration of hydrogen peroxide thereafter remains below 10 ppm for the remainder of the process cycle (some additional 40 min). In order to enable personnel to gain access to the environments that have been subject to treatment, the concentration of the HPV needs to fall below the exposure limit value of 1 ppm.

Integrating the hydrogen peroxide concentration–time data for the commercial decontamination cycles (Figure 7) coupled with the regression model obtained by fitting *C. difficile* inactivation data (Figure 6) allowed assessment of the potential inactivation achieved at select time points for the two commercial process cycles (shown as open diamonds (ASP) and open circles (Bioquell)). Integrating the concentration–time curves reported by Fu et al. (2012) suggested a possible 6-log inactivation of *C. difficile* spore BIs could be achieved after 60 min and 30 min with the ASP process and the Bioquell process, respectively. These results are in agreement with those of Fu et al. (2012), who reported a 6-log reduction of *C. difficile* spores (using BI spore discs prepared in-house) at the end of both process cycles. The results are also in agreement with those of Otter and French (2009) and Barbut et al. (2012).

In a comparison of eight methods for terminal disinfection aimed specifically at the elimination of *C. difficile*, Doan et al. (2012) concluded that the release of HPV was the most effective method, but was also the most expensive, confirming the earlier statements of Vonberg et al. (2008) and Davies et al. (2011). Doan et al. (2012) claimed that disinfection with a chlorine-releasing agent, although marginally less effective than HPV, was considerably cheaper and therefore should be considered the method of choice. However, these authors did not specify precisely how the chlorine-releasing agent was applied to surfaces and whether the researchers themselves or cleaning staff conducted the decontamination with these agents. This is significant, as although the use of HPV generators requires specially trained personnel, its application would be more consistent, unlike manual cleaning, in which inaccessible places tend to get overlooked by the cleaning staff. Indeed, Unger-Bimczok et al. (2011) recently demonstrated how well HPV could penetrate cavities that liquid disinfectants would be unable to. In a study conducted with *S. aureus*, Exner et al. (2004) showed firstly that certain liquid disinfectants and the methods by which they are deployed onto surfaces, e.g., by mopping, possess a low, but quantifiable, risk of disseminating pathogenic organisms, and secondly, that some of these agents induce the sporulation of *C. difficile*.

Conclusions

The work described here enabled estimates to be made of *D* values for spores of *C. difficile* over a clinically relevant range of HPV concentrations currently employed in the decontamination of healthcare facilities. We demonstrate the suitability of a method for integrating the temporal unsteady HPV concentration versus time curves (currently being used in healthcare facilities) to estimate the inactivation efficacy of HPV for *C. difficile* spores. The work

presented here should enable decontamination processes based on this particular oxidant to be rigorously designed and would lend the predictions obtained a higher level of confidence.

Notwithstanding, there exists a need to extend the work reported here to more closely emulate the true state in which *C. difficile* spores are found in healthcare environments. Typically, spores of *C. difficile* are associated with fecal matter, and whilst the use of feces in experimental studies would have a deleterious effect on spore recovery (although the binomial count-based method used in the present study would permit reliable estimation of spore survival), previous workers have used a variety of simulants. For example, Wren et al. (2008) used horse serum, whilst more recently Ali et al. (2016) employed a menstruum of bovine serum albumin.

We should acknowledge the limitations of this study. In this work no assessment has been made of the impact of soiling (presence of biological fluid of fecal contamination) on *C. difficile* spore survival upon exposure to HPV at relevant concentrations and exposure times. In healthcare environments it is highly likely that *C. difficile* spores will be associated with organic matter. Empirical evidence has been presented in the literature that high spore loadings ($>10^5$ spores per BI) associated with biological material survive commercial hydrogen peroxide decontamination cycles (Fu et al., 2012; Ali et al., 2016). This finding reinforces the need for a thorough cleaning of the environment to reduce biological contamination prior to hydrogen peroxide treatment regardless of the type of hydrogen peroxide device or specific exposure cycle parameters used. In this work only *C. difficile* spores were employed. It would be pertinent to conduct further tests with *G. stearothermophilus* spores as well as catalase-producing organisms including meticillin-resistant *Staphylococcus aureus* (MRSA). Cycle time optimization and selection require studies conducted in actual healthcare settings showing the spatial and temporal distribution of HPV within the clinical environment and proof of inactivation of indicator organisms achieved thereof, e.g., *G. stearothermophilus* and MRSA at relevant loadings in the presence of associated organic matter.

Funding

The authors would like to acknowledge EPSRC support for this work (Grant no. EP/D039614/1) *via* the Health and Care Infrastructure Research and Innovation Centre (HaCIRIC).

References

Ali, S., Muzslay, M., Bruce, M., Jeanes, A., Moore, G., and Wilson, A. P. R. (2016). Efficacy of two hydrogen peroxide vapour aerial decontamination systems for enhanced disinfection of meticillin-resistant *Staphylococcus aureus, Klebsiella pneumonia* and *Clostridium difficile* in single isolation rooms, *J. Hosp. Infect.*, **93**, 70–77.

