

Mathematically modelling the inactivation kinetics of *Geobacillus stearothermophilus* spores: Effects of sterilization environments and temperature profiles

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ABSTRACT

In this study, inactivation kinetics of *Geobacillus stearothermophilus* spores were evaluated in different sterilization environments. The kinetics were analysed and mathematically modelled based on experimental data collected. The inactivation kinetics were measured precisely in moist heat environments using different sterilization temperatures and holding times. All measured inactivation times were shorter than the inactivation time indicated by the Biological Indicator (BI) manufacturer. Increasing sterilization efficiency was found in the following environments: air, saturated steam, wet steam, liquid water, dialysis solutions. Applying first- and second-order reaction kinetics approaches, formulas were derived from measured data that enabled bacterial inactivation to be modelled. A mathematical first-order reaction kinetic modelling approach could be taken to effectively predict inactivation kinetics for *G. stearothermophilus* spores based on the experimentally measured data collected in wet steam and air environments. A second-order reaction kinetics approach could be taken, however, to model measured data more accurately in liquid water and dialysis-solution environments. The mathematical models presented here can be applied to describe inactivation kinetics for *G. stearothermophilus* spores in different sterilization test environments or for any given sterilization temperature profile. These findings can be used to improve the quality of sterilization processes.

1. Introduction

Medical devices and pharmaceuticals are typically subjected to sterilization processes to ensure patient safety [1–4]. A Sterility Assurance Level (SAL) of 10^{-6} is currently required for these processes, meaning that the device or product can contain no more than one viable microorganism per one million sterilized items, before these can come in contact with patients in order to ensure that the products are safe [5]. The term ‘sterilization processes’ refers to procedures used to inactivate microorganisms on both the surfaces and inside these items.

Sterilization processes performed with moist heat (e.g. steam sterilization) are often used to sterilize medical devices and pharmaceuticals. Many studies have been carried out to investigate how efficiently these moist heat sterilization processes inactivate bacteria [6–9], while others have been performed to examine how effectively they sterilize specific products [10–14].

Specific investigations have been carried out under particular conditions or on specific aspects of the sterilization process. For example, Mazzola et al. [15] determined the decimal reduction time (D -value) for chemical agents used in hospitals for disinfection purposes. Rodriguez et al. [16] developed a model that could be applied to predict bacterial inactivation under conditions of moist heat and high pressures and Geeraerd et al. [17] developed a free-ware tool that could be used to assess non-log-linear microbial survival curves. Van Doornmalen and Kopinga [18] investigated the temperature dependence of F -, D - and z -values in steam sterilization processes, while Hossain et al. [19] measured the inactivation of different types of bacteria in clinical solid waste using these same processes. Other authors studied the kinetics of bacterial spore inactivation at elevated temperatures [20–22].

All of these studies were performed to study bacterial inactivation processes under laboratory conditions, following special sterilization protocols in which the samples were heated and cooled occurred rapidly

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(i.e. using resistometer vessels). According to ISO 11 138–3:2017 [23], the heating and cooling time in a resistometer should not exceed 10 s, but the heating and cooling times used in steam sterilizers in the industry are much longer (i.e. from several minutes up to a half hour). Another difference is that the steam in a resistometer is saturated. Steam saturation in other autoclaves can not be guaranteed by only measuring pressure and temperature [24,25]. Furthermore, in nearly all of these studies, bacterial inactivation was investigated with linear inactivation curves (e.g. first-order reaction kinetics) for one specific sterilization environment (e.g. saturated steam). The literature review conducted for the current study revealed two interesting gaps in knowledge: Although environments with dialysis solutions, liquid water, or wet steam are often used for sterilization purposes, little is known about the influence of these environments on bacterial inactivation. Furthermore, the inactivation curves that occur during the sterilization process in these environments are not precisely known.

For this reason, the aim of this study was to determine the inactivation curves for spores of the gram-positive bacterium *G. stearothermophilus* under real moist heat sterilization cycle conditions and in different sterilization environments. This organism was used because it is extremely resistant to moist sterilization processes [26,27] and, for that reason, it is commonly used in sterilization validation studies. However, the experimental values and inactivation kinetics cited in the literature [15,28–30] do not allow researchers to model the real inactivation kinetics for this organism, because measurements were usually made with resistometers under conditions of nearly saturated steam. Moreover, second-order reaction kinetics have apparently rarely ever been applied to model the inactivation kinetics. To address these gaps, in this work, we measured the inactivation of *G. stearothermophilus* in all sterilization environments that can be present inside a peritoneal dialysis bag system (PDBS) (steam, liquid water and different dialysis solutions) as well as in air.

To investigate the inactivation kinetics, we used a commercially available steam autoclave to guarantee that all measurements were performed using typical scenarios and conditions for real-world sterilization processes. These experimental measurements were used to mathematically model the bacterial inactivation processes based on the current norms and standards. To ensure significance of the modelling, the coefficient of determination (r^2) must be 0.8 or higher (according to the ISO 11 138–7:2019 [31]). Therefore, the inactivation curves were not only modelled using first-order reaction kinetics – as has commonly been done [32] – but also using second-order reaction kinetics [33]. In this paper, we present and explain the complex mathematical models developed in this study. These enable other researchers to accurately model the inactivation of *G. stearothermophilus* spores in the investigated sterilization environments as well as for any sterilization temperature

profile. In addition, the study findings provide us with a more thorough and clearer understanding of real bacterial spore inactivation kinetics that occur during sterilization processes. The presented kinetics can be applied by other researchers to further validate and optimize sterilizers and sterilization cycles. The authors of this study for example, implemented the developed inactivation kinetics models to simulate the inactivation of *G. stearothermophilus* spores using Computational Fluid Dynamics (CFD) [13].

2. Methods

2.1. Steam sterilizer

All experiments in this study were performed with a commercially available steam sterilizer from Systec® with a volume of 162 L (Systec HX-150 [34]), schematic sketch shown in Fig. 1), a sterilizer that is typically used in real-world laboratories. It has a cylindrical chamber (diameter: 500 mm, length: 750 mm) with two steam inlets which are located on the top of the chamber and the outlet, which is located on the bottom, near the door. The cooling water inlets are located between the two steam inlets.

In all steam sterilization cycles carried out in this study, at least one external laboratory sensor (independent of the sterilizer's pre-installed sensors) was fitted inside the chamber near the front (see Fig. 1). The fluid temperature inside the steam autoclave was measured with at least with two thermocouples, located at the top of the sterilization chamber and near the outlet (Fig. 1: T1 and T2). To measure the bacterial inactivation kinetics in different sterilization environments, more pressure and temperature loggers were added to the measurement set-up.

2.2. Modelling the bacterial inactivation based on sterilization parameters

The inactivation of bacteria by steam sterilization follows an exponential decrease curve, which can be expressed using first-order inactivation kinetics [35]. Thus, the number of bacteria at any given time can be calculated as follows:

$$N(t) = N_0 \cdot e^{-k_d(T) \cdot t} \quad (1)$$

where $N(t)$ represents the number of currently active bacteria and $k_d(T)$, the inactivation rate of the bacteria, which is a function of the temperature (T) and the sterilization environment. The temperature dependence of the inactivation rate $k_d(T)$ is usually modelled using the Arrhenius equation (see Eq. (2)) [20]:

$$k_d(T) = k_{d0} \cdot e^{\frac{-E_a}{R \cdot T(t)}} \quad (2)$$

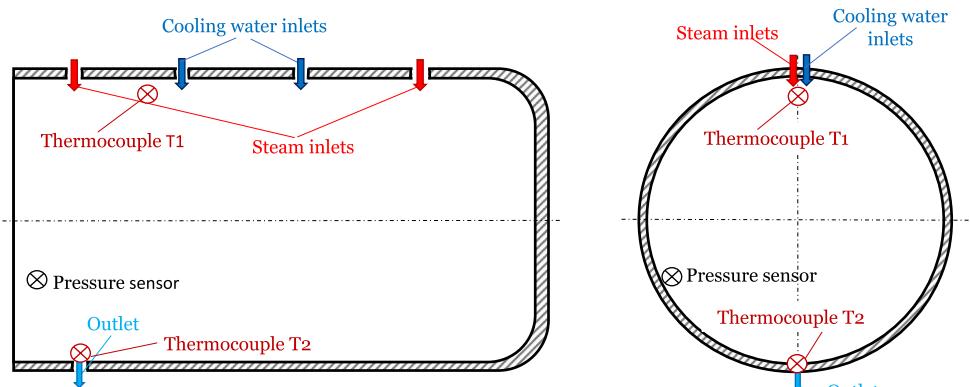


Fig. 1. Schematic sketch of the steam autoclave with the locations of the steam and cooling water inlets, the outlet and the positions of the pressure sensor and the two thermocouples (T1 and T2) are shown.

where k_{d0} is a pre-exponential factor, E_d is the activation energy of the bacteria, R is the universal gas constant, and $T(t)$ is the temperature at a given time t .

