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RESEARCH

Nitrogen Dioxide Sterilization Follows Log-Linear Microbial Inactivation Kinetics Using *Geobacillus stearothermophilus* Biological Indicators

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ABSTRACT: The primary purpose of this study was to determine the inactivation kinetics of *Geobacillus stearothermophilus* biological indicators (BIs) exposed to nitrogen dioxide (NO_2) gas in the presence of humidity. BIs inoculated with 6 $\log_{10}$ *G. stearothermophilus* spores were used as a test substrate. Three BI lots manufactured from each of three different BI spore crops were evaluated. Test cycles were run at room temperature with approximately 80% relative humidity. Direct enumeration methods were used to quantify the resistance of spores with surviving populations greater than approximately 50 colony forming units (CFU). Fraction negative methods were used to estimate the surviving spore populations in the quantal region. The methods were combined in order to show spore inactivation from 6 $\log_{10}$ to approximately −2 $\log_{10}$. The *D* value and coefficient of determination (r^2) were calculated. Over 100 direct enumeration and fraction negative cycles were completed at a fixed NO_2 concentration varying only exposure time. Process parameters were maintained over all cycles. Empirical data confirmed a log-linear relationship over an 8 $\log_{10}$ population range with r^2 values greater than 0.8, allowing for extrapolation of the curve to achieve a sterility assurance level (SAL) of 10^{-6}. Study outcomes were comparable for all manufactured BI lots. NO_2 sterilization follows first-order log-linear microbial inactivation kinetics, which is consistent with a mechanism of action based on a single active species. This is the first study to report on the microbial inactivation kinetics of NO_2 sterilization.

KEYWORDS: Nitrogen dioxide, Terminal sterilization, Inactivation kinetics, Sterility assurance, Sterility test.

Introduction

Nitrogen dioxide (NO_2) is a highly effective gas sterