J Pharm Innov (2008) 3:123–133
DOI 10.1007/s12247-008-9027-1

RESEARCH ARTICLE

The Influence of Humidity, Hydrogen Peroxide Concentration, and Condensation on the Inactivation of *Geobacillus stearothermophilus* Spores with Hydrogen Peroxide Vapor

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Published online: 8 May 2008

Abstract The study presented here examined the factors influencing the effectiveness of surface decontamination with hydrogen peroxide vapor. The impact of relative humidity and hydrogen peroxide gas concentrations was investigated and compared to a dew point analysis of these various sterilant atmospheres. For this purpose, a series of different H_2O_2 decontamination cycles were developed and tested for antimicrobial effectiveness using biological indicators inoculated with greater than 10^6 spores of *Geobacillus stearothermophilus.* The results indicate that an increasing concentration of hydrogen peroxide in the gas phase and higher humidity levels result in a faster inactivation of the test organisms. The higher the H_2O_2 gas phase concentration was, the more independent the inactivation effect from the humidity level. At lower H_2O_2 concentrations, the same kill was achieved with higher humidity. Subvisible condensation was found to be necessary for short inactivation times, but condensation in the visible range did not further enhance the sporicidal activity. The molecular deposition of water and hydrogen peroxide on the target surface represents the determining factor for microbial inactivation, whereas the hydrogen peroxide concentration in the gas phase is of secondary importance.

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Keywords Condensation · Decontamination · *D* value · *Geobacillus stearothermophilus* · Hydrogen peroxide · Inactivation · Isolator · Relative humidity · Vapor

Introduction

Hydrogen peroxide is a strong oxidizing agent, and aqueous H_2O_2 solutions have been used for sterilization and disinfection purposes for more than a century. Since it was discovered that the antimicrobial effectiveness of the sterilant is significantly increased in the gaseous phase [1], hydrogen peroxide vapor is commonly used for surface decontamination. Due to its environmentally friendly and nontoxic decomposition products, hydrogen peroxide is being increasingly substituted for vapor phase sterilants like ethylene oxide and formaldehyde, which are toxic, carcinogenic, and potentially explosive [2]. Vapor phase hydrogen peroxide was shown to have effective broad-spectrum antimicrobial properties and to inactivate bacteria, fungi, viruses, and highly resistant spores [2–5].

In the pharmaceutical industry, hydrogen peroxide vapor has been used in the decontamination of barrier systems for more than 10 years [4, 6]. Today, the use of isolator technology is increasing, and hydrogen peroxide vapor is gaining additional importance as a decontamination agent [7–10]. Several parameters are known to affect the decontamination success of rooms and chambers with hydrogen peroxide vapor. Besides the construction materials that are to be decontaminated [11, 12], the environmental conditions play an important role. However, the cycle parameters that affect microbial inactivation by hydrogen peroxide vapor are still under investigation and remain controversial.

The literature reveals disagreement on the optimum conditions concerning relative humidity, hydrogen peroxide concentration, and temperature. There is also debate concerning whether condensation is needed for the mechanism of microbial inactivation or if a dry cycle is preferential. Several publications propose a direct correlation between the concentration of the gaseous sterilant in the chamber air and the antimicrobial efficacy [5, 13, 14]. In contrast, some authors report high inactivation rates with comparatively low hydrogen peroxide concentrations [12], while others question a correlation between measured H_2O_2 concentration and the absolute antimicrobial efficacy [15]. Associated with the issue of hydrogen peroxide concentration is the question regarding appropriate levels of relative humidity. Watling et al. [16] investigated flash vaporization of 35% aqueous hydrogen peroxide solution in a closed-loop system. The authors demonstrated that the concentration of gaseous hydrogen peroxide at the point when condensation first occurs is higher when the initial relative humidity inside the chamber is low. Therefore, it is a common procedure to dehumidify the sealed enclosure prior to the introduction of the hydrogen peroxide vapor to reach high sterilant concentrations in the gas phase and to prevent condensation [5, 14]. With this method, the microbial inactivation is regarded as a dry process. Condensation is considered uncontrollable and unwanted because of potential problems caused by material corrosion, prolonged aeration time, and inhomogeneous decontamination [5, 14].

In a different approach, it is claimed that condensation cannot be avoided during standard decontamination processes [17] and that the occurrence of condensation is the critical factor affecting antimicrobial efficacy [17]. In this context, Imai et al. [15] suggested that relative humidity is a less critical factor for vapor phase hydrogen peroxide decontamination and proposed eliminating the dehumidification phase. According to surveys conducted by Watling [18], invisible microcondensation is responsible for the rapid microbial inactivation.

As described, published data regarding the influence of operating conditions on the effectiveness of hydrogen peroxide vapor decontaminations are not conclusive. Until now, no standard system or method has been established for the evaluation of the sporicidal effect of hydrogen peroxide. This paper provides an analysis of the parameters affecting sporicidal activity of hydrogen peroxide vapor for an open-loop system. The objective of this study is to deepen the understanding of the inactivation mechanism and to optimize decontamination cycles. The article describes the evaluation of sterilant atmospheres with different levels of hydrogen peroxide and water vapors, varied independently, and the resulting condensation levels. The various sterilant atmospheres were systematically examined for the capability of inactivating certain microbial test populations. Biological indicators (BIs) were exposed to the various sterilant atmospheres, and subsequently, both *D* values and inactivation kinetics were determined.

Materials and Methods

Study Design

The following investigations were carried out during the study:

- End-point *D* value studies with BIs sealed in Tyvek (for four humidity levels [HLs] and three hydrogen peroxide levels). Investigation into the influence on *D* value of the following parameters:
  - Water and hydrogen peroxide concentrations
  - Hydrogen peroxide consumption
  - Condensation
- End-point *D* value studies with BIs sealed in Tyvek and unsealed (for four HLs and one hydrogen peroxide level)
- Inactiviation kinetics using the most probable number (MPN) method (for four HLs and two hydrogen peroxide levels)

Decontamination System

Isolator and Vapor Generator

The experiments were conducted in a five-glove, rigid-wall isolator with an internal volume of 7.5 m^3. The machine was located within a clean room environment with the compartment air temperature controlled at 22±2°C. The barrier system was connected to a Bosch SafeVAP vapor generator operated in open-loop mode. The gas generator was modified from standard operation to enable simultaneous flash vaporization of water and hydrogen peroxide by two parallel vaporizers. This provided the means to develop customized cycles and to produce test atmospheres with any combination of humidity and hydrogen peroxide concentration in the isolator during the decontamination cycle. The sporicidal agent used was 35% hydrogen peroxide by weight (Interox SG-35, Solvay Chemicals GmbH, Rheinberg), and the water vaporized for the regulation of the humidity was high-performance liquid chromatography Gradient Grade Water, Carl Roth, Karlsruhe.

