TECHNOLOGY/APPLICATION

Development and Quantification of H_2O_2 Decontamination Cycles

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ABSTRACT: Whereas correlation of physical process parameters with bacterial reduction is well established in thermal sterilisation, such a method is currently neither generally recognised nor possible for H_2O_2 decontamination. As a result, the efficiency and reproducibility of H_2O_2 decontamination and the course of the process over time can at present only be ascertained, verified, and documented using a microbiological system.

Based on the "Fractional Negative" method of determining the D-values of Biological Indicators (BIs), which is contained in the ISO 11138-1 and EN 866-3 standards, a complete and systematic method is presented that enables the parameters for each cycle phase to be determined and verified, and the effectiveness of the process to be quantified. The method also enables differences in bacterial reduction between positions which can be effectively decontaminated and "worst case" positions to be quantified, so that, using the results, the process can be individually adjusted to specific overall bacterial reduction requirements. The new method also specifies the procedure for assessing the suitability of the microbiological system used prior to qualification and validation a conditio sine qua non if process parameter studies are to be used to establish and document a decontamination cycle.

With the aid of practical experimental data, this paper presents in detail the individual stages involved in the method proposed for decontamination cycle development, and interpretation of the results and their implications for the process parameters. In particular, it is shown that bacterial reduction is only stable over time under certain conditions, and that doubling the decontamination time does not result in doubling of kill effect. Moreover, the method makes it possible to react to any fluctuations in resistance in the microbiological system employed, which occur during requalification of the process.

Keywords: Hydrogen Peroxide, H_2O_2 decontamination, cycle development, biological indicator (BI), biological indicator calibration, sterility assurance, survival time, kill time, Fractional Negative Method, Limited Spearman Karber Method (LSKM), minimized LSKM, reactive pattern recognition, Skan integrated sterilization system (SIS).

Introduction

When working on the development of H_2O_2 decontamination cycles and their requalification, there is a recurring problem of obtaining random

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results that casts doubt on the procedure and cannot be properly explained. The usual procedure is as follows:

- Choose a BI with a recognised bacterial spore type and known number of spores (typically 10^6 spores/carrier).
- Place a large number of BIs in the zone to be decontaminated in a range of different positions.
- Perform a number of iterations aimed at killing the microorganisms.
- If all the BIs test negative in the growth test, then the parameters for the destruction of 10^6 spores are given.
- The reaction time is doubled to increase safety level.

In practice, isolated positive samples may be found, or else the target overall bacterial reduction cannot be demonstrated. The technique used up to now to develop decontamination cycles does not produce procedural transparency (it is not possible to attribute influence and effect clearly), nor does it permit quantification of the different factors.

For this reason, one of the objectives in developing the Skan Integrated Sterilisation system (SIS) H_2O_2 decontamination system was to establish an easy and traceable cycle development procedure. The method described below demonstrates how to quantify the resistance and the influencing factors using known models and statistical analysis. Applying the Limited Spearman Karber Method (LSKM), the BI is first of all tested for its suitability as a sensor. Only then is it used to develop decontamination cycles. To improve transparency, cycle development is broken down into a number of sub-steps that are analysed and verified individually. The data derived from this procedure allow overall bacterial reduction to be specified reliably and enable simple evaluation in cases of requalification.

Principles

Survival time model

The resistance of BIs to a defined inactivation method is expressed as a decimal reduction per unit of time [mins], the D-value. The D-value thus specifies the time it takes to reduce the population of the test organism by 90% (1, 2, 3).

If N_0 is defined as the initial number of test organisms at time t = 0 and $N_{(t)}$ is the number of surviving test organisms at time t, then the survival time model of the BI is defined as:

[1] $N(t) = N_0 10^{-t/D}$ *(where D = D-value)*

When the population is expressed on a log scale, as is customary, this produces:

[2] $\log N(t) = \log N_0 - t/D$

When displayed graphically in semi-logarithmic form, the survival curve appears as a straight line whose origin is {t = 0 [min], log N_0} and whose slope is $\frac{-1}{D-value}$ (6, 7) (Figure 1).

The D-value is used to define a time window (survival/kill window) inside which the transition from reliably positive to reliably negative results takes place (1, 2, 4).

The survival time is defined as:

[3] $< (\log N_0 - 2)$ *x specified D – value [*min*]*

The kill time is defined as:

[4] $> (\log N_0 + 4)$ *x specified D – value [*min*]*

This means that Bis, which are exposed for less than the survival time to the specified inactivation, then test reliably positive in the subsequent growth test. Exposure for longer than the kill time produces reliably negative results. Between the survival time and the kill time lies the "fractional field." This time window represents the later stages of microbial inactivation; i.e., only a small number of surviving microorganisms are left on the carrier, some of which test positive and some negative in a growth test (4) (Figure 1).

The definition of the survival/kill window, and hence the transition from reliably positive results through the fractional field to reliably negative results, is based on a probability distribution (6, 7).