Andersen, B. M., Syversen, G., Thoresen, H., Rasch, M., Hochlin, K., Seljordslia, B., Snevold, I., and Berg, E. (2010). Failure of dry mist of hydrogen peroxide 5% to kill *Mycobacterium tuberculosis*, *J. Hosp. Infect.*, **76**, 80–83.

Barbut, F., Yezli, S., and Otter, J. A. (2012). Activity in vitro of hydrogen peroxide vapour against *Clostridium difficile* spores, *J. Hosp. Infect.*, **80**, 85–87.

Barbut, F., Yezli, S., and Otter, J. A. (2013). Hydrogen peroxide room disinfection: there is no elephant in the room but there's plenty of evidence in the trunk, *J. Hosp. Infect.*, **83**, 355–356.

Bayliss, D. L., Walsh, J. L., Iza, F., Shama, G., Holah, J., and Kong, M. G. (2012). Complex responses of microorganisms as a community to a flowing atmospheric plasma, *Plasma Process Polym.*, **9**, 597–611.

Davies, A., Pottage, T., Bennett, A., and Walker, J. (2011). Gaseous and air decontamination technologies for *Clostridium difficile* in the healthcare environment, *J. Hosp. Infect.*, **77**, 199–203.

Destrez, P. (2012). Reply to Fu et al.: Efficacy, efficiency and safety aspects of hydrogen peroxide vapour and aerosolized hydrogen peroxide room disinfection systems, *J. Hosp. Infect.*, **82**, 68.

Doan, L., Forrest, H., Fakis, A., Craig, J., Claxton, L., and Khare, M. (2012). Clinical and cost effectiveness of eight disinfection methods for terminal disinfection of hospital isolation rooms contaminated with *Clostridium difficile* 027, *J. Hosp. Infect.*, **82**, 114–121.

Exner, M., Vacata, V., Hornei, B., Dietlein, E., and Gebel, J. (2004). Household cleaning and surface disinfection: new insights and strategies, *J. Hosp. Infect.*, **56**, S70–S75.

Fawley, W. N., Underwood, S., Freeman, J., Baines, S. D., Saxton, K., Stephenson, K., Owens, R. C., and Wilcox, M. H. (2007). Efficacy of hospital cleaning agents and germicides against epidemic *Clostridium difficile* strains, *Infect. Control Hosp. Epidemiol.*, **28**, 920–925.

Fu, T. Y., Gent, P., and Kumar, V. (2012). Efficacy, efficiency and safety aspects of hydrogen peroxide vapour and aerosolized hydrogen peroxide room disinfection systems, *J. Hosp. Infect.*, **80**, 199–205.

He, M., Miyajima, F., Roberts, P., Ellison, L., Pickard, D. J., Martin, M. J., Connor, T. R., Harris, S. R., Fairley, D., Bamford, K. B., D'Arc, S., Brazier, J., Brown, D., Coia, J. E., Douce, G., Gerding, D., Kim, H. J., Koh, T. H., Kato, H., Senoh, M., Louie, T., Michell, S., Butt, E., Peacock, S. J., Brown, N. M., Riley, T., Songer, G., Wilcox, M., Pirmohamed, M., Kuijper, E., Hawkey, P., Wren, B. W., Dougan, G., Parkhill, J., and Lawley, T. D. (2012). Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*, *Nat. Genet.*, **45**, 109–113.

Lewis, J. C. (1956). The estimation of decimal reduction times, *Appl. Microbiol.*, **4**, 211–221.

Holmdahl, T., Lanbeck, P., Wullt, M., and Walder, M. H. (2011). A head-to-head comparison of hydrogen peroxide vapor and aerosol room decontamination systems, *Infect. Control Hosp. Epidemiol.*, **32**, 831–836.

Jeffery, G. H., and Vogel, A. I. (1989). *Vogel's textbook of Quantitative Chemical Analysis*, Longman Scientific and Technical, New York.

Johnston, M. D., Lawson, S., and Otter, J. A. (2005). Evaluation of hydrogen peroxide vapour as a method for the decontamination of surfaces contaminated with *Clostridium botulinum* spores, *J. Microbiol. Methods*, **60**, 403–411.

Kim, K. H., Fekety, R., Batts, D. H., Brown, D., Cudmore, M., Silva, J., and Waters, D. (1981). Isolation of *Clostridium difficile* from the environment and contacts of patients with antibiotic-associated colitis, *J. Infect. Dis.*, **143**, 42–50.

Klapes, N. A., and Vesley, D. (1990). Vapor-phase hydrogen-peroxide as a surface decontaminant and sterilant, *Appl. Environ. Microbiol.*, **56**, 503–506.

Kuijper, E. J., Coignard, B., and Tüll, P. (2006). Emergence of *Clostridium difficile*-associated disease in North America and Europe, *Clin. Microbiol. Infect.*, **12**, 2–18.

Maillard, J. Y. (2011). Innate resistance to sporicides and potential failure to decontaminate, *J. Hosp. Infect.*, **77**, 204–209.

Malik, D. J., Shaw, C. M., Rielly, C. D., and Shama, G. (2013). The inactivation of *Bacillus subtilis* spores at low concentrations of hydrogen peroxide vapour, *J. Food Eng.*, **114**, 391–396.