Decontamination Cycle

The decontamination cycle included four successive cycle steps. The four stages of the decontamination cycle that are to specify the single-decontamination phases are illustrated in Fig. 1. During the preparation phase (I), the relative

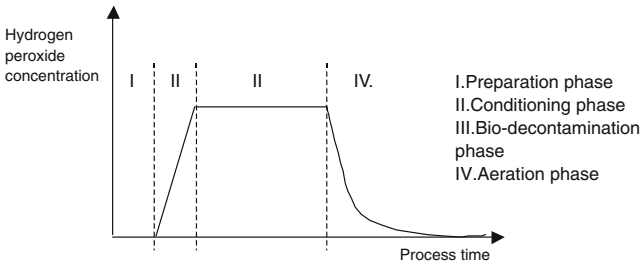

Fig. 1 Schematic example of the hydrogen peroxide concentration during a decontamination cycle

humidity of the isolator atmosphere was reduced to 20%, and the ductwork and evaporators were heated for the subsequent introduction of the hydrogen peroxide. In the conditioning phase (II), hydrogen peroxide (and in this configuration, water) is introduced at a high rate to reach the desired concentration levels within the isolator. During the bio-decontamination phase (III), hydrogen peroxide is introduced at a reduced rate such that only the consumed hydrogen peroxide is replaced, and a constant hydrogen peroxide concentration is maintained within the isolator. The terminal phase of the decontamination cycle is the aeration (IV), during which the hydrogen peroxide is removed from the system by dilution with fresh air.

For this study, customized decontamination cycles were developed to produce 12 distinct sterilant atmospheres during the bio-decontamination phase. Table 1 shows the 12 sterilant atmospheres developed: Three distinct hydrogen peroxide concentrations (400, 600, and 800 ppm H_2O_2) were developed, each at four distinct HLs. HL 1 (for 400, 600, and 800 ppm H_2O_2) was created by the introduction of the hydrogen peroxide solution only and provided a water concentration range of about 3,500 to 8,500 ppm. For HLs 2 through 4, vaporized water was introduced to the system in increasing amounts. This resulted in water concentration ranges of 8,500 to 13,000 (HL 2), 11,500 to 17,000 (HL 3), and 14,500 to 21,000 ppm (HL 4). Figure 2 is an example of the composite hydrogen peroxide and water concentration curves during the decontamination cycles with hydrogen peroxide concentrations of 400, 600, and 800 ppm at HL 2. The varying water content in the isolator atmosphere for HLs 1 to 4 is demonstrated in Fig. 3, which shows the trend of the water concentration during the bio-decontamination phase with a H_2O_2 concentration of 600 ppm.

Measuring Instruments

Inside the isolator chamber, a near-infrared spectrometer probe (H_2O_2 Vapor Monitor, Guided Wave, Rancho Corvo, CA, USA) was installed to record water and hydrogen peroxide concentrations during the decontamination cycles. The Vapor Monitor provided a direct in situ measurement of hydrogen peroxide and water concentrations in the gas phase [19]. For qualitative verification of the measurement, an additional electrochemical H_2O_2 sensor (Analytical Technology, Collegeville, PA, USA) was used to monitor the H_2O_2 concentration. A capacitive dew sensor (BTF 11356A, CIS Institut für Mikrosensorik gGmbH, Erfurt) was installed in the test chamber for the detection and quantification of condensation during the decontamination cycles. The dew point detector consisted of a silicon-integrated, stray field capacitor connected to a capacitance–frequency converter. Due to its dielectric constant, condensed vapor causes a rise in capacitance and therefore an increase in the frequency output signal in proportion to the level of condensation.

Biological Test Procedure

Test organisms were exposed to the different test atmosphere combinations in the isolator to evaluate the inactivation efficiency of the various decontamination conditions. BIs were exposed to the decontamination cycle solely during the bio-decontamination phase and removed from the test atmosphere at predetermined time intervals. The commercially available BIs were composed of grade 304 stainless steel carriers, each inoculated with 2.4×10^6 spores of *Geobacillus stearothermophilus* American Type Culture Collection no. 12980. The BIs, sealed in 1073B medical-grade Tyvek™, were manufactured by Apex Laboratories, Sanford, NC, USA. The certified BI population was tested and verified according to the acceptance criteria specified in US Pharmacopoeia (USP) [20]. All BIs used throughout the study were from a single production lot.

Table 1 Decontamination cycles with different combinations of hydrogen peroxide concentration and humidity levels during bio-decontamination phase

HL: range of H_2O concentration	H_2O_2 concentration		
	400 ppm	600 ppm	800 ppm
HL 1 (3,500 to 8,500 ppm H_2O)	400 ppm H_2O_2, HL1	600 ppm H_2O_2, HL1	800 ppm H_2O_2, HL1
HL 2 (8,500 to 13,000 ppm H_2O)	400 ppm H_2O_2, HL2	600 ppm H_2O_2, HL2	800 ppm H_2O_2, HL2
HL 3 (11,500 to 17,000 ppm H_2O)	400 ppm H_2O_2, HL3	600 ppm H_2O_2, HL3	800 ppm H_2O_2, HL3
HL 4 (14,500 to 21,000 ppm H_2O)	400 ppm H_2O_2, HL4	600 ppm H_2O_2, HL4	800 ppm H_2O_2, HL4