If N_0 is defined as the initial number of microorganisms, the average number of surviving microorganisms $N_{(t)}$ after t minutes exposure is given by (from [1]): (7)

[5] $$N(t) = 10^{\left(\log N_0 - t/D\right)}$$

If $N_{(t)}$ represents the average number of surviving microorganisms, the probability $P(N_{(t)})$ that a very small $N_{(t)}$ will produce a negative result is given by: (7)

[6] $$P_{(N(t))} = e^{-N(t)}$$

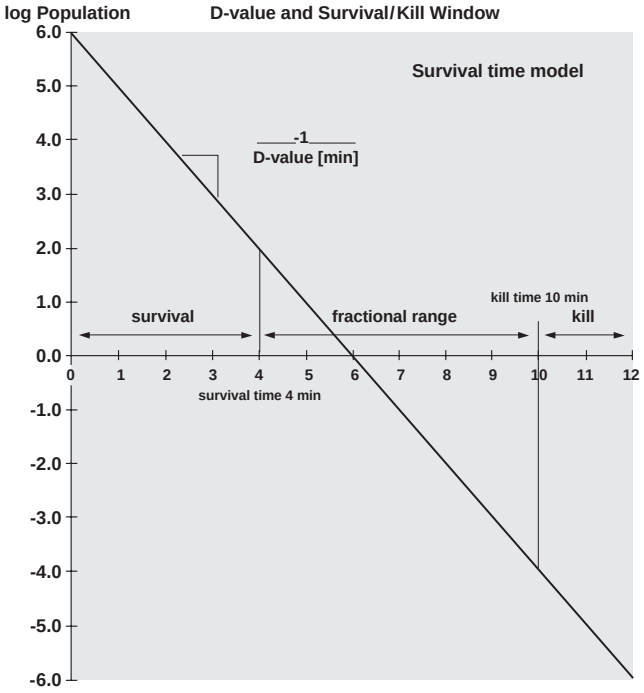
On this basis the survival curve is now obtained, a probability distribution of positive and negative results expressed as a function of exposure time.

Example

If we assume an initial number of microorganisms N_0 of 1x 10^6 and a D-value of 1 min, the probability of observing a negative result is near 0% for exposures equal to the duration of the defined survival time, and near 100% for exposures equal to the duration of the kill time. It is interesting to consider an exposure duration of 6 mins, which in this example corresponds to a bacterial reduction of six orders of magnitude. From this we obtain the following:

Exposure time	6 mins
$N_{(t)}$:	$10^0 = 1$
$P(N_{(t)})$:	0.367

Figure 1: Survival time model, N_0 1 x 10^6 [CFU], D-value 1 [mins].



BIs with an average of one surviving microorganism have a 37% probability of testing negative in the growth test and a 63% probability of testing positive. The evaluation of BIs using the growth test provides no basis for quantifying the residual number of microorganisms on the carrier and hence the bacterial reduction obtained. In order to be able to observe

reliably negative results, the initial population of a BI must be reduced by more than the number of inoculated microorganisms. If the range of sizes of the initial population is reduced, positive and negative results occur in the ratio 2:1.

The exponential survival time model (the survival curve as a semi-logarithmic straight line) is generally recognised as a means of describing the inactivation of microorganisms (6, 7). The reduction rate, which an initial population experiences during a decontamination cycle, is a measure of the resistance of a BI to the selected cycle parameters, with the D-value as the slope of the survival curve being the defining parameter. If a strong decontamination effect is shown on the BI, the slope of the survival curve is steeper; i.e., the D-value drops and reliably negative results are observed earlier. Conversely, with a weak decontamination effect, negative results occur only very late, the survival curve is flat, and hence the D-value is high.

If a BI behaves as predicted by the survival time model during inactivation with gaseous H_2O_2, the D-value for this BI and the changes in its D-value over the course of the decontamination cycle can be used to assign parameters to, describe and quantify the decontamination capacity of the cycle.

Model behaviour, calculation of D-value

The LSKM referenced in the standards allows a D-value to be calculated for BIs in modern isolator systems and comparable applications utilising H_2O_2 decontamination methods. With the LSKM, the final stages of microbial inactivation are captured. In this time window, the fractional field, a ratio of positive to negative results, is observed as described on the model above. When the initial population and the time for which the test organism is exposed to the inactivation method are known, these results are used to calculate the "mean time to sterility"(U_{sk}), from which the D-value can be derived.

For the LSKM in practice, several groups of BIs are exposed simultaneously to bacterial reduction. The groups are sequentially removed from the decontamination cycle at a constant time interval *d* and then evaluated using the growth test. The exposure times for the individual BI groups, and hence the intervals at which they are removed, are chosen so that the results of the LSKM provide a "map" or "reactive pattern" of the entire survival time model, from reliably positive results through the fractional field to reliably negative results.

Figure 2 shows the results of a LSKM, which complies with USP (5) requirements as to its execution, and the trend of the individual group results. The experiment was carried out using a defined H_2O_2 decontamination cycle and commercially available BIs.

Group 1 shows an overall positive result preceding the fractional field, which is formed by groups 2 to 6. Then come four all-negative groups. Based on these results, a D-value of 2.06 $\pm$ 0.2 mins can be estimated with 95% confidence limits.

The results shown for the LSKM reflect the survival time model extremely well and permit accurate estimation of the D-value and of the 95% confidence limits for it. In order to be able to achieve such a result, the expected D-value must be known prior to perform the LSKM, so that the time window for removal of the BIs can be precisely set. The narrow confidence limits for the calculated D-value results from the large number of groups and the short removal interval, which requires more than 100 BI samples.

Figure 2:Result of the LSKM.