Furthermore, the inactivation kinetics were also studied by taking a second-order reaction kinetic approach. The derived number of live or dead bacteria, depending on the time, is expressed as follows [36]:

$$\frac{dN}{dt} = k_d(T) \cdot N^2 \quad (3)$$

Integrating Eq. (3) with $N = N_0$ at $t = t_0$ leads to:

$$N(t) = \frac{1}{k_d(T) \cdot t + \frac{1}{N_0}} \quad (4)$$

where again $N(t)$ represents the number of currently active bacteria and $k_d(T)$, the bacterial inactivation rate. The bacterial inactivation rate was also modelled here using the Arrhenius equation (see Eq. (2)). The $k_d(T)$ values used in the first-order reaction kinetic approach (see Eq. (1)) and the second-order reaction kinetic approach (see Eq. (4)) differ.

2.3. D- and z-value

To describe the temperature resistance of bacteria, the D - and z -values can be used. The D -value represents the time (in minutes) it takes to inactivate 90% of the bacteria at a given temperature (T) [15]. The higher the D -value, the higher the temperature resistance of the bacteria. The z -value describes the temperature dependence of the D -value; namely, it represents the temperature increase (in $^{\circ}\text{C}$) which is needed to reduce the D -value by a factor of 10.

To calculate the bacterial inactivation using a first-order reaction kinetics approach (see Eq. (1)), the inactivation rate $k_d(T)$ is needed. With Eq. (5), the inactivation rate for the Arrhenius equation can be calculated from the D -value of the bacteria. Jones [37] and Mann et al. [38] provide more detailed information about this equation.

The kinetics of the inactivation shown in Eq. (5) are considered to be valid in any situation in which these kinetics follow the pattern of first-order kinetics (e.g. saturated steam sterilization).

$$D(T) = \frac{2.303}{k_d(T)} \quad (5)$$

In this study, we investigated bacterial inactivation kinetics in different sterilization environments that represent real operational conditions present in steam sterilizers. Therefore, the inactivation kinetics in liquid water and two dialysis solutions were also modelled using a second-order reactions kinetic approach (see Eq. (4)). With the inactivation kinetics based on first order (see Eq. (1)) or second order (see Eq. (4)) the number of the active bacteria can be calculated as a function of the sterilization time ($N(t)$). Due to comparison of the calculated time-dependent number of active bacteria ($N(t)$) with the measured number of active bacteria at given times (t), the coefficient of determination of the inactivation kinetics models can be calculated.

It should be mentioned that Eq. (5), however, is not valid for use with the second-order reaction kinetics approach, because the D -value is based on first-order reaction kinetics. In the case of second-order reactions kinetics, the inactivation rate ($k_d(T)$) has to be obtained using other methods.

2.4. Description of the lethality of a steam sterilization process

The F_{phys} value is used to describe the efficiency of a steam sterilization process. This value is defined as the equivalent number of minutes that a device or product is exposed to a steam sterilization process (assuming saturated steam conditions), as compared to its exposure to a steam sterilization process at a constant reference temperature T_{ref} . The higher the F_{phys} value, the higher the theoretical bacterial inactivation rate [28,39]. Eq. (6) is used to calculate the F_{phys} value for a given

sterilization process (generally with saturated steam):

$$F_{\text{phys}} = \int_{t_0}^{t_n} 10^{\frac{T(t)-T_{\text{ref}}}{z}} dt \quad (6)$$

where $T(t)$ is the temperature at the time t , z is the z -value, and t_0 and t_n are the respective start and times for the sterilization process. Since all the bioindicators (BIs) used in this study were defined using a D -value at $121.1\ ^{\circ}\text{C}$ (established by the manufacturer), all F_{phys} values were calculated with reference to this temperature. The z -values used to calculate the F_{phys} values were set according to the z -value of the BIs. The authors are aware that the changes in the z -value during the sterilization process in different sterilization environments (e.g. liquid water, dialysis solutions, air) would have to be assumed. Nevertheless, the authors calculated the F_{phys} values for all the sterilization cycles performed in this study using the z -value provided by the BI's manufacturer and the established manufacturer's reference temperature of $121.1\ ^{\circ}\text{C}$. This assumption serves as base for the further development of inactivation kinetics.

For reasons of comparability, the F_0 value is also given for elected cycles in this study. The F_0 value is a special form of the F_{phys} value and can also be used to describe the lethality of the investigated steam sterilization processes. The F_0 value is calculated using a reference temperature of $121.1\ ^{\circ}\text{C}$ and a z -value of $10\ ^{\circ}\text{C}$ (Eq. (7)) [40].

$$F_0 = \int_{t_0}^{t_n} 10^{\frac{T(t)-121.1\ ^{\circ}\text{C}}{10}} dt \quad (7)$$

A detailed description about the investigated sterilization cycles in this study can be found in Section 3.1.

2.5. Biological Indicators

Biological Indicators (BIs or spore strips) of *G. stearothermophilus* ATCC 7593 were obtained from SIMICON GmbH [41] and were in compliance with ISO 11 138-1:2017 [23] and ISO 11 138-3:2017 [42]. Each BI had a starting population of $2 \cdot 10^6$. All measurements in this study were performed using BIs from three different batches. The D -values and z -values of the spores differed between the batches. Table 1 shows the D -value and the z -value of the different batches for the BIs used to obtain measurements in this study. The respective D -values and the z -values were taken from the manufacturer's certificate and used as a basis for the comparison and evaluation of the results of the performed measurements. BIs from the three batches (see Table 1) were randomly used for all these measurements to investigate the inactivation kinetics in all specific sterilization environments.

2.6. Preparation of the BIs for the measurements

To evaluate the reaction kinetics in liquid water and the different dialysis solutions, the BIs were prepared for the test runs as follows:

The BI strips were removed from the paper slips and placed in a semi-permeable cuprophane tube, which then was knotted on the two open sides to ensure that the solution could reach the BIs through the cuprophane membrane and, at the same time, that the BIs could not diffuse into the surrounding liquids.

For the tests carried out in wet steam and air, the BI paper slips were placed directly into the sterilization environment.

Table 1

D - and z -values for the three batches of *G. stearothermophilus* BIs used for the measurements in this study.