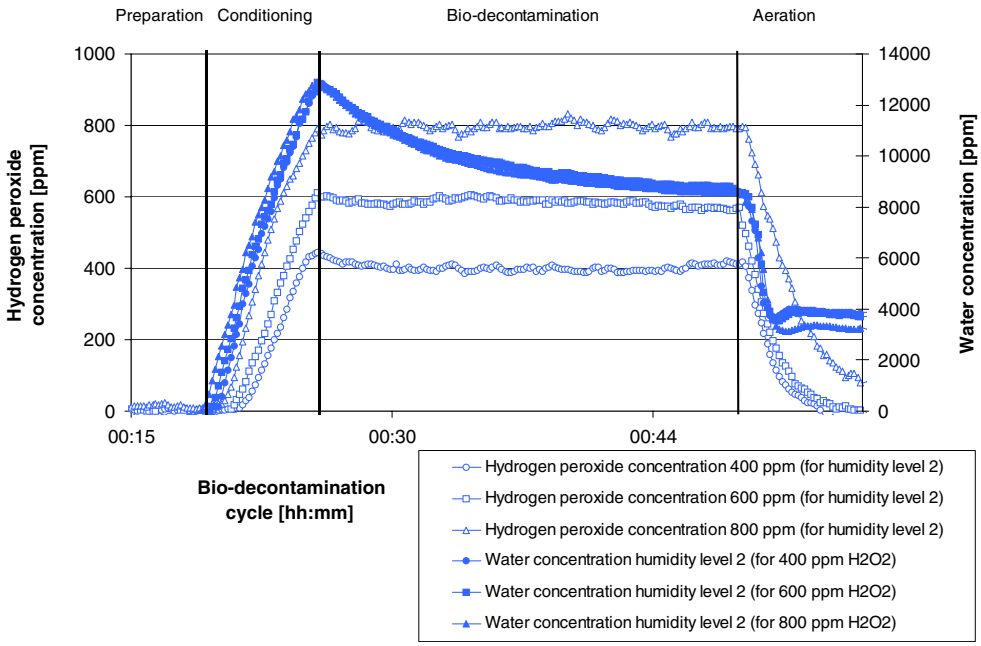

Fig. 2 Water and hydrogen peroxide concentrations during decontamination cycles with 400, 600, and 800 ppm H_2O_2 concentrations at humidity level 2 (measured with the NIR probe)

D Value

The *D* value is the time (in minutes) of exposure at given sporicidal conditions, which causes a one log or 90% reduction in the population of a specific test microorganism [21]. At the beginning of the bio-decontamination phase, BIs were exposed to the sporicidal conditions in the isolator. For the determination of *D* values, five BIs were removed from the isolator at each exposure time. The samples were aseptically transferred into test tubes with 3 ml of sterile soybean casein digest broth (Caso-Bouillon, Merck KGaA, Darmstadt) and incubated at 55°C for 7 days. The test tubes were evaluated for growth daily, and after the expiration of the incubation period, *D* values were calculated according to the fraction-negative approach of the Holcomb–Spearman–Karber method with at least two exposure times in the lethal area [22, 23]. The initial spore population, certified by the manufacturer of the BIs, was used for the calculation of the *D* values. For each test atmosphere, the cycle was repeated several times. One *D* value determination was performed per cycle run (five BIs per takeout interval) to obtain at least three values for every test atmosphere. In some experiments, *D* values were determined for unsealed BIs. In these cases, the bare indicators were exposed without the Tyvek envelope; all other steps were identical.

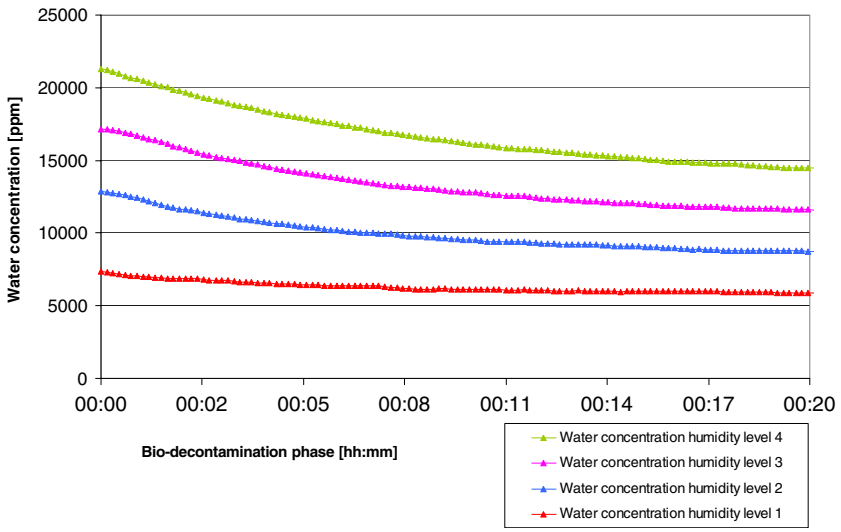

Fig. 3 Water concentration during the bio-decontamination phase for humidity levels 1–4 and 600 ppm H_2O_2 (measured with the NIR probe)

Most Probable Number

The MPN procedure is a standard method for the estimation of densities of viable microorganisms in liquid samples. It does not rely on a direct count of single cells or colonies but on the statistical probability of diluting to extinction [24–26]. In this study, the MPN method was used to establish inactivation kinetics of the test populations exposed to the test atmospheres. At each exposure interval, one BI was removed from the chamber and eluted with 10 ml of sterile medium (Caso-Bouillon, Merck KGaA). To remove the spores from the carrier and singularize them in the suspension, samples were treated in an ultrasonic bath for 30 min (VWR Ultrasonic Cleaner USC200T, VWR International GmbH, Darmstadt). A three-tube MPN procedure with aseptic serial tenfold dilutions was used to enumerate the density of viable microorganisms subsequent to vapor treatment. The samples were incubated for 7 days at 55°C, and the tubes were observed daily for the presence or absence of growth. The resulting data were interpreted by the means of MPN tables [27]. The determination of the inactivation kinetics was repeated three times for every cycle parameter set.

Results and Discussion

D Value Studies with BIs Sealed in Tyvek

Decontamination Cycle

The sterilizing atmospheres created for the *D* value studies were found to be very repeatable. Figure 2 demonstrates that for the cycles with HL 2 (for 400, 600, and 800 ppm H_2O_2), the water concentration was reproducible. The same repeatability was also found for the other cycles. Cycle repeatability was crucial because the duration of the study required 2 months, with each cycle performed several times for the determination of repeated *D* values. As shown in Fig. 2, the hydrogen peroxide concentration in the isolator atmosphere during the bio-decontamination phase was constant as a function of time for all cycles. Due to reduced injection rates in the bio-decontamination phase and increasing chamber temperature during the cycle, from 22°C at the beginning of the cycle to around 27°C at the end of the bio-decontamination phase, the water concentration decreased slightly over time. In Fig. 3, the trend of the water concentration during the bio-decontamination phase was plotted against time for the four distinct HLs. Water concentration follows a similar trend for HLs 1 to 4, reaching a steady-state condition under each condition. Although the exposure conditions for the BIs were not completely fixed in terms of water concentration, the *D* values for the different HLs can be compared because of the similar trend of the water concentration in HLs 1 to 4.