Method parameters	
Number of groups	10
Number of BIs per group	10
Removal interval d [mins]	2.5
Initial population N_0 [CFU]	2.0×10^6

+ = growth
— = no growth

Exposure time (mins)		6.0	8.5	11.0	13.5	16.0	18.5	21.0	23.5	26.0	28.5		
Result	**1**	+	+	+	+	+	+	—	—	—	—	+	—
	2	+	+	+	+	+	+	—	—	—	—	+	—
	3	+	+	+	+	—	—	—	—	—	—		
	4	+	+	+	+	—	—	—	—	—	—		
	5	+	+	+	—	—	—	—	—	—	—		
	6	+	+	+	—	—	—	—	—	—	—		
	7	+	+	+	—	—	—	—	—	—	—		
	8	+	+	+	—	—	—	—	—	—	—		
	9	+	+	—	—	—	—	—	—	—	—		
	10	+	—	—	—	—	—	—	—	—	—		

For the purposes of cycle development, a precise D-value for the BI used is less important than the mapping of its reactive pattern towards the bacterial reduction throughout the cycle. Figure 3 shows the results of a minimized LSKM. Three BIs were used per group and a removal interval of 3 mins was selected. The BIs and cycle parameters used were identical to those previously described for the untrimmed LSKM in Figure 2.

Groups 1 and 2 both test all-positive, groups 3 and 4 constitute the fractional field, and then come six groups with all-negative results. Using the LSKM formulae, a D-value of 2.0 mins can be estimated from these results.

A LSKM trimmed in this way maps the reactive pattern of the BI very clearly and produces a good estimate of the D-value, even when compared with the complete LSKM. All in all, it provides a comprehensive description of the resistance behaviour of the BI. Because of the lower number of groups and the larger intervals at which BIs are removed, the resolution in the fractional field is not so detailed; however, the rapid transition and the large number of all-negative groups provide clear evidence of the effectiveness of the decontamination. The reduction rate obtained can be quantified from the estimated D-value.

Figure 2:Result of minimizes LSKM.

Method parameters	
Number of groups	10
Number of BIs per group	3
Removal interval d [mins]	2.5
Initial population N_0 [CFU]	2.0×10^6

+ = growth
— = no growth

Group		1	2	3	4	5	6	7	8	9	10	Pos.	Neg.
Exposure time (mins)		6.0	9.0	12.0	15.0	18.0	21.0	24.0	27.0	30.0	33.0		
Result	**1**	+	+	+	+	—	—	—	—	—	—	+	—
	2	+	+	+	—	—	—	—	—	—	—	+	—
	3	+	+	—	—	—	—	—	—	—	—		

The minimized LSKM is a suitable tool for visualising the reactive pattern of BIs and for estimating and quantifying the bacterial reduction rate obtained. Further on in this paper, the minimized LSKM will be shown to be sensitive to changes in the cycle parameters and to the resistance behaviour of BIs. In practice, performing the minimized LSKM is easily accomplished in any modern system utilising H_2O_2 decontamination without great microbiological lab resources. The results obtained from a minimized LSKM with 30 BIs score well in terms of cost versus benefit. To put this experimental methodology into practice and be in a position to interpret the results of the minimized LSKM, it is only necessary to expose the BI groups in the chamber to the decontamination process at a defined time and to remove them at selected time intervals.

Experiment 0: Reactive pattern recognition

If the BI is to be used as a sensor for specifying the decontamination process, its resistance behaviour must be known in advance. The resistance behaviour is determined prior to the development of the process in a defined and reproducible decontamination cycle of a test chamber. Performing a *reactive pattern recognition*, the model behaviour of the BI is evaluated using the minimized LSKM, its D-value is estimated, and hence its suitability for use in development of the cycle is assessed.

The results of *reactive pattern recognition* performed on different BIs are presented and interpreted below. All BIs used are commercially available and are specified for gaseous H_2O_2. The *reactive pattern recognitions* were all carried out in a test chamber with identical cycle parameters. Where necessary, the LSKM parameters (exposure time, removal interval) were adjusted to the specific resistance of the BIs.

Examples of reactive pattern recognitions:

Example 1: BI A

Test microorganism/ATCC no.	B.stearothermophilus / 7953
Initial population [CFU]	3.5×10^6
Carrier material	CrNi steel

+ = growth
— = no growth

Group		1	2	3	4	5	6	7	8	9	10	Pos.	Neg.
Exposure time (mins)		4.0	8.0	12.0	16.0	20.0	24.0	28.0	32.0	36.0	40.0		
Result	1	+	+	+	+	—	—	—	—	—	—	+	—
	2	+	+	+	+	—	—	—	—	—	—		
	3	+	+	+	—	—	—	—	—	—	—		

BI *A*'s reactive pattern is consistent with the model, and a D-value of 2.6 mins can be estimated from the results of the LSKM.

Example 2: BI B

Test microorganism/ATCC no.	B.stearothermophilus/ 12980
Initial population [CFU]	2.0×10^6
Carrier material	CrNi steel

+ = growth
— = no growth

Group		1	2	3	4	5	6	7	8	9	10	Pos.	Neg.
Exposure time (mins)		6.0	9.0	12.0	15.0	18.0	21.0	24.0	27.0	30.0	33.0		
Result	1	+	+	+	—	—	—	—	—	—	—	+	—
	2	+	+	+	—	—	—	—	—	—	—		
	3	+	+	+	—	—	—	—	—	—	—		

BI *B* shows an acceptable reactive pattern with a D-value of 2.0 mins. The lack of fractional groups results from minimising the LSKM. However, the suitability of the BI for cycle development can nevertheless be assessed.