Mayfield, J. L., Leet, T., Miller, J., and Mundy, L. M. (2000). Environmental control to reduce transmission of *Clostridium difficile*, *Clin. Infect. Dis.*, **31**, 995–1000.

O'Brien, J. A., Lahue, B. J., Caro, J. J., Davidson, D. M. (2007). The emerging infections challenge of Clostridium difficile – associated disease in Massachusetts Hospitals: Clinical and Economic Consequences, *Infect. Cont. Hosp. Epidemiol.*, **28**, 1219–1227.

Otter, J. A., and French, G. L. (2009). Survival of nosocomial bacteria and spores on surfaces and inactivation by hydrogen peroxide vapor, *J. Clin. Microbiol.*, **47**, 205–207.

Otter, J. A., and Yezli, S. (2012). Are commercially available *Geobacillus stearothermophilus* biological indicators an appropriate standard for hydrogen peroxide vapour systems in hospitals? *J. Hosp. Infect.*, **80**, 272–273.

Passaretti, C. L., Otter, J. A., Reich, N. G., Myers, J., Shepard, J., Ross, T., Carroll, K. C., Lipsett, P., and Perl, T. M. (2013). An evaluation of environmental decontamination with hydrogen peroxide vapor for reducing the risk of patient acquisition of multidrug-resistant organisms, *Clin. Infect. Dis.*, **56**, 27–35.

Perdigao, J., Lambrechts, P., Van Meerbeek, B., Vanherle, G., and Lopes, A. L. (1995) Field emission SEM comparison of four postfixation drying techniques for human dentin, *J. Biomed. Mater. Res.*, **29**, 1111–1120.

Perez, J., Springthorpe, V. S., and Sattar, S. A. (2011). Clospore: A liquid medium for producing high titers of semi-purified spores of *Clostridium difficile*, *J. AOAC Int.*, **94**, 618–626.

Pottage, T., Macken, S., Walker, J. T., and Bennett, A. M. (2012). Meticillin-resistant *Staphylococcus aureus* is more resistant to vaporized hydrogen peroxide than commercial *Geobacillus stearothermophilus* biological indicators, *J. Hosp. Infect.*, **80**, 41–45.

Rogers, J. V., Sabourin, C. L. K., Choi, Y. W., Richter, W. R., Rudnicki, D. C., Riggs, K. B., Taylor, M. L., and Chang, J. (2005). Decontamination assessment of *Bacillus anthracis, Bacillus subtilis*, and *Geobacillus stearothermophilus* spores on indoor surfaces using a hydrogen peroxide gas generator, *J. Appl. Microbiol.*, **99**, 739–748.

Rupnik, M., Wilcox, M. H., and Gerding, D. N. (2009). Clostridium difficile infection: new developments in epidemiology and pathogenesis. Nat. Rev. Microbiol., **7**, 526–536.

Rutala, W. A., and Weber, D. J. (2011). Are room decontamination units needed to prevent transmission of environmental pathogens? *Infect. Cont. Hosp. Epidemiol.*, **32**, 743–747.

Schumb, W. C., Satterfield, C. N., and Wentworth, R. L. (1955). *Hydrogen Peroxide*, Reinhold Publishing Corporation, New York.

Shan, J. Y., Patel, K. V., Hickenbotham, P. T., Nale, J. Y., Hargreaves, K. R., and Clokie, M. R. J. (2012). Prophage carriage and diversity within clinically relevant strains of *Clostridium difficile*, *Appl. Environ. Microbiol.*, **78**, 6027–6034.

Unger-Bimczok, B., Kosian, T., Kottke, V., Hertel, C., and Rauschnabel, J. (2011). Hydrogen peroxide vapor penetration into small cavities during low-temperature decontamination cycles, *J. Pharm. Innov.*, **6**, 32–46.

Vonberg, R. P., Reichardt, C., Behnke, M., Schwab, F., Zindler, S., and Gastmeier, P. (2008). Costs of nosocomial *Clostridium difficile*-associated diarrhoea, *J. Hosp. Infect.*, **70**, 15–20.

Watson, H. E. (1908). A note on the variation of the rate of disinfection with change in the concentration of the disinfectant, *J. Hyg.*, **8**, 536–542.

Wiencek, K. M., Klapes, N. A., and Foegeding, P. M. (1990). Hydrophobicity of *Bacillus*, and *Clostridium* Spores, *Appl. Environ. Microbiol.*, **56**, 2600–2605.

Wren, W. M. D., Rollins, M. S. M., Jeanes, A., Hall, T. J., Coen, P. J., and Gant, V. A. (2008). Removing bacteria from hospital surfaces: a laboratory comparison of ultramicrofibre, and standard cloths, *J. Hosp. Infect.*, **70**, 265–271.

Yu, H., Perni, S., Shi, J. J., Wang, D. Z., Kong, M. G., and Shama, G. (2006). Effects of cell surface loading and phase of growth in cold atmospheric gas plasma inactivation of *Escherichia coli* K12, *J. Appl. Microbiol.*, **101**, 1323–1330.