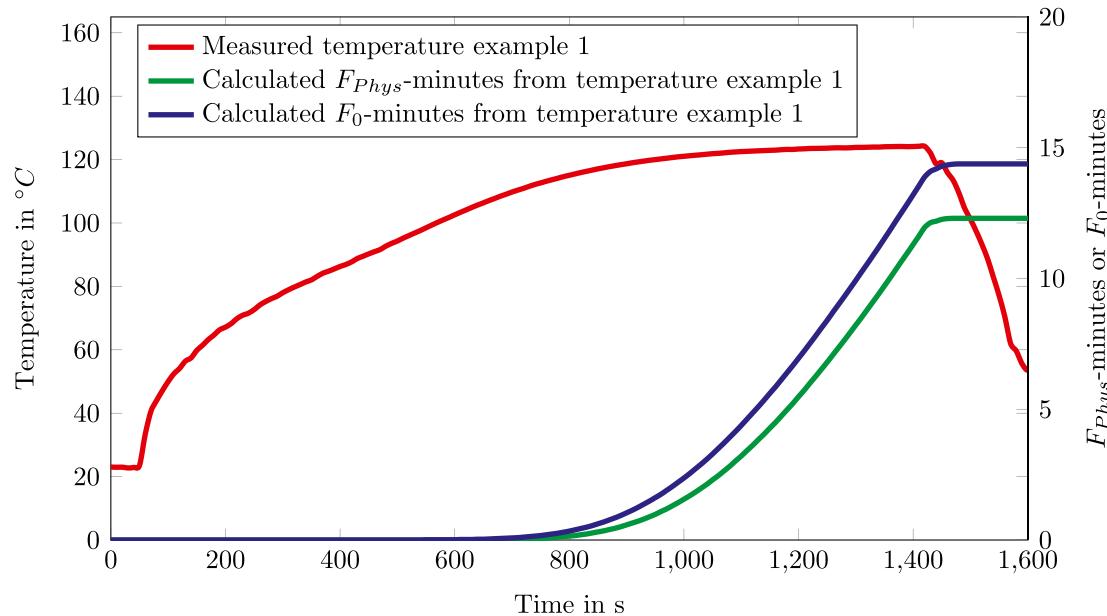
	D -value at $121.1\ ^{\circ}\text{C}$ [min]	z -value [$^{\circ}\text{C}$]
Batch 1	2.4	7
Batch 2	2.3	8.1
Batch 3	2.5	8.5

2.7. Counting the active bacteria on the BIs

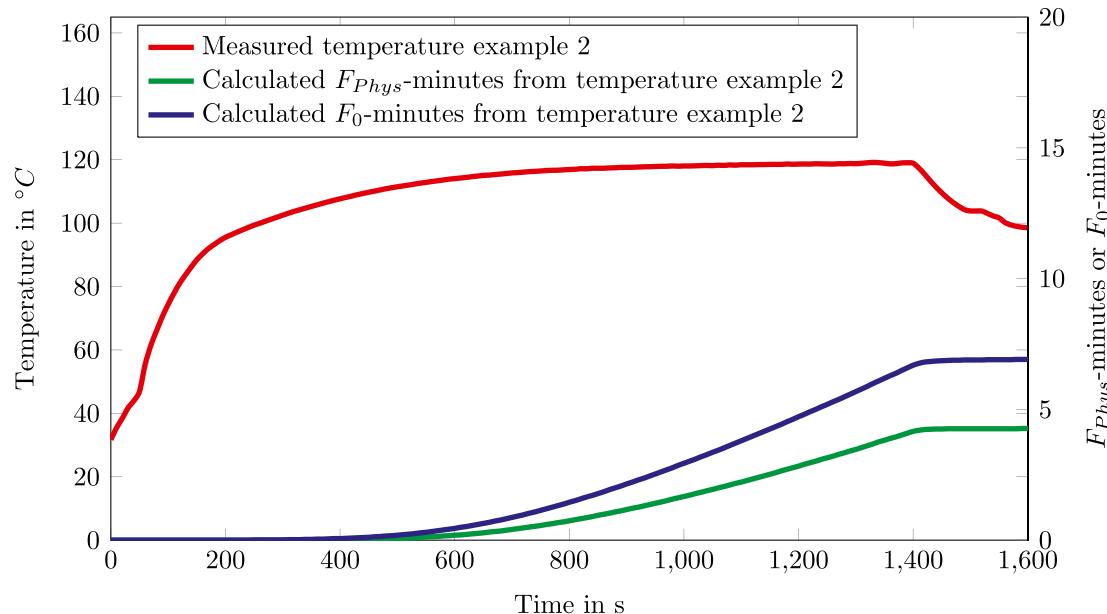
When counting colonies on plates, only values between 30 and 300 colonies per plate are usually considered as statistically valid (in compliance with ISO 11 138–1:2017 [23]). The detailed counting process of the active bacteria on the BIs is described in the supplementary material. Because the experiments were designed to show the inactivation kinetics of commercially available BIs, small CFU numbers – as low as 1 – also had to be assessed. The focus of the study was not to determine the influence of any carrier material of the bacterial spores on the inactivation kinetics. We evaluated smaller values than 30 colonies and

added the corresponding error bars, enabling us to show the variation in the numbers of counted colonies and, therefore, the method's level of inaccuracy.

In this study, 5 up to 10 BIs were used for each measurement to investigate the inactivation kinetics of *G. stearothermophilus*. Error bars represent the maximum and minimum measured population numbers on the BIs used for the respective measurement. Therefore, the variation in the measured results is represented by the error bars; however, when the counts approached 0, the error bars enter far into the negative range due to the logarithmic scale used. For this reason, we chose not to display error bars below 10^{-2} . Conversely, the logarithmic representation of the



(a) Measured temperature and calculated F_{Phys} and F_0 minutes of an investigated sterilization cycle with approximately 12.3 F_{Phys} minutes.



(b) Measured temperature and calculated F_{Phys} and F_0 minutes of an investigated sterilization cycle with approximately 4.2 F_{Phys} minutes.

Fig. 2. Measured temperature and calculated F_{Phys} and F_0 minutes of two investigated sterilization cycles with a F_{Phys} minutes of (a) approximately 12.3 and (b) approximately 4.2.

error bars makes its upper end appear lower.

The measured population values shown in the diagrams represent the mean value of the counted surviving bacteria on all BIs used in the respective measurement. Naturally, this included the possibility of a measured value below 1 (e.g. 8 BIs with 1 surviving bacteria and 2 BIs with 0 surviving bacteria).

3. Measurement set-up

3.1. Investigated steam sterilization cycles

Different steam sterilization cycles were investigated to characterize the bacterial inactivation kinetics under real sterilization conditions. The following parameters were varied: the sterilization temperature, holding time of the sterilization temperature and environments (fluid). The goal was to measure the number of surviving bacteria for different F_{Phys} minutes. To calculate the number of F_{Phys} minutes, we considered the complete temperature profile, including the heating and the cooling phases of the sterilization cycles. Overall, 100 different steam sterilization cycles were investigated, with the F_{Phys} minutes ranging from 0.01 to over 500. This corresponded to a range from 0.01 to over 400 in F_0 minutes. Fig. 2 shows two examples of the investigated sterilization cycles. In the first sterilization example, a maximum sterilization temperature of approximately 124 °C was reached, and a holding time of approximately 90 s was used. This sterilization cycle resulted in an F_{Phys} of approximately 12.3 min (see Fig. 2(a)). The second sterilization example had a maximum sterilization temperature of approximately 119 °C and a holding time of approximately 360 s. This sterilization cycle resulted in an F_{Phys} of approximately 4.2 min (see Fig. 2(b)). All steam sterilization cycles were measured at least twice. The results of all measurements performed showed high reproducibility.

3.2. Inactivation of bacteria in steam

To measure the BI inactivation kinetics under real steam sterilization conditions (wet steam), the BIs were placed directly into the steam autoclave chamber. Eleven BIs were used for each measurement: Ten BI strips were placed into the steam autoclave, and one was taken as an untreated positive control. The pressure and temperature inside the autoclave were measured with external loggers in close proximity to the BIs. The loggers measured the pressure to an accuracy of ±30 mbar and the temperature to an accuracy of ±0.5 K. Due to the fact that only the pressure and the temperature of the steam was measured, it is possible that a very small amount of air is present as well [24].

The F_0 minutes and the F_{Phys} minutes of the steam sterilization cycle were calculated from the measured temperatures. The numbers of remaining viable bacteria were counted on all eleven BIs and then plotted against the F_{Phys} minutes. The F_0 minutes were used to provide an overview of the sterilization cycles investigated.