Example 3: BI C

Test microorganism/ATCC no.	B.stearothermophilus/ 12980
Initial population [CFU]	4.5×10^5
Carrier material	Glass fibre pad

+ = growth
— = no growth

Group		1	2	3	4	5	6	7	8	9	10	Pos.	Neg.
Exposure time (mins)		5.0	10.0	15.0	20.0	25.0	30.0	35.0	40.0	45.0	50.0		
Result	1	+	+	+	+	+	—	+	—	—	—(*)	+	—
	2	+	+	—	—	—	—	—	—	—	—		
	3	+	—	—	—	—	—	—	—	—	—		

BI *C* has a large fractional field, extending from group 2 to group 7, which, along with the estimated D-value of 2.6 mins, departs from the model. This casts doubts on the reliability of the all-negative groups 8 to 10. In an additional experiment using a larger time window, positive results were also obtained for this BI at exposure times of up to 50 mins (*).

Example 4: BI D

Test microorganism/ATCC no.	B.stearothermophilus / 7953
Initial population [CFU]	1.0×10^6
Carrier material	Glass fibre pad

+ = growth
— = no growth

Group		1	2	3	4	5	6	7	8	9	10	Pos.	Neg.
Exposure time (mins)		6.0	9.0	12.0	15.0	18.0	21.0	24.0	27.0	30.0	33.0		
Result	1	+	—	+	+	+	+	+	—	+	+	+	—
	2	+	—	+	+	+	+	+	—	—	+		
	3	—	—	—	+	—	+	—	—	—	+		

BI *D* shows the first negative result after exposures of 6 mins in group 1; however, a clear transition to all-negative results is not observed even after exposure times of 33 mins in group 10. In additional trials to determine the resistance of this BI, this stochastic pattern of positive and negative results was found at exposure times of up to 70 mins.

It is not possible to either specify or develop a decontamination cycle with BIs of type *C* and *D*. When using BIs with such reactive pattern, random late-positive results are found over the whole course of cycle development, preventing unambiguous interpretation of the experimental data observed. Changes to the cycle variables do not produce any reliable effect on the bacterial reduction. On the other hand, BI *A* and *B* show a reactive pattern that is in line with the model specified in the referenced standards. Randomly late-positive results that are attributable to the model behaviour of the BI can be excluded with these BIs. Hence the experimental results can be projected directly to the bacterial reduction obtained. Thus it is possible to detect insufficient bacterial reduction and, if appropriate, inhomogeneities in the distribution of bacterial reduction.

Two main systems determine the decontamination cycle:

1. The microbiological system with BI and culture media.
2. The system consisting of the chamber to be decontaminated, H_2O_2 evaporation apparatus, and the decontamination procedure.

If the behaviour of one of the two systems is known, then that system can be used to describe and define the unknown system. With the *reactive pattern recognition*, the behaviour of the microbiological system is described and evaluated in a known decontamination cycle. In this sense, the *reactive pattern recognition* serves to calibrate the BI. Only then can the microbiological system thus defined be used to describe a corresponding decontamination system and to develop a cycle.

Decontamination procedure, variables, influencing factors

In H_2O_2 surface decontamination, the overall bacterial reduction is obtained from the release of gaseous H_2O_2 and the effect of the lethal dose over time. H_2O_2 decontamination is subdivided into four cycle phases, as follows:

Phase 1: Preconditioning
In the preconditioning phase, the initial conditions required for decontamination are created in the chamber air.

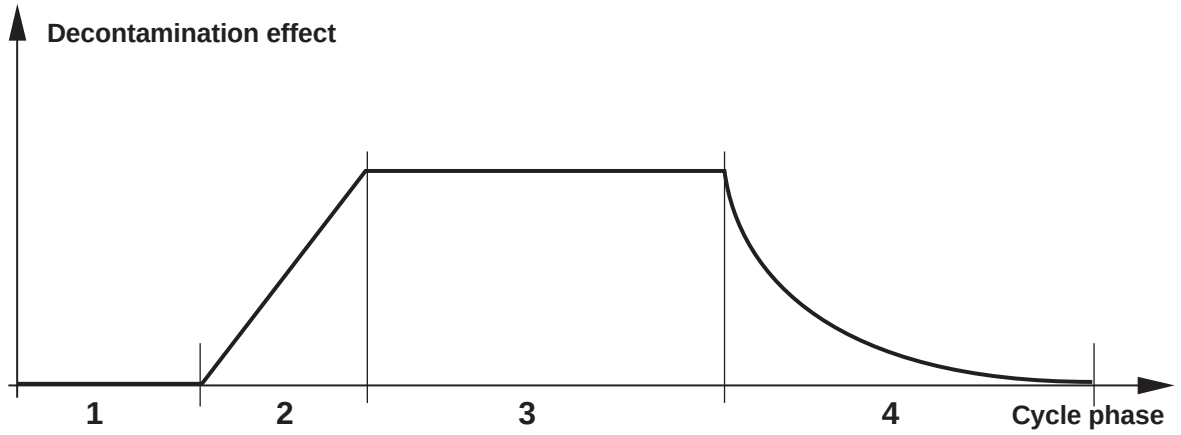
Phase 2: Conditioning
In this phase, the dose of gaseous H_2O_2 necessary to reach the desired decontamination effect is generated in the chamber. For this purpose, an initial quantity of H_2O_2 is vaporised from aqueous solution.