Water and Hydrogen Peroxide Concentration

In this study, the *D* values provide a means of comparing the inactivation efficiency of the different sterilant atmospheres. Table 2 summarizes all *D* values determined for the various combinations of hydrogen peroxide and water. For the comparison of the different HLs, the water concentration of every decontamination cycle was averaged for the first 15 min of the bio-decontamination phase and listed together with the respective *D* values. In Figs. 4, 5, and 6,

Table 2 *D* values and average water concentration for decontamination cycles with 400, 600, and 800 ppm hydrogen peroxide during bio-decontamination phase

	HL1 *D* value (min)	*c* (water) (ppm)	HL2 *D* value (min)	*c* (water) (ppm)	HL3 *D* value (min)	*c* (water) (ppm)	HL4 *D* value (min)	*c* (water) (ppm)
400 ppm H_2O_2	14.83	3,612	4.27	10,177	1.38	13,588	0.68	18,151
	14.33	3,919	4.27	10,081	1.13	13,880	0.78	17,469
	14.70	3,919	3.14	10,109	1.43	13,880	0.93	17,469
	–	–	–	–	–	–	0.88	18,383
	–	–	–	–	–	–	0.78	18,383
600 ppm H_2O_2	2.87	6,414	0.87	10,413	0.75	13,924	0.75	18,272
	2.56	5,518	0.83	11,497	0.53	12,898	0.35	18,126
	2.14	7,349	0.88	11,497	0.58	12,898	0.45	18,126
	–	–	1.13	9,992	–	–	0.58	20,052
800 ppm H_2O_2	0.56	6,741	0.48	11,250	0.25	14,046	0.38	17,184
	0.53	7,213	0.65	9,550	0.28	14,525	0.48	17,378
	0.53	8,633	0.65	9,550	0.28	14,525	0.43	17,378
	–	–	0.45	9,386	–	–	0.43	17,378

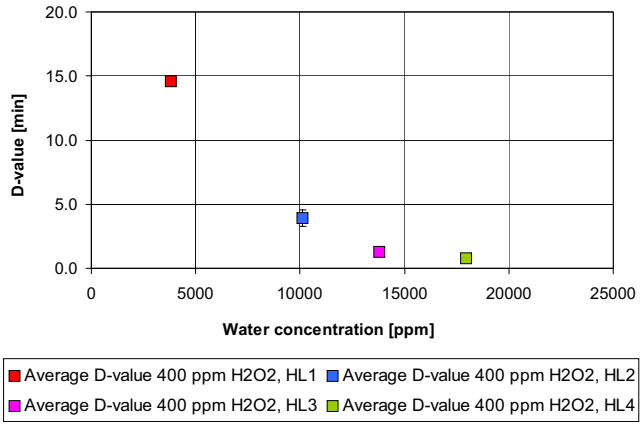

Fig. 4 Effect of increasing relative humidity on sporicidal activity at 400 ppm of hydrogen peroxide (water concentration averaged for first 15 min of bio-decontamination phase)

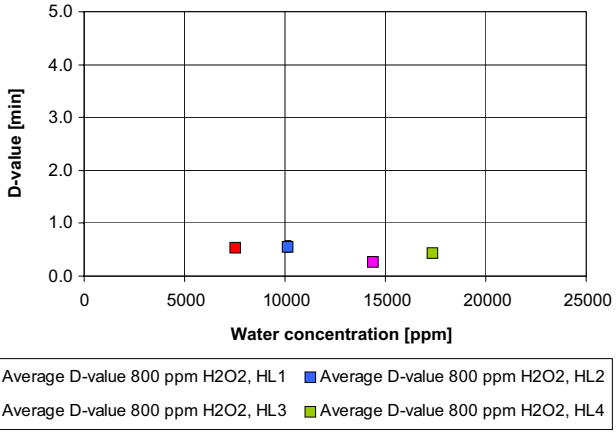

Fig. 6 Effect of increasing relative humidity on sporicidal activity at 800 ppm of hydrogen peroxide (water concentration averaged for first 15 min of bio-decontamination phase)

the determined *D* values are plotted against the water concentration for the 400, 600, and 800 ppm peroxide cycles. The data points represent a mean of the *D* values and the average water concentration determined for every set of water and hydrogen peroxide concentration. The *y*-axis error bars show the standard deviation of the calculated *D* values.

The reported data is valid for the tested decontamination equipment in combination with the according environmental conditions and the biological test system used. The results presented in Table 2 and Figs. 4, 5, and 6 show clearly that, for an identical HL, an increasing concentration of hydrogen peroxide vapor results in a faster inactivation of the test organisms. A correlation between the hydrogen peroxide vapor concentration and the death rate of the microorganisms is especially apparent for HL 1: The average *D* value for 400 ppm hydrogen peroxide is 14.6 min, for 600 ppm 2.5 min, and for 800 ppm only 0.5 min. For HL 2, the experiments show the same qualitative outcome but with diminished *D* value intervals between the different hydrogen peroxide concentration levels (3.9 [400 ppm], 0.9 [600 ppm], and 0.6 min [800 ppm H_2O_2]).

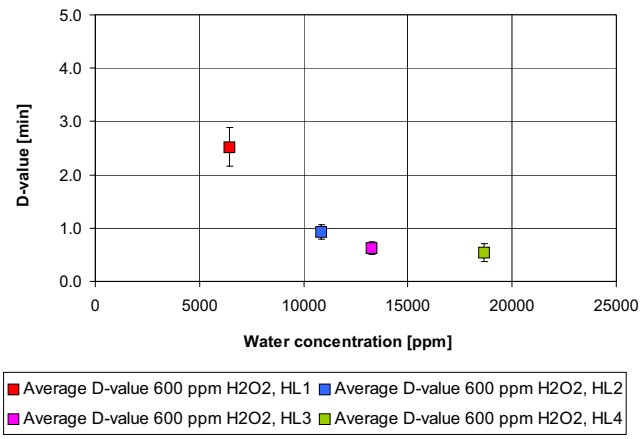

Fig. 5 Effect of increasing relative humidity on sporicidal activity at 600 ppm of hydrogen peroxide (water concentration averaged for first 15 min of bio-decontamination phase)

The linear dependency of H_2O_2 vapor concentration and microbial kill has been the subject of controversial discussion in the literature. While some authors have the opinion that antimicrobial efficacy increases with peroxide concentration [13], others doubt a correlation between the measured H_2O_2 concentration and the absolute antimicrobial efficacy [15] and have provided inactivation studies conducted at low hydrogen peroxide concentrations [12].