3.3. Inactivation of bacteria in water

To investigate the inactivation of the bacteria in water, a dialysis bag (Stay safe Balance®) with two compartments was filled with WFI. A schematic sketch of the measurement set-up is shown in Fig. 3. Each compartment had a volume of 1000 ml and the following dimensions: length = 240 mm, width = 80 mm and height = 55 mm. Five BIs were put into each compartment so that they were surrounded by water. Two temperature loggers were put into each compartment to measure the water temperature. One temperature logger measured the temperature in the compartment's centre, and the other measured the temperature on the compartment's outer surface (see green text and arrows in Fig. 3). After the sterilization process, the F_0 minutes and the F_{Phys} minutes were

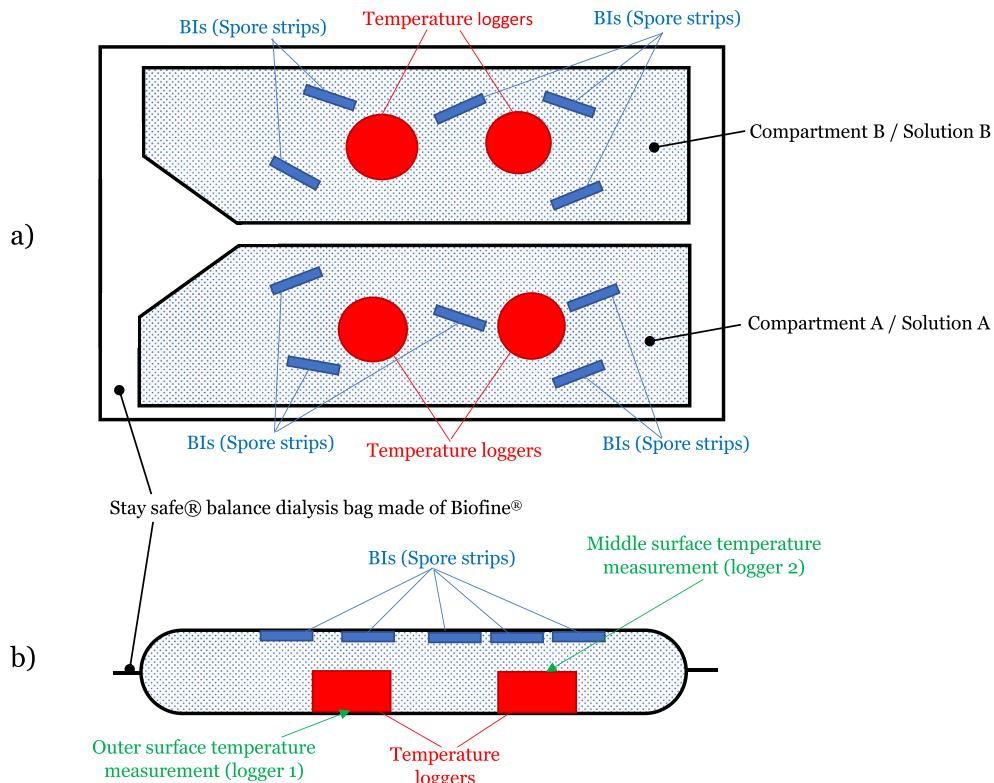


Fig. 3. Schematic sketch of (a) the top view and (b) the cross-section of dialysis bag system (Stay safe®) with the positions of the temperature loggers and the position of the BIs (spore strips) within the two compartments shown. This measurement set-up was used for liquid water as well as dialysis solution.

calculated using the average of the temperatures measured by these two loggers in each compartment. Differences between the temperatures measured by these two loggers were found to be below 0.5 K during the sterilization process. The number of surviving bacteria on each of the five BIs was counted. This measurement was later repeated with another sterilization cycle with different F_0 minutes and F_{phys} minutes.

3.4. Inactivation of bacteria in dialysis solution

The inactivation rate of bacteria in the dialysis solution was assessed using a similar set-up as used for inactivation in water (see Fig. 3), but the compartments of the dialysis bag were filled with two different dialysis solutions instead of water. Compartment A was filled with solution A, whereas compartment B was filled with solution B; these solutions had different chemical properties. Solution A contained glucose and, therefore, possessed an acidic pH value of approximately 3, whereas solution B included lactate and had a basic pH value of approximately 8.5. Detailed information about the chemical compositions of the two dialysis solutions is shown in Table 2.

The bacterial inactivation in the dialysis solution was also measured for different sterilization cycles with different F_0 and the F_{phys} minutes. After each sterilization cycle, the numbers of surviving bacteria on the BIs were measured, and, once again, the number of the surviving bacteria was plotted against the F_0 and the F_{phys} minutes of the corresponding sterilization cycle.

3.5. Set-up to measure the bacterial inactivation in air

The bacterial inactivation in air was measured using a metal container with a lid that could be opened to put in the measurement equipment (inner dimensions: 75 mm × 75 mm × 140 mm, inner volume: approx. 0.6 L). Before the sterilization process, the opened metal container was dried for 1 h in an oven at 105 °C to remove any surface moisture. Temperature and pressure loggers were placed inside the metal container (see Fig. 4).

The ten BIs were hung with tape at regular intervals in the middle of the metal container. Afterwards, the gas-tight container was placed inside the steam sterilizer. Since no humidity was present inside the metal container at the beginning of the sterilization cycle, the pressure inside the container can be directly calculated from the measured temperature (isochoric change of state). Again, the measured temperature inside the metal container was used to calculate both the F_0 minutes and the F_{phys} minutes of each investigated sterilization.

4. Results

4.1. Measured bacterial inactivation in steam

Plotting the measurements taken to record the inactivation of *G. stearothermophilus* during different steam sterilization cycles yielded an exponential inactivation curve (see Fig. 5). Each measured point in Fig. 5 represents the mean number of viable bacteria after the sterilization of 10 BIs. As already mentioned, the measured point could have a

Table 2

Chemical composition of the two investigated dialysis solutions (Solution A and Solution B).

	Solution A	Solution B
Na^+	195 mM	75 mM
Lactate	–	70 mM
HCO_3^-	–	5.0 mM
Ca^{2+}	2.5 mM	–
Mg^{2+}	1.0 mM	–
Cl^-	201 mM	–
Glucose	45.46 mM	–
pH value	2.8–3.1	8–8.8

value of less than 1 (e.g. 5 BIs with 1 surviving bacteria and 5 BIs with 0 surviving bacteria). The error bars in Fig. 5 represent the minimum and maximum numbers of surviving bacteria on the BIs used for this measurement. Overall, more than 200 BIs and 15 different sterilization cycles were measured, and the measurements were plotted to create this inactivation curve. Besides demonstrating that the inactivation follows an exponential decrease curve, the results show that a F_{phys} value of only approximately 10 min is needed to inactivate the initial number of $2 \cdot 10^6$ bacteria. The black line in Fig. 5 represents the theoretically calculated bacterial inactivation, based on the average D -values and z -values of the BIs provided by the manufacturer (see Table 3). These results indicate that an F_{phys} value of approximately 15 min is needed to inactivate $2 \cdot 10^6$ bacteria.

An inactivation curve based on a first-order reaction kinetic approach was obtained using the measured data for the bacterial inactivation in wet steam (see dark green line in Fig. 5). The coefficient of determination (r^2) exceeded 0.95. The obtained values from the measured inactivation curve for the pre-exponential factor (k_{d0}), activation energy (E_a), as well as the inactivation rates at 100 °C and 121 °C ($k_d(100\text{ }^\circ\text{C})$ and $k_d(121\text{ }^\circ\text{C})$) are shown in Table 3. By entering the pre-exponential factor (k_{d0}) and activation energy (E_a) into the Arrhenius equation (see Eq. (2)) and then applying a first-order reaction kinetics approach (see Eq. (1)), it is possible to accurately mathematically model the inactivation of *G. stearothermophilus* spores during the sterilization process in wet steam. Due to the high coefficient of determination using first-order reaction kinetics, we conclude that this approach is the most appropriate one.