Phase 3: Decontamination
In this phase, the obtained effective dose is kept stable for the period of time that is necessary in order to achieve the desired decontamination result. In addition, the quantities of gaseous H_2O_2 that are no longer available in the chamber air due to adsorption and absorption on surfaces, used in bacterial reduction and decomposition are continuously made up.

Phase 4: Purging
In this phase, the required maximum residual concentration of H_2O_2 in the chamber is achieved through purging with fresh air.

Figure 4 illustrates the decontamination effect as a function of cycle phase.

Figure 4: Decontamination cycle phases.



Phase influence / parameters and variables

- The preconditioning phase generates defined initial conditions in the chamber air to ensure a reproducible decontamination cycle. During this phase, the parameters humidity [%rH] and temperature [°C] of the chamber air must be monitored.
- The conditioning phase is responsible for achieving a maximum bacterial reduction rate. The vaporised initial quantity (q1) of liquid H_2O_2 [g] per chamber volume [m^3] is the critical process variable in this phase.
- The decontamination phase has two important process parameters. First is the rate (q2) of continuously vaporised H_2O_2 [% q1/h] (q2 is stated as a percentage of the initial quantity q1 per hour). This rate ensures that the bacterial reduction rate previously achieved in the conditioning phase remains stable over the entire duration of the decontamination. Second is the duration of the decontamination [mins]. It ensures the total bacterial reduction at a known reduction rate.
- The purging phase [mins] ensures that the maximum residual concentration of H_2O_2 [ppm] is reached in the chamber.

Accordingly, the following parameters must be ascertained and defined during cycle development, see Table 1.

Table 1

Cycle Phase	Parameters [units]	Effect
Preconditioning	Air humidity [%rH] Air temperature [°C]	Reproducibility of the decontamination cycle
Conditioning	q1 [g/m^3] Initial quantity of liquid H_2O_2 per volume unit	Bacterial reduction rate obtained
Decontamination	q2 [%q1/h] Rate of making up	Maintain stability of the bacterial reduction rate obtained
Decontamination	Duration of the decontamination [mins]	Overall bacterial reduction obtained [log scale] during decontamination
Purging	Purge time [mins]	Residual concentration of H_2O_2 [ppm] obtained in the chamber

A complete decontamination cycle is developed below. The influence of the individual cycle parameters is explained and discussed. Based on the survival time model, the cycle parameters are set and the achieved decontamination capacity is quantified.

Preconditioning, chamber air initial conditions

Air humidity [%rH]

The humidity of the chamber air is lowered to a defined value prior to starting H_2O_2 vaporisation in order to ensure that the chamber air is capable of absorbing the H_2O_2 vapour that will subsequently be introduced. Starting from a defined moisture content, a defined partial pressure ratio of gaseous H_2O_2 and water vapour is then set, and hence a defined effective dose in the chamber. Starting humidities of 10 - 20 %rH at normal chamber temperatures are usually adequate.

Air temperature [°C]

To avoid incurring extra power consumption and time on preconditioning the chamber, it is recommended that the initial temperature of the chamber air is either the working or operating temperature of the chamber. H_2O_2 decontamination is used over a broad range of temperatures, so the optimal process temperature is the one that incurs the lowest cost for the operator.

For the bacterial reduction rate achieved in the subsequent conditioning phase, the initial chamber air conditions constitute secondary effects which are only small compared with the main effect, the quantity of H_2O_2 vaporised. If the quantity of vaporised H_2O_2 is held constant and the initial humidity is varied (10%rH, 20%rH), slightly better bacterial reduction rates are obtained at higher initial humidities than with lower ones. If the temperature is varied (30°C, 40°C), better bacterial reduction rates are obtained at lower temperatures than at higher initial temperatures (8).

It will be shown below that if a suitable initial quantity of H_2O_2 (q1) is chosen in the conditioning phase, the impacts of the side-effects, in the range stated above, on the bacterial reduction rate achieved are so low that they can be ignored (8).

Experiment 1: Bacterial reduction rate achieved, quantity q1

The initial quantity of H_2O_2 per chamber volume (q1) [g/m^3] vaporised during the conditioning phase establishes a killing effect per unit of time [mins] in the chamber. This bacterial reduction rate can be visualised through a minimized LSKM and quantified through the estimated D-value. To perform the minimized LSKM, a position is chosen in the chamber at which a good killing effect can be expected. The influence of local gradients in the decontamination effect on the experimental results is thus excluded at the beginning of cycle development, so that the bacterial reduction rate obtained can be assessed under optimum conditions. Based on the reduction rate thus observed, a relationship can be established between positions where decontamination is good and positions where bacterial reduction is poor. To establish the bacterial reduction rate with the selected quantity q1, the BIs are exposed immediately following the end of the conditioning phase.

The next two experimental results show the effect of the initial quantity q1 on the bacterial reduction rate to be determined. In experiment 1.1, q1 was 5 g/m^3, in experiment 1.2, it was 7.5 g/m^3. All other cycle parameters were held constant.