Figures 4, 5, and 6 demonstrate that the hydrogen peroxide concentration is an important factor in the inactivation efficacy but that the *D* values converge with increasing HLs. With a low hydrogen peroxide gas concentration (400 ppm), a higher humidity is necessary to achieve the same kill rates than with a high gas concentration (800 ppm). All test cycles show increasing sporicidal activity with increasing water concentration in the chamber. This could be explained by the intensified condensation events occurring due to the saturation of the isolator atmosphere with water and hydrogen peroxide [14, 16]. The process of condensate deposition on a surface is based on a series of successive steps starting with the adsorption of gas molecules, followed by the formation and growth of a thin film, and finally the development of droplets. The process of the formation and the microscopic deposition of aqueous hydrogen peroxide solution on the chamber surfaces can be referred to as "microcondensation."

As seen in Table 2, the cycles consisting of HL 1 with 800 ppm hydrogen peroxide concentration showed an average water concentration of 7,500 ppm. The water content of the cycles consisting of HL 1 with 400 and 600 ppm hydrogen peroxide exhibited water concentrations

of 3,800 and 6,400 ppm water, respectively. The data suggest that for high hydrogen peroxide concentrations (equal to or exceeding 800 ppm), the additional humidity becomes less important with respect to the kill. This is demonstrated in Fig. 6, where the amount of water transferred into the system by evaporation of the 35% liquid hydrogen peroxide at HL 1 enables a highly efficient kill with a resulting average *D* value of 0.5 min. Higher amounts of water in the system with around 10,100, 14,400, and 17,300 ppm water (HLs 2, 3, and 4) do not result in further significant reductions in the *D* value. Consequently, for the highest tested hydrogen peroxide concentration (800 ppm H_2O_2), the inactivation effect is independent of the HL.

Hydrogen Peroxide Consumption

For a cost-effective decontamination cycle, it is necessary to minimize the use of hydrogen peroxide. This produces a savings of the consumable sterilant, hydrogen peroxide, and a savings in the time and energy used for the aeration of the isolator following the bio-decontamination phase. Figure 7 shows the amount of hydrogen peroxide consumed during the conditioning phase for every combination of H_2O_2 concentration and humidity tested. The H_2O_2 consumption is plotted versus the average *D* value determined for the particular decontamination cycle.

It can be seen from Fig. 7 that the decontamination cycles with a consumption between 150 and 250 g hydrogen peroxide during the conditioning phase have a noticeably economic ratio between the amount of hydrogen peroxide vaporized and the achieved kill rate, compared to the other cycles.

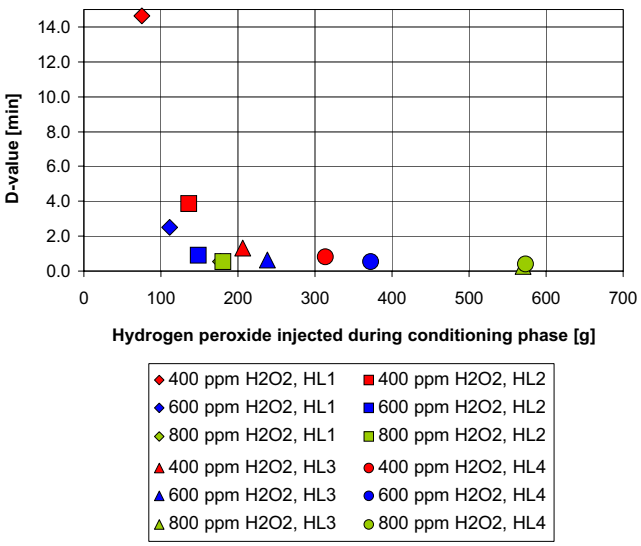

Fig. 7 Amount of hydrogen peroxide injected into the decontamination chamber during conditioning phase plotted against the average *D* value

The most economical decontamination cycles were those with a high peroxide concentration combined with a low humidity (800 ppm H_2O_2, HLs 1 and 2), cycles with a medium peroxide concentration and a medium humidity (600 ppm H_2O_2, HL 2), or cycles with a low hydrogen peroxide concentration and a high humidity (400 ppm H_2O_2, HL 3). Both H_2O_2 vapor concentration and humidity determine the kill efficacy.

The results for the other decontamination cycles shown in Fig. 7 indicated either (a) insufficient inactivation of the test organisms (*D* values greater than 2 min for H_2O_2 vaporization less than 150 g) or (b) that unreasonably high amounts of peroxide (H_2O_2 vaporization greater than 300 g) were expended without a decrease of the *D* value. In the test system, a vaporization of greater than or equal to 200 g peroxide during the conditioning phase resulted in a *D* value below 1 min for every cycle. An increase in the mass of hydrogen peroxide vaporized did not result in any further decrease in the inactivation rate.

The decontamination cycles in Fig. 7 that were economical in their use of H_2O_2 all had high vaporization rates with hydrogen peroxide consumption ranging between 25 and 28 g/min. This indicates that less peroxide is needed to achieve a good kill when the H_2O_2 concentration in the isolator atmosphere is rapidly increased during the conditioning phase. By the use of an efficient flash vaporizer, high gas concentrations and an efficient decontamination process can be achieved. Additionally, a minimized H_2O_2 injection volume also has the positive secondary effect that the load of peroxide on the barrier system (e.g., filters) is reduced, resulting in the preservation of the construction materials and a shortening of the cycle time.

Condensation

According to the prevailing laws of physics, a certain amount of condensation cannot be avoided when injecting hydrogen peroxide vapor and water vapor into a system at conditions typically present in a pharmaceutical isolator (20% relative humidity, 25°C to 30°C) [16, 17].

However, there is disagreement about the mechanism of microbial inactivation by the hydrogen peroxide vapor. Some investigators hold the opinion that decontamination is a dry vapor process and condensation should be avoided [14], while others state that only condensation induces the sporicidal activity of the hydrogen peroxide vapor [15, 17].