4.2. Measured bacterial inactivation in liquid water

To measure the bacterial inactivation in liquid water, each compartment of the dialysis bag was filled with liquid water, five BIs and two temperature loggers (see Fig. 3). Each measured point shown in Fig. 6 represents the mean value of 5 BIs of active bacteria for one specific sterilization cycle. Again, the error bars represent the maximum and the minimum numbers of surviving bacteria found on these five BIs.

Fig. 6 shows the measured inactivation of *G. stearothermophilus* by sterilization in liquid water, as well as the inactivation curves constructed based on first- and second-order reaction kinetics approaches, based on these measured data. The measured values show that the bacterial inactivation is strongly under-predicted in liquid water. In contrast to the manufacturer's information, which indicates that 15 F_{phys} minutes are needed for inactivation (as measured in saturated steam), our experimental results indicate that only approximately 5 F_{phys} minutes are needed to inactivate $2 \cdot 10^6$ bacteria in liquid water. Fig. 6 shows that the fit of the modelled first-order inactivation curve (depicted as a dark blue line) is not as close to the measured data as the fit seen in the steam environment (see Fig. 5). The coefficient of determination (r^2) of the first-order reaction kinetics was approximately 0.3. These results may be due to the fact that bacteria are inactivated at much lower temperatures in water environments than in wet and saturated steam environments. Measurements showed that approximately 1 log-ratio of bacteria can be inactivated merely by heating up the water to approximately 90 °C. At such low temperatures, hardly any F_{phys} minutes are generated. We also modelled the bacterial inactivation in liquid water by taking a second-order reaction kinetics approach (see light blue line in Fig. 5), since this approach is used to model many other chemical reactions in liquids [36]. The modelled second-order inactivation curve shows a much better coefficient of determination ($r^2 > 0.85$) and, thus a better fit, to the measured points as compared to the fit achieved when modelling the inactivation using a first-order reaction kinetics curve. Therefore, the second-order reaction kinetics approach is considered the most appropriate and is in accordance with ISO 11 138–7:2019 [31], where an $r^2 > 0.8$ for a reaction kinetic curve was applied. The parameters set to model the inactivation of *G. stearothermophilus* during the sterilization in a liquid water environment and using first-order reaction

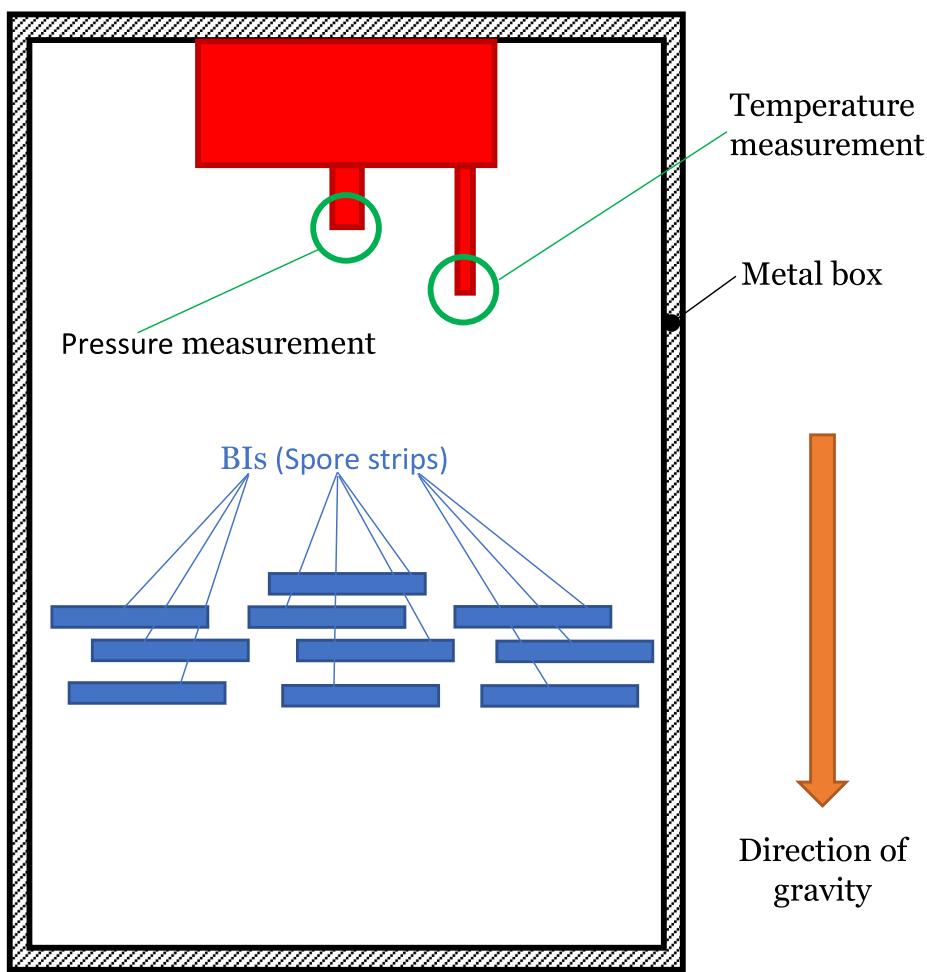


Fig. 4. Schematic sketch of the metal box with the BIs (spore strips) and the pressure and temperature loggers used to measure the inactivation of the bacteria in air shown.

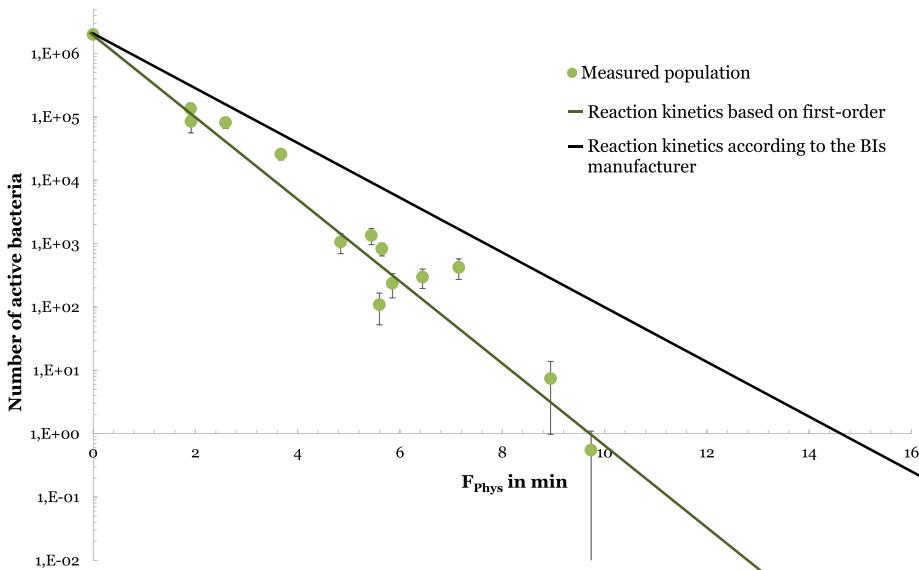


Fig. 5. Measured populations, indicating the inactivation of *G. stearothermophilus* spores during steam sterilization, based on the number of bacteria calculated using a first-order reaction kinetic approach and the number of bacteria calculated according to the values provided by of the manufacturer.

Table 3

Obtained pre-exponential factor (k_{d0}), activation energy (E_a) as well as the inactivation rates at 100 °C and 121 °C ($k_d(100\text{ }^\circ\text{C})$ and $k_d(121\text{ }^\circ\text{C})$) to model the inactivation of *G. stearothermophilus* spores during the sterilization process in wet steam, liquid water and two dialysis solutions using a first-order reaction kinetics approach.