Experiment 1.1: Bacterial reduction rate achieved with quantity q1 = 5 g/m³

+ = growth
— = no growth

Group		1	2	3	4	5	6	7	8	9	10	Pos.	Neg.
Exposure time (mins)		6.0	9.0	12.0	15.0	18.0	21.0	24.0	27.0	30.0	33.0		
Result	1	+	+	+	+	+	+	+	+	—	—	+	—
	2	+	+	+	+	+	+	—	—	—	—		
	3	+	+	+	+	—	—	—	—	—	—		

Experiment 1.2: Bacterial reduction rate achieved with quantity q1 = 7.5 g/m³

+ = growth
— = no growth

Group		1	2	3	4	5	6	7	8	9	10	Pos.	Neg.
Exposure time (mins)		6.0	9.0	12.0	15.0	18.0	21.0	24.0	27.0	30.0	33.0		
Result	1	+	+	+	—	—	—	—	—	—	—	+	—
	2	+	+	—	—	—	—	—	—	—	—		
	3	+	—	—	—	—	—	—	—	—	—		

Both experiments show no irregularities in the reactive pattern of the BI and therefore may be used to estimate the D-value. Based on the results of experiment 1.1, a D-value of 3.5 mins is estimated, while the results of experiment 1.2 produce an estimated D-value of 1.6 mins.

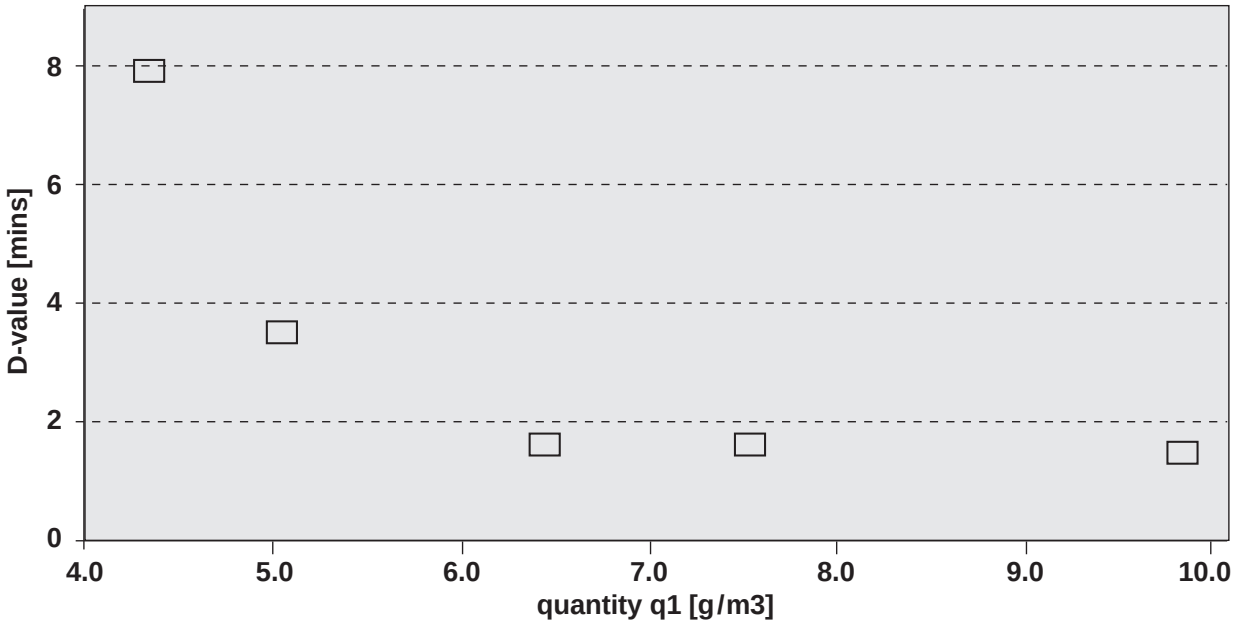
Taken together, the two experiments show the clear dependence of the bacterial reduction rate obtained on the initial quantity q1. An increase in quantity q1, as in experiment 1.2, more than doubles the bacterial reduction rate. These experiments visualize the BI model behaviour and also show how simple and easy it is to interpret the results obtained from the minimized LSKM. The D-value estimation makes results and parameter effects quantifiable.

The dependence of the D-values on quantity q1 is characterized by a non-linear relationship as shown below (Figure 5), (8).

As the initial quantity q1 of H_2O_2 is increased, the D-value falls to a minimum. At this point, the curve shows a sharp bend, and a further increase in quantity q1 does not improve the bacterial reduction rate significantly; the observed D-values remain stable.

At bacterial reduction rates below the maximum bacterial reduction rate, small changes in quantity q1 result in large changes of the D-value. In this region, secondary effects (chamber conditions) are observed to have an influence on the bacterial reduction rate, so that

Figure 5: D-value as a function of quantity q1.



decontamination cannot be considered as stable. If the initial quantity q1 is set so that the bacterial reduction rate comfortably reaches its maximum, changes in quantity q1 have no further effect on the D-value, and the decontamination effect is insensitive to secondary effects. H_2O_2 decontamination is robust and the reproducibility of the bacterial reduction rate is thus ensured.

Experiment 2: Stability of decontamination effect, quantity q2

By making up the H_2O_2 quantity in the decontamination phase at the rate q2 [%q1/h], the stability of the bacteria-reducing effect over the entire duration of the decontamination phase is ensured. Parameter assignment for quantity q2 is likewise performed using the minimized LSKM. To record data on the stability of the bacterial reduction rate, two minimized LSKMs (LSKM 1 and LSKM 2) are carried out over the maximum duration of the decontamination. The BIs for LSKM 1 are exposed immediately following the end of the conditioning phase. The results should reproduce the bacterial reduction rate obtained from the previous experiment, using q1. Exposure of LSKM 2 takes place towards the end of the decontamination phase. The duration of the decontamination phase is set to the maximum, and the positioning of the LSKMs in the chamber is performed in analogous fashion to the determination of quantity q1. The number of groups and removal intervals can be adjusted to the expected results (q1).