To learn more about the basic relationship between condensation and microbial inactivation, the condensation level was recorded for every cycle in this study. Figure 8 illustrates the correlation between the micro-condensation level and *D* value, while Fig. 9 exhibits the relationship between the hydrogen peroxide concentration and humidity. The asterisks in each plot represent

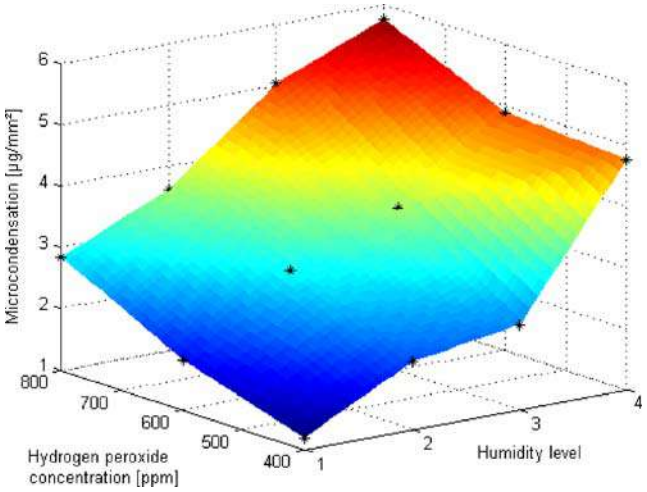

Fig. 8 Average microcondensation measured during bio-decontamination phase plotted against average hydrogen peroxide concentration during the first 15 min of bio-decontamination and humidity level (linear interpolation)

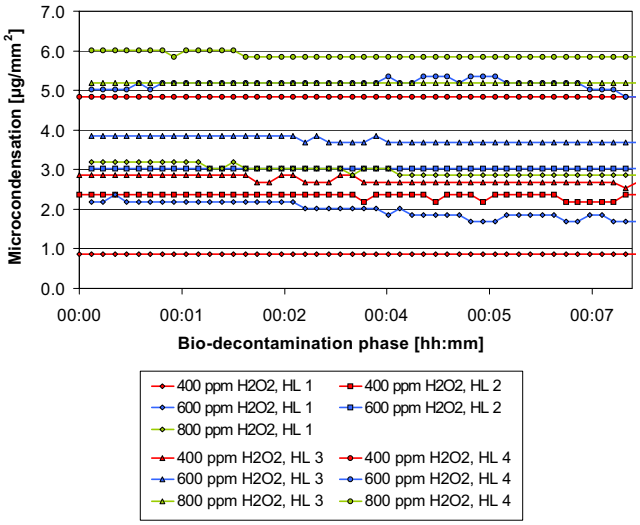

Fig. 10 Microcondensation measurement during bio-decontamination phase for cycles with different combinations of water and hydrogen peroxide concentrations in the isolator atmosphere

individual data points in the three-dimensional coordinate system. A linear interpolation is displayed by the colored traces. It was found that microcondensation occurred at all tested humidity and hydrogen peroxide levels. The condensation sensor detected condensation amounts considerably beneath the range where fogging becomes visible. Condensation was reproducibly found to become visible at about 2.9 µg/mm^2 for the given ambient conditions.

Condensation during the bio-decontamination phase of the test cycles, as detected by the dew sensor, is shown in Fig. 10. The amount of condensate deposited on the surfaces during the various cycles remained stable during the bio-decontamination phase when the BIs were exposed for the determination of *D* values. The bar chart in Fig. 11 shows that the microcondensation levels averaged over the bio-decontamination phase for all test cycles. As expected, the lowest condensation level was found for the cycle with 400 ppm H_2O_2 and HL 1 and the highest for 800 ppm H_2O_2 and HL 4. Within each set of cycles with the same hydrogen peroxide concentration, condensation increases with rising HL. Similarly, for the same HL, the condensation grows with increasing hydrogen peroxide concentration.

In Fig. 12, the condensation during the bio-decontamination was averaged for every set of cycles and plotted against the average *D* value, providing another way to evaluate the impact of condensation on the microbial inactivation. The diagram indicates that a low amount of microcondensation results in a noticeable decrease in the microbial inactivation rate. This is demonstrated, most obviously, by the cycle with 400 ppm H_2O_2 and HL 1. The extremely low humidity (1 µg/mm^2 average microcondensation) during this cycle results in a very low inactivation of the test organisms. An increase in the microcondensation level from 1 to 2 µg/mm^2

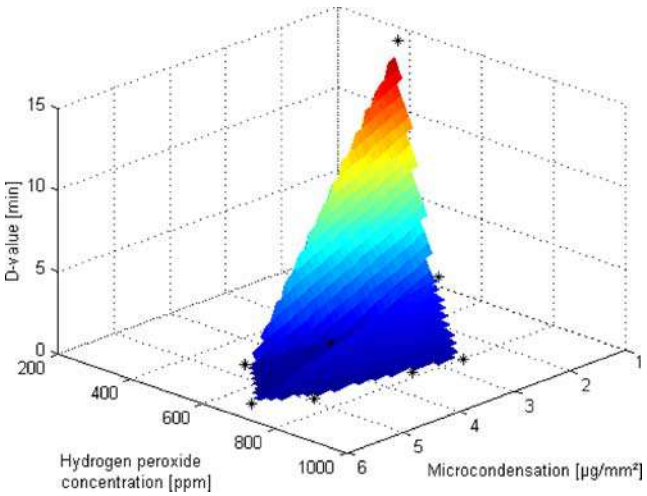

Fig. 9 Average *D* value plotted against average hydrogen peroxide concentration and microcondensation during the first 15 min of bio-decontamination phase (linear interpolation)

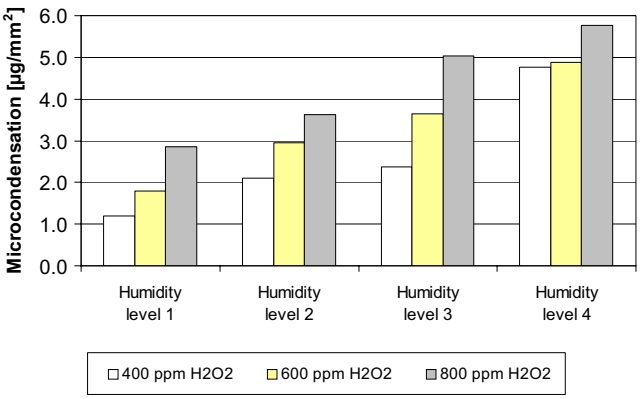

Fig. 11 Comparison of average condensation during bio-decontamination for cycles with 400, 600, and 800 ppm H_2O_2 and humidity levels 1 to 4 measured with the NIR probe