	$k_{d0} [\text{s}^{-1}]$	$E_a [\text{J} \cdot \text{mol}^{-1}]$	$k_d(100\text{ }^\circ\text{C}) [\text{s}^{-1}]$	$k_d(121\text{ }^\circ\text{C}) [\text{s}^{-1}]$
Wet steam	$1.018\ 42 \cdot 10^{48}$	$3.744\ 07 \cdot 10^5$	$3.966\ 21 \cdot 10^{-5}$	$2.458\ 74 \cdot 10^{-2}$
Liquid water	$2.855\ 48 \cdot 10^{43}$	$3.375\ 94 \cdot 10^5$	$1.486\ 05 \cdot 10^{-4}$	$4.912\ 06 \cdot 10^{-2}$
Dialysis solution A (pH-value 3)	$5.170\ 04 \cdot 10^{43}$	$3.377\ 88 \cdot 10^5$	$2.690\ 97 \cdot 10^{-4}$	$8.889\ 49 \cdot 10^{-2}$
Dialysis solution B (pH-value 8.5)	$6.831\ 82 \cdot 10^{43}$	$3.377\ 88 \cdot 10^5$	$3.555\ 92 \cdot 10^{-4}$	$1.175\ 39 \cdot 10^{-1}$

kinetics (dark blue line in Fig. 6) are shown in Table 3, whereas the parameters set to model this inactivation using second-order reaction kinetics (see light blue line in Fig. 6) are shown in Table 4.

4.3. Measured bacterial inactivation in dialysis solutions

The inactivation of *G. stearothermophilus* in two different kinds of dialysis solutions was measured using the same procedure as described in previous sections. The two different dialysis solutions differed in their pH values and their chemical compositions (see Table 2). Results of the measured number of viable bacteria for dialysis solution A (pH value approximately 3) are shown in Fig. 7(a). At an F_{Phys} value of approximately 3 min, $2 \cdot 10^6$ bacteria could already be inactivated. In this case, an F_{Phys} of 3 min corresponds to an F_0 of approximately 3.5 min. As in the case mentioned previously, the exponential inactivation curve (first-order reaction kinetics curve) (dark red line in Fig. 7(a)) does not fit the measured inactivation values for *G. stearothermophilus* adequately ($r^2 \approx 0.2$). Therefore, the bacterial inactivation was again modelled using a second-order reaction kinetic approach (see orange line in Fig. 7(a)). This second-order reaction kinetic curve again showed a better fit with respect to the measured data ($r^2 \approx 0.86$). The parameter values used in

the Arrhenius equations to calculate the bacterial inactivation in dialysis solution A, taking the first- and second-order approaches, can be found in Tables 3 and 4, respectively.

Fig. 7(b) shows the measured and simulated numbers of viable bacteria in the sterilization experiments with dialysis solution B (pH value 8.5). The bacterial inactivation in this environment was faster than that seen with dialysis solution A. At an F_{Phys} value of approximately 2.5 min (F_0 value of approximately 3 min), $2 \cdot 10^6$ bacteria were inactivated. Again, the inactivation curve created by taking a second-order reaction kinetics approach (see yellow line in Fig. 7(b), $r^2 \approx 0.88$) shows a better fit to the measured data, as compared to the fit seen when taking a first-order reaction kinetics approach (see dark yellow line in Fig. 7(b), $r^2 \approx 0.15$). The parameters used for the Arrhenius equations for the investigated inactivation kinetics in dialysis solution B are shown in Table 3 (first-order reaction kinetics) and Table 4 (second-order reaction kinetics).

To calculate the number of active bacteria during the sterilization process in the two investigated dialysis solution environments by taking first-order or second-order reaction kinetics approaches, the pre-exponential factor (k_{d0}), activation energy (E_a) shown in Tables 3 and 4 must be substituted into the Arrhenius equation (Eq. (2)). Afterwards, the calculated inactivation rate (k_d) must be substituted in Eq. (1) to calculate the inactivation based on first-order reaction kinetics and

Table 4

Obtained pre-exponential factor (k_{d0}), activation energy (E_a) as well as the inactivation rates at 100 °C and 121 °C ($k_d(100\text{ }^\circ\text{C})$ and $k_d(121\text{ }^\circ\text{C})$) to model the inactivation of *G. stearothermophilus* spores during the sterilization process in liquid water and two dialysis solutions using a second-order reaction kinetics approach.

	$k_{d0} [\text{s}^{-1}]$	$E_a [\text{J} \cdot \text{mol}^{-1}]$	$k_d(100\text{ }^\circ\text{C}) [\text{s}^{-1}]$	$k_d(121\text{ }^\circ\text{C}) [\text{s}^{-1}]$
Liquid water	$7.312\ 93 \cdot 10^{166}$	$1.272\ 86 \cdot 10^6$	$7.922\ 79 \cdot 10^{-12}$	$2.402\ 78 \cdot 10^{-2}$
Dialysis solution A (pH-value 3)	$2.246\ 71 \cdot 10^{154}$	$1.173\ 06 \cdot 10^6$	$1.401\ 01 \cdot 10^{-10}$	$7.855\ 09 \cdot 10^{-2}$
Dialysis solution B (pH-value 8.5)	$1.368\ 54 \cdot 10^{154}$	$1.168\ 67 \cdot 10^6$	$3.518\ 87 \cdot 10^{-10}$	$1.829\ 50 \cdot 10^{-1}$