Two sets of LSKMs are shown, with the number of groups reduced to five:

- In each case LSKM 1 was exposed 5 mins following the end of conditioning.
- In each case LSKM 2 was exposed 30 mins following the end of conditioning.

In experiment 2.1, the made-up quantity q2 was set at 25% q1/h, and in experiment 2.2, at 100% q1/h. All other parameters were held stable.

Experiment 2.1: Stability of decontamination, quantity q2 = 25% q1/h

*LSKM 1, exposure **5** mins following the end of conditioning:*

Group		1	2	3	4	5	Pos.	Neg.
Exposure time (mins)		7.5	10.0	12.5	15.0	17.5		
Result	1	+	—	—	—	—	+	—
	2	+	—	—	—	—		
	3	—	—	—	—	—		

+ = growth
— = no growth

*LSKM 2, exposure **30** mins following the end of conditioning:*

Group		1	2	3	4	5	Pos.	Neg.
Exposure time (mins)		7.5	10.0	12.5	15.0	17.5		
Result	1	+	+	+	+	+	+	—
	2	+	+	+	+	+		
	3	+	+	+	+	+		

A D-value of approx. 1.3 mins was estimated for LSKM 1 in both sets of experiments. This reproduces well the good bacterial reduction rate obtained in the previous experiment with quantity q1 in the conditioning phase. The results obtained from each of the LSKM 2s and their implications for the stability of the decontamination effect are evident. In experiment 2.1, LSKM 2 produced only all-positive groups and no killing effect was observed. In experiment 2.2, the LSKM 2 reproduced the results of the previous LSKM 1 very well, with an estimated D-value of approx. 1.3 mins.

All in all, the results obtained from the minimized LSKM in the two sets of experiments reveal clearly the importance of the made-up quantity q2 for the *stability* of the bacterial reduction rate. In experiment 2.1, the made-up quantity q2 was not sufficient to sustain the bacterial reduction rate obtained during conditioning. The decontamination effect collapsed and no further bacterial reduction could be observed. The bacterial reduction rate obtained in experiment 2.2, and its stability over time, provide the basis for a decontamination cycle.

The development of an H_2O_2 decontamination cycle with the objective of certifying a defined decontaminating effect is only possible if the bacterial reduction rate is known and stable over time. If conditioning does not produce a stable bacterial reduction rate all over the cycle, the reproducibility of the bacterial reduction, and

Experiment 2.2: Stability of decontamination, quantity q2 = 100% q1/h

*LSKM 1, exposure **5** mins following the end of conditioning:*

Group		1	2	3	4	5	Pos.	Neg.
Exposure time (mins)		7.5	10.0	12.5	15.0	17.5		
Result	1	+	—	—	—	—	+	—
	2	+	—	—	—	—		
	3	+	—	—	—	—		

+ = growth
— = no growth

*LSKM 2, exposure **30** mins following the end of conditioning:*

Group		1	2	3	4	5	Pos.	Neg.
Exposure time (mins)		7.5	10.0	12.5	15.0	17.5		
Result	1	+	—	—	—	—	+	—
	2	+	—	—	—	—		
	3	—	—	—	—	—		

hence of the entire decontamination process, cannot be assured. In practice, random results may then be observed in identical decontamination cycles when the BIs are evaluated.

It is essential to prove the stability of the bacterial reduction rate over time in order to design a decontamination cycle. If the rate is not stable and certified, it cannot be assumed that extending the decontamination phase will have the effect of increasing the overall achievable bacterial reduction. When assessing the decontamination capacity of the process on the basis of a *single* set of exposures of BIs, the bacterial reduction can only be assured by repeating the entire process and *not* by doubling the duration of decontamination.

Changes in the reduction rate over time result directly in changes of the overall bacterial reduction. If these changes are not revealed, it is not possible to draw any firm conclusions as to the finally achievable bacterial reduction.

Estimation of D-value $_{best\ place}$

As the basis for the next steps in cycle development, the reduction rate achieved in experiment 1 was calculated and reproduced with the LSKM 1 from experiment 2. The stability of this reduction rate was confirmed in experiment 2 with LSKM 2. We therefore have three D-value estimations available, and from their mean we obtain the D-value $_{best\ place}$, see Table 2.

The D-value $_{best\ place}$ describes the bacterial reduction rate observed at a position in the chamber, which can be well decontaminated:

Table 2

Experiment	Estimated D-value (mins)
Experiment 1.2	1.6
Experiment 2.2, LSKM 1	1.3
Experiment 2.2, LSKM 2	1.3
D-value $_{best\ place}$ (mean)	**1.4**

Continuing the process of cycle development, the decontamination duration is calculated on the basis of the D-value $_{best\ place}$ and the survival time model. Positions in the chamber with poor decontamination effect (worst cases) are identified and tested. The results are then used to adjust the decontamination to the duration required to guarantee the target bacterial reduction.

Experiment 3: Worst case study, duration of decontamination

In the worst case study, the bacterial reduction is determined at positions in the chamber that are difficult to decontaminate. This is based on the calculated D-value $_{best\ place}$ and the BI survival time model.