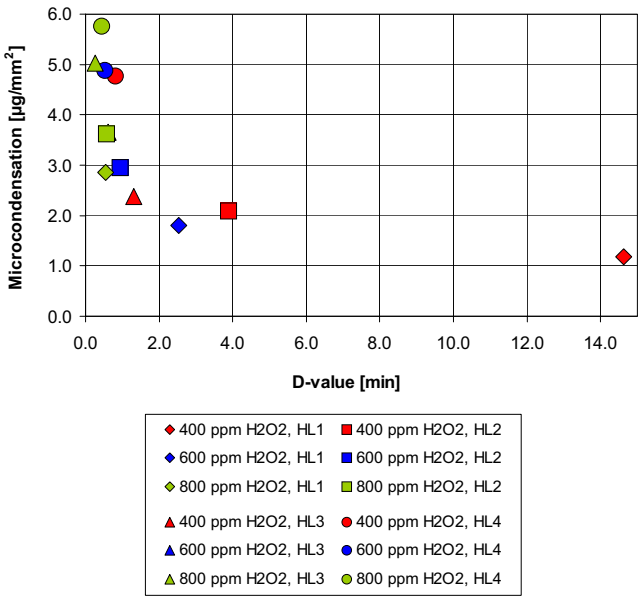

Fig. 12 Average microcondensation during first 15 min of biodecontamination phase plotted against average *D* value

reduces the *D* value dramatically from 14.6 (400 ppm H_2O_2, HL 1) to 3.9 min (400 ppm H_2O_2, HL 2).

Figure 12 also demonstrates that, at similar $H2O_2$ levels, higher water concentration and increased microcondensation promote enhanced microbial inactivation. Beyond microcondensation levels of 2.9 µg/mm^2, however, further condensation does not improve inactivation. Another important finding that supports the results of Watling [18] is that visible condensation is apparently not necessary to achieve good sporicidal conditions. Subvisible microcondensation is effectual for short inactivation times.

It is known that hydrogen peroxide, particularly in the liquid phase, can cause corrosion of construction materials. In addition, the aeration phase can be prolonged and problems with irregular decontamination can arise when dripping condensation occurs in the system [14]. Strong condensation with the formation of visible droplets during decontamination is therefore undesirable and should be prevented. The experiments conducted in this study showed that microcondensation is part of the sporicidal cycle with vaporous hydrogen peroxide and that some condensation is required for an efficient kill. Therefore, it is reasonable to develop decontamination cycles with the required amount of condensation needed to achieve quick microbial inactivation but not sufficient to allow excessive condensation that can result in material and aeration problems.

D Value Studies with BIs Sealed in Tyvek™ and Unsealed

D values were determined for unsealed BIs exposed to HLs 1 to 4 for the 600-ppm hydrogen peroxide level. The results are illustrated in Fig. 13 together with *D* values for indicators sealed in Tyvek™ derived from the same set of decontamination cycles.

As expected, the spores on the unsealed carriers were inactivated faster than those sealed in Tyvek™, and a higher HL resulted in a faster kill for all indicators. Tyvek™ hinders the diffusion of the vapor to the BIs. For higher HLs, the *D* value difference between unsealed and sealed indicators decreased from 0.8 (HL 1) to 0.2 min (HL 4). The influence of the Tyvek™ barrier on the *D* value appears to diminish with increased humidity and more significant condensation. Increasing humidity appears to promote the penetration of the Tyvek™ barrier.

Spore Survivor Curves (MPN Method)

Decontamination cycles at 400 and 600 ppm H_2O_2 were studied further to evaluate inactivation kinetics. The cycles with 800 ppm hydrogen peroxide were omitted from this part of the study because a preliminary experiment showed rapid kill and no significant differences between the inactivation curves of the four HLs.

The number of spores surviving after predefined exposure intervals was quantified by MPN procedures. For each condition, three consecutive decontamination runs were performed. The mean value of surviving spores from the three enumerations was plotted in Fig. 14 versus the respective exposure time, and the *y*-axis error bars represent the standard deviation. In case no growth occurred in all test tubes of a dilution series, the resulting number of surviving spores in the original sample according to the applied MPN table [27] was three viable microorganisms. This lower limit of detection for the MPN method is drawn as a horizontal line in the charts of Fig. 14. Data points for which no growth occurred in the experiment were plotted on the limit of detection line in the diagram.

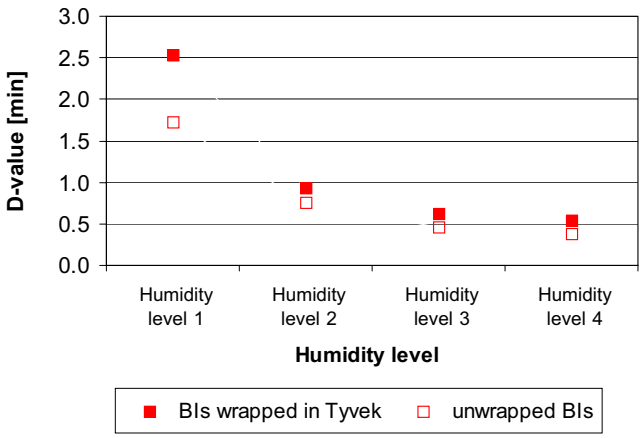

Fig. 13 *D* values for biological indicators with and without a Tyvek™ envelope at 600 ppm H_2O_2