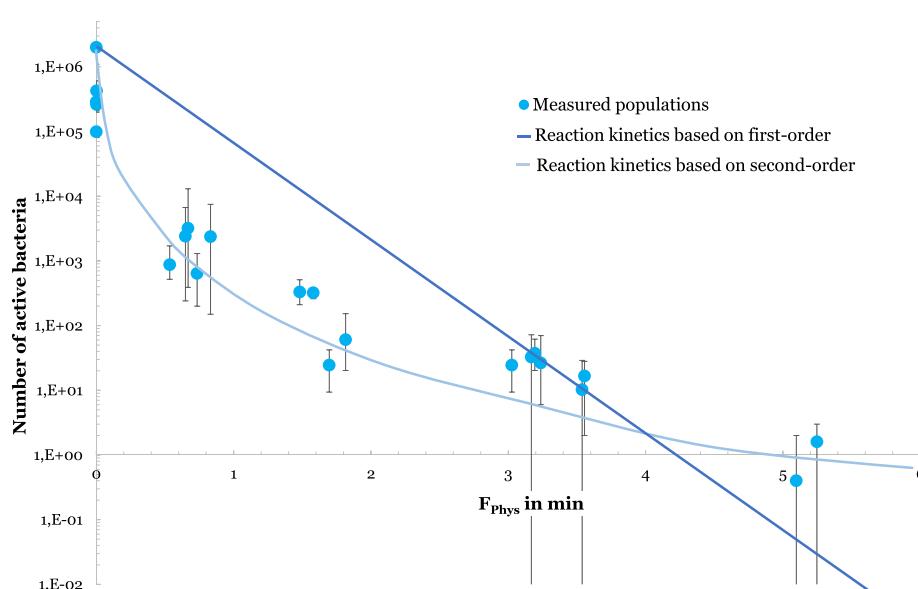
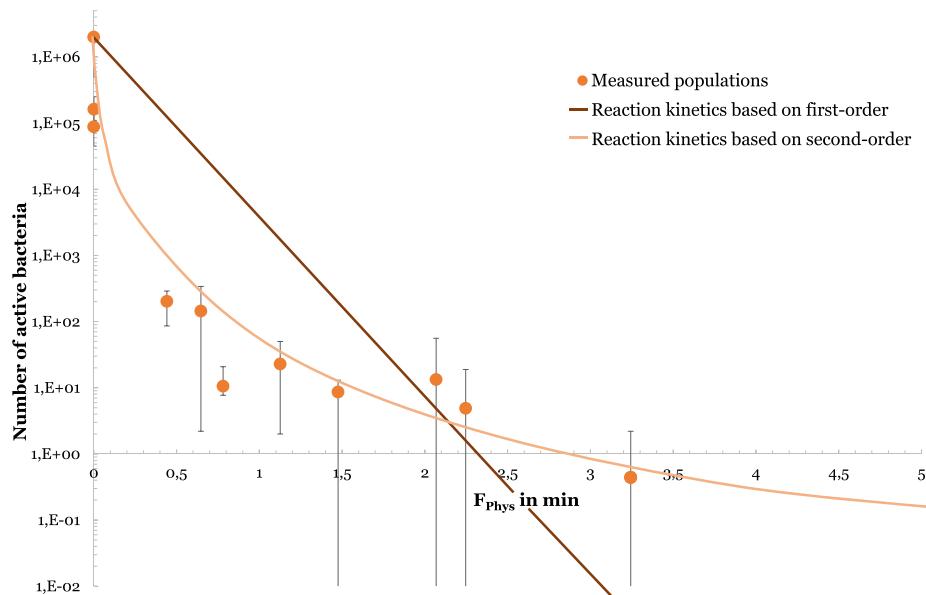
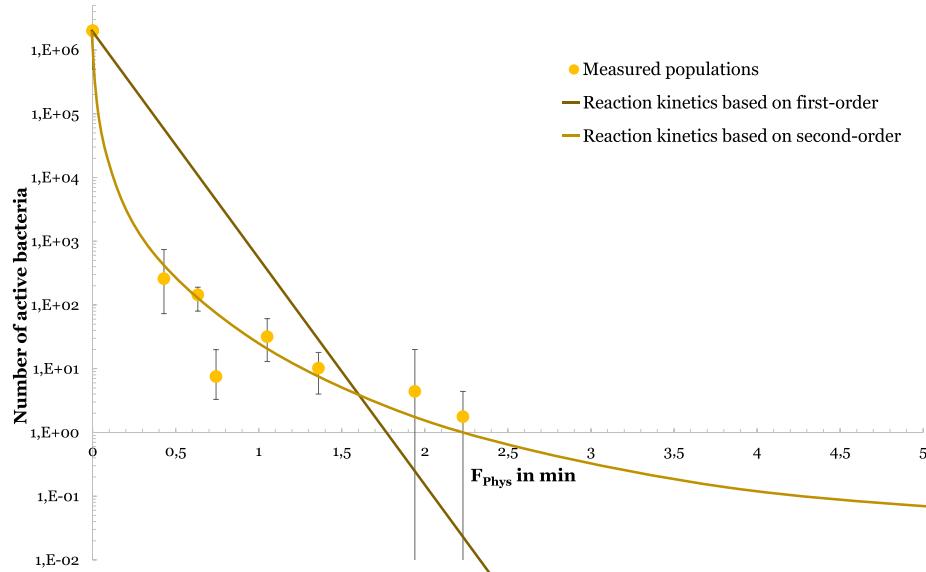


Fig. 6. Measured populations, indicating the inactivation of *G. stearothermophilus* spores during the sterilization in water, based on the number of bacteria calculated using a first-order reaction kinetic approach and the number of bacteria calculated using a second-order reaction kinetic approach.



(a) Measured and calculated active bacteria in the dialysis solution A (pH value 3).



(b) Measured and calculated active bacteria in the dialysis solution B (pH value 8.5).

Fig. 7. Measured populations, indicating the inactivation of *G. stearothermophilus* spores during the sterilization process in (a) dialysis solution A (pH value 3) and (b) dialysis solution B (pH value 8.5), based on the number of active bacteria calculated using a first-order reaction kinetic approach and the number of active bacteria calculated using a second-order reaction kinetic approach.

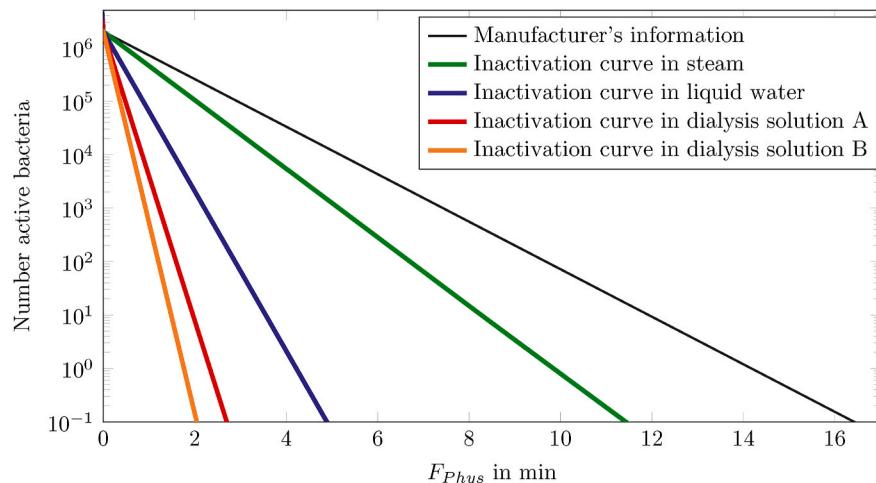
respectively in Eq. (4) to calculate it using second-order reaction kinetics.

4.4. Measured bacterial inactivation in air

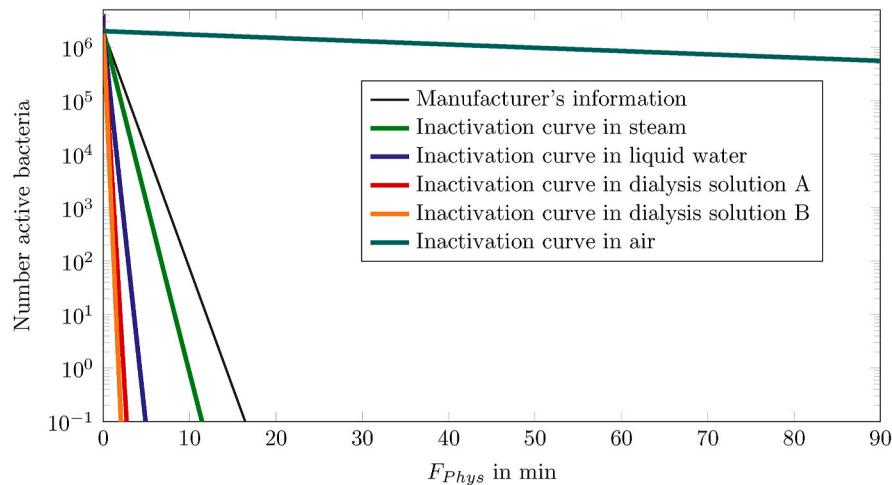
Inactivation of *G. stearothermophilus* in air was measured in a metal container (see Fig. 4). Fig. 8(b) shows the exponential decrease curve (first-order reaction kinetics) of the bacteria for the sterilization experiments in air. The modelled first-order reaction kinetic curve fit closely with the measured values ($r^2 > 0.95$). As one can see, very high F_{Phys} values are needed to inactivate all $2 \cdot 10^6$ bacteria spores of *G. stearothermophilus* in the air environment.

4.5. Comparison of the measured inactivation kinetics

In Fig. 8, we compare all the first-order inactivation curves created from measurements taken in the different sterilization environments. The inactivation of $2 \cdot 10^6$ *G. stearothermophilus* spores took $2.5 F_{\text{Phys}}$ minutes in solution B, $3 F_{\text{Phys}}$ minutes in solution A, $5 F_{\text{Phys}}$ minutes in liquid water, $10 F_{\text{Phys}}$ minutes in the measured wet steam and $15 F_{\text{Phys}}$ minutes according to the BI manufacturer (as measured in saturated steam, see Fig. 8(b)). The inactivation rate of *G. stearothermophilus* measured in air is completely different from the rate measured in wet environments, as is commonly known. At the investigated temperature levels (approx. 115 – 130 °C), it is hardly impossible to inactivate a sufficiently large number of *G. stearothermophilus* spores within a few hours.



(a) Measured inactivation curves of the inactivation kinetics of *Geobacillus stearothermophilus* during the sterilization in all atmospheres except air.