The kill time is derived from the definitions of the survival time model. For a given D-value it defines the exposure time in minutes after which the BIs used have to show reliably negative results in the growth test. For the D-value $_{best\ place}$ in above example (1.4 mins) and the initial population of microorganisms of 1×10^6 [CFU], the required kill time is calculated to be 14 mins (corresponding to 10 D-values). This means that BIs that are exposed to a bacterial reduction rate equal to the calculated D-value $_{best\ place}$ will show reliably negative results after a decontamination period of 14 mins. If now isolated positions in the chamber show D-values greater than the calculated D-value $_{best\ place}$, the decontamination period of 14 mins will no longer assure sufficient reduction of the test microorganism population. In the subsequent growth test, these BIs do show fractional and/or all-positive results.

Definition of worst case positions

For the worst case study, the critical positions to be considered in the chamber are first identified. Particular attention should be paid here to places where large deviations from the average physical conditions in the chamber may be expected.

Worst case study procedure

Three BIs are placed in each of the previously defined worst case positions. This allows observation of all-positive, fractional, and all-negative results at the individual position and, based on the result, to estimate the decontamination effect achieved at that specific position. In the worst case study, the BIs are subjected to a complete decontamination cycle with preconditioning, conditioning, and decontamination. The duration of the decontamination phase, as explained above, is set equal to the kill time of the BI used based on the mean D-value $_{best\ place}$. For all other cycle parameters, the previously determined values apply.

Interpretation of the results

On the basis of the survival time model, the results of the worst case study are interpreted as follows:

- If all three BIs used in a specific position test negative, then this chamber position assures a

bacterial reduction similar to that calculated for the best place position; the D-value at that position therefore corresponds to the D-value $_{best\ place}$. Given the bacterial reduction obtained, this position does not constitute a worst case.

- The bacterial reduction at positions at which the BIs produce a fractional result (positive and negative results in the ratio 2:1 or 1:2) is estimated to be equal to the test microorganism population.
- Positions with all-positive results show no or only poor bacterial reduction, corresponding to the definition of the survival time, and therefore a quantification is not possible.

On this basis the bacterial reduction rate achieved for positions with fractional results is estimated to be the D-value $_{worst\ case}$:

$$D\text{-}value_{Worst\ Case}\ [mins] = \frac{Duration\ of\ decontamination\ [mins]}{log\ N_0}$$

In the above example, with a decontamination duration of 14 mins, a test microorganism population of 1x 10^6 [CFU] and a D-value $_{best\ place}$ of 1.4 mins, the estimated bacterial reductions based on the results are as follows, see Table 3.

To confirm the estimates made, a second worst case study is carried out on the basis of the first D-value $_{worst\ case}$ calculated, but this time only positions previously found to correspond to the worst case are considered. With this iterative procedure, the duration of decontamination is adjusted to the worst case positions observed, and, at the same time, the maximum D-value $_{worst\ case}$ is determined.

The final duration of the decontamination phase depends on the overall bacterial reduction the process should guarantee, and is derived from the maximum D-value $_{worst\ case}$ and the target bacterial reduction.

$$Duration\ of\ decontamination\ [mins] = D\text{-}value_{WorstCase}\ [mins]$$

$$x$$

$$target\ bacterial\ reduction\ [log\text{-}scale]$$

Once the worst case study has been completed and the parameter decontamination duration has been established, all parameters of the decontamination cycle affecting kill rate are described and quantified.

Experiment 4: Determination of purge time

It is appropriate to generate a purge curve to calculate the purge time. Suitable measuring methods are gas test tubes and H_2O_2 gas sensors with appropriate measuring range. The residual gas concentration required [ppm] of H_2O_2 in the chamber is determined with reference to the system application. If the chamber will be opened or entered by personnel after decontamination, the residual concentration must satisfy legal

Table 3

Biological indicator results	Bacterial reduction [log scale]	Estimated D-value [mins]
All-negative (3:0)	≥ 10	1.4 (D-value $_{best\ place}$)
Fractional (2:1, 1:2)	≥ 4, ≤ 10, (evaluated with 6)	2.3
All-positive (0:3)	≤ 4	Cannot be estimated

requirements for personal safety and limit values before the chamber is opened. Where the systems to be decontaminated are used in the manufacture and testing of products, the residual concentration achieved must not affect the quality of the product or the test to be carried out. The maximum permitted H_2O_2 residual concentration in these applications can only be established through appropriate tests.

Experiment 5: Determination of D-value

As a final step of the cycle development, a LSKM is performed to determine the definite D-value using the final decontamination cycle parameters. The procedure adopted here is similar to the minimized LSKM in experiment 1. The D-value thus calculated with its 95% confidence limits supports the D-values estimated throughout the course of the cycle development and, as the characteristic figure for the process in the specific equipment, describes the bacterial reduction rate obtained by the complete system.

When the decontamination process is requalified, the reactive pattern recognition used to check the BI batches can be based on this figure D-value for the equipment / process. In this way, in the event of fluctuations in the D-value and irregularities in model behaviour of the BIs, appropriate action can be taken prior to starting any qualification work.

Summary

The method presented here to develop H_2O_2 decontamination cycles describes and quantifies the influence of every process parameter relevant to the decontamination effect using a defined microbiological system. The chronology of the series of experiments systematically excludes any secondary effects on the results, and thus ensures that the experimental data can be properly interpreted. On the basis of well-accepted microbiological and statistical methods, this H_2O_2 decontamination process development becomes transparent and contributes to sterilisation process validation.

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