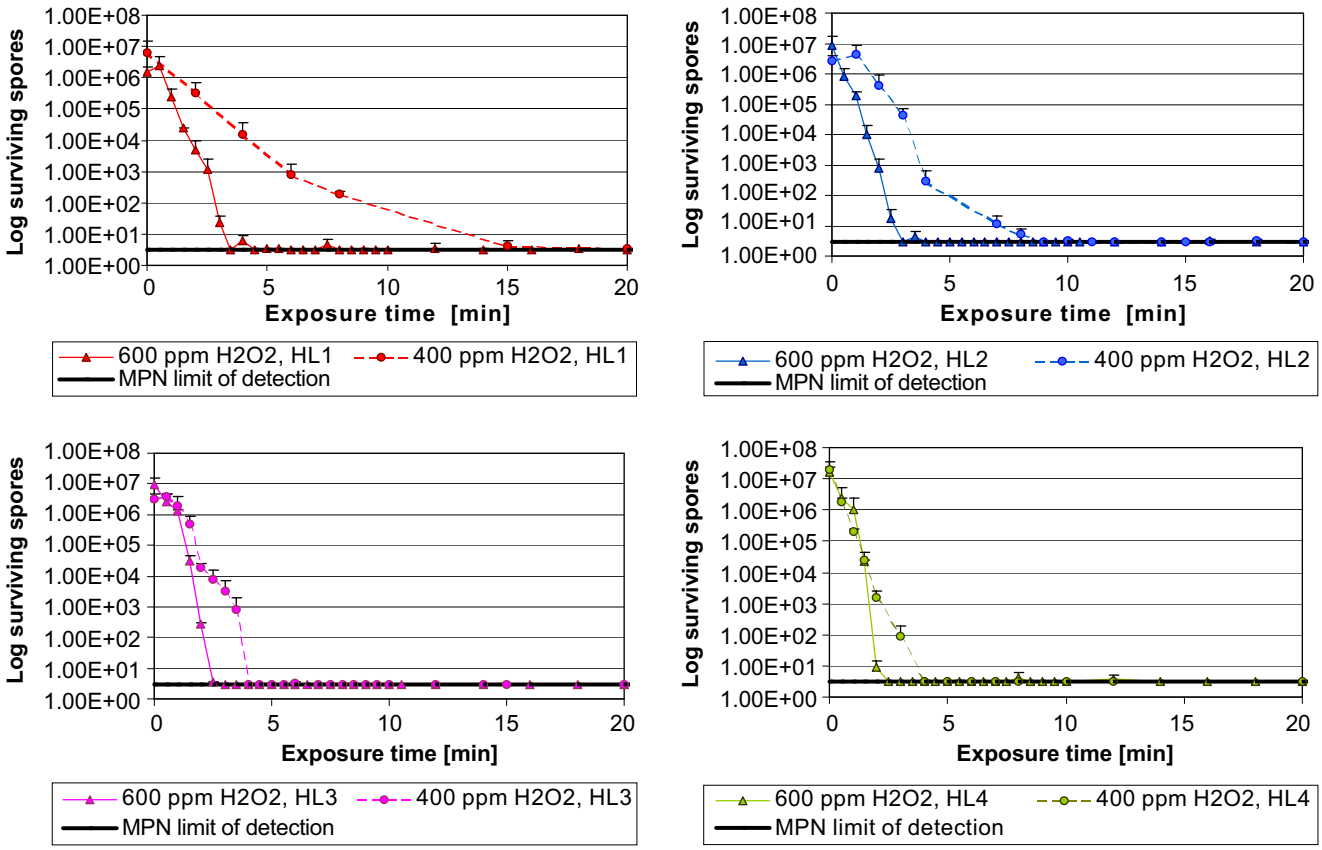

Fig. 14 Inactivation kinetics for humidity levels 1 to 4 for 400 and 600 ppm hydrogen peroxide (limit of detection=3.00E+00 spores)

The initial spore population on the inoculated discs presented in Fig. 14 was found to be slightly higher with the MPN method than the 2.4×10^6 cfu per carrier, which was certified by the manufacturer of the BIs and verified according to USP [20]. The maximum value detected by the MPN procedure was 1.9×10^7, and the average value was 8.3×10^6 cfu per carrier. This indicates that the MPN procedure tends to overestimate the number of microorganisms. The finding corresponds to several articles that report a built-in positive bias of the MPN method [28, 29]. Nevertheless, the overall results of the spore survivor curves provide valuable information concerning the inactivation pattern for the various sporicidal atmospheres, and the conclusions derived from the end-point *D* value data are confirmed.

Figure 14 shows that the kill proceeds faster for atmospheres with a higher peroxide concentration (600 ppm compared to 400 ppm H_2O_2) and that an increased HL results in a faster inactivation of the spores. The differences between the HLs are more distinct for the 400-ppm H_2O_2 cycles as compared to the 600-ppm cycles. The inactivation rates of the cycles with 600 ppm H_2O_2, HL 1 and 400 ppm H_2O_2, HL 4 demonstrate that a lower hydrogen peroxide concentration can be compensated by a higher humidity.

Additional information is gained from the slope of the inactivation curves shown in Fig. 14. For the 600-ppm H_2O_2 condition, the slopes of all inactivation curves are very similar, indicating that decontamination follows similar kinetics at various humidity levels. In contrast, the inactivation rates for 400 ppm H_2O_2 varies with HL. The plot indicates that the 600-ppm H_2O_2 atmospheres provide nearly decontamination rates, whereas the 400-ppm conditions show a lag phase and distinctive tailing, especially for HLs 1 and 2. This correlates with the data in Figs. 10 and 12, which show a clearly lower microcondensation level for these two atmospheres than for the other test atmospheres. The water and hydrogen peroxide vapor would appear to be inadequate to assure sufficient sterilant deposition on the surfaces. This finding indicates that the deposition of water and hydrogen peroxide on the surface is primarily responsible for the microbial inactivation, whereas the hydrogen peroxide vapor concentration is of secondary significance. During cycle development, it is common practice to extrapolate the cycle time needed to achieve a complete kill from the determined *D* values. From the calculated time needed for a one log reduction, the time needed for a 6 log reduction is extrapolated. The results of the MPN survey, shown in Fig. 14, imply that, for a nonlinear inactivation curve, such an extrapolation might not be acceptable. It should, therefore, first be demonstrated that the test organisms show a linear inactivation behavior under the given conditions.

Conclusions

From the results presented in this investigation, it can be seen that a combination of several parameters is required for the successful inactivation of *G. stearothermophilus* spores with hydrogen peroxide vapor. The process was found to be sensitive to relative humidity, hydrogen peroxide vapor concentration, and condensation levels. Higher humidity and hydrogen peroxide concentration were found to promote the microbial inactivation rate. Based on the observations, it was suggested that the dominant kill factor is the overall deposition of water and hydrogen peroxide on the material surface to be decontaminated. For the 800-ppm hydrogen peroxide concentration, the inactivation rate was independent from the HL. At lower H_2O_2 concentrations, higher humidity provided decontamination that was comparable to higher H_2O_2 concentrations at low humidity. This suggests that effective microbial inactivation can be achieved with different approaches of cycle development. Subvisible condensation was discovered to increase the inactivation rate, but dripping condensation should be avoided due to potentially negative effects on materials, aeration time, and uniform decontamination efficiency.

Acknowledgment We acknowledge Benjamin Stelzner for his assistance with the preparation and execution of the experiments.

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