(b) Measured inactivation curves of the inactivation kinetics of *Geobacillus stearothermophilus* during the sterilization in all atmospheres including air.

Fig. 8. Measured inactivation of *Geobacillus stearothermophilus* during the sterilization in (a) all atmospheres except air and (b) in all atmospheres including air.

To inactivate *Geobacillus stearothermophilus* spores in air, temperatures of over 160 °C are needed to achieve the same number of viable bacteria achieved when using steam sterilization at approximately 121 °C [29, 43].

5. Discussion and conclusion

In this study, we assessed the inactivation kinetics of *G. stearothermophilus* spores in environments containing wet steam, air, liquid water and dialysis solutions. We mathematically described the inactivation kinetics by taking first- and second-order reaction kinetics approaches. If we carefully examine the sterilization process, the reason taking these approaches to analyse these inactivation kinetics becomes clearer.

Our results show that maintaining wet conditions during the sterilization process inside a real steam sterilizer more efficiently inactivates bacteria than sterilizing the samples in a resistometer and exposing them to a saturated steam environment. Steam saturation inside a resistometer can be verified by additional measurements, but not only by measuring pressure and temperature [24]. In our experimental set-up, we observed a large amount of condensation on all the inner walls and trays in the steam sterilizer. In a resistometer, the amount of condensation is

significantly lower. In our set-up, some of this condensation was partly absorbed by the BIs and led to more rapid inactivation. Similar results were found in previous studies [13, 44, 45]. Consequently, the amount of moisture absorbed by the BIs is primarily responsible for the differences seen between the inactivation curve constructed on the basis of the manufacturer's information and the curve based on values measured in this study (10 measured F_{Phys} minutes found in our experiments as compared to 15 F_{Phys} minutes according to the manufacturer's information, see Fig. 5). A minor part of this difference is caused by the fact that we calculated an equivalent sterilization time (F_{Phys} minutes) as compared to the real sterilization time measured in a resistometer. Overall, our results show that wet steam reduces the D -value to approximately 1.6 min, which is nearly 0.8 min less than the D -value measured by the BI's manufacturer (saturated steam in a resistometer). Furthermore, applying the developed formula (based on first-order reaction kinetics), we can mathematically model the bacterial inactivation during the sterilization in wet steam with a high accuracy ($r^2 \approx 0.95$). Square values of more than 0.8 show sufficient accuracy to be considered valid (according to the ISO 11 138-7:2019 [31]). Similar results were cited by Brown [46] and Z. Tang [47].

Our experimental series in liquid water showed that this environment is highly effective for bacterial inactivation. Only 5 F_{Phys} minutes

were required to inactivate $2 \cdot 10^6$ bacterial spores of *G. stearothermophilus* in liquid water. This is at least 3 times faster than the period indicated by the manufacturer (as measured in a resistometer under nearly saturated steam conditions). The authors are aware that, in this case, steam sterilization by direct contact [48] was compared with the indirect sterilization of liquids [27]. It was necessary to assume that the z-values remain the same in all investigated sterilization environments in order to mathematically model the inactivation curves, and it is clear that the sterilization environment influences the z-value. For example, increasing the z-value results in shorter inactivation times (i.e. F_{phys} minutes) at temperatures above 121.1 °C and to longer inactivation times (i.e. higher F_{phys} minutes) at temperatures below 121.1 °C. Decreasing the z-value has the opposite influence on the F_{phys} minutes. Nevertheless, without making this assumption, it would not have been possible to directly compare the inactivation curves based on measurements made in different sterilization environments. We took additional measurements to confirm our initial observations and could show that the bacterial inactivation in liquid water also begins to take place at lower temperatures (approximately 90 °C) where hardly any F_{phys} minutes are generated. We expect that the bacterial strain used on the BIs in a validated test has a widely homogenous heat susceptibility. Therefore, these additional results underline and support the results presented in this study, i.e. that the use of a liquid water environment (liquid fluids) in the sterilization process is highly efficient for bacterial inactivation. In any case, the results presented in Fig. 6 show that the measured inactivation curve follows a second-order reaction kinetics for the inactivation of *G. stearothermophilus* in liquid water ($r^2 > 0.85$). A non-linear bacterial inactivation curve was also found in the investigations of Hiatt [49] and Xiao et al. [50] for different sterilization environments. These results lead us to generally conclude that the inactivation curve created by taking a second-order reaction kinetics approach describes the sterilization process accurately. Nevertheless, due to the asymptotic course of the curve, over 530 F_{phys} minutes would be needed to reach a Sterility Assurance Level (SAL) of 10^{-6} . This exorbitantly high number of F_{phys} minutes can obviously not describe the real behaviour during the sterilization process. According to the first-order reaction kinetics curve, approximately 9 F_{phys} minutes would be needed to reach an SAL of 10^{-6} . All measurements made in this study indicate that this value is much more accurate. To investigate the phenomena of the asymptotic course of the curve in more detail, it would be necessary to make many additional measurements, since microbiological evaluations of colonies below 30 CFU are not considered statistically valid. Therefore, this topic exceeds the scope of the current study but could be addressed in a future study.

Overall, the sterilization process in a dialysis solution represents a 'best case' scenario for bacterial inactivation. Sterilization in dialysis solution was significantly faster as compared to water (3 F_{phys} minutes for solution A and 2.5 F_{phys} minutes for solution B vs. 5 F_{phys} minutes in water, see Fig. 7 vs Fig. 6). We assume that the pH value is the most important factor, ensuring the more rapid inactivation of the bacterial spores in the dialysis solution [51,52]. In addition, it may be that the ion concentration is crucial for a more rapid inactivation of bacterial spores [53]. The underlying mechanism is outside the scope of our study.

As in water, the inactivation curves modelled with the second-order reaction kinetics approach fit the measured points more accurately than the exponential decrease curve (first-order reaction kinetics approach). We also found that the reaction kinetics curves created by taking the second-order kinetics approach more accurately reflect the curves based on the measured values for the two investigated dialysis solutions. Nevertheless, exorbitantly high values (over 130) of F_{phys} minutes would also be needed in the dialysis solutions to achieve a SAL of 10^{-6} . The same problem occurs: The decease curve can no longer be adequately measured in the asymptotic region. This practice was justified because no active bacteria were found in the dialysis solutions during the study of PDBS. After a sterilization with 30 F_{phys} minutes no viable bacteria were ever found. This suggests that the inactivation curve in the

asymptotic region has a completely different cause and, therefore, must be further investigated in the future.

Sterilization in air took much longer than sterilization in all other investigated fluids. As a result, the sterilization in air represents the 'worst case' scenario due to the significantly slower bacterial inactivation in the air as compared to the measured bacterial inactivation in the wet steam environment (see the more than 600 F_{phys} minutes shown in Fig. 8(b) for air vs. the 10 F_{phys} minutes shown in Fig. 5 for wet steam). Our findings re-emphasize the well-known fact that sterilization in pure air (i.e. without any moisture) is not successful. Nevertheless, the modelled inactivation curve (first-order) can be used to predict the inactivation of *G. stearothermophilus* during sterilization in air with high accuracy.

The results presented in this study allow us to reach several conclusions. It is possible to mathematically model the inactivation of *G. stearothermophilus* during the sterilization process in different environments (steam, liquid water, dialysis solution and air) with high accuracy. Researchers and developers of sterilization processes can use the information presented in this paper to model the inactivation of *G. stearothermophilus* for any temperature profile for a sterilization process using the investigated sterilization environments. Furthermore, these results may help to improve the overall efficiency of the sterilization processes.

Further studies on the inactivation of *G. stearothermophilus* spores could include making additional measurements with more measured data and in other sterilization environments. The inactivation kinetics presented here may be fruitfully applied to numerically simulate the inactivation of *G. stearothermophilus* spores as a function of the local temperature and the local sterilization environment using CFD [54–58].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.phmed.2021.100046>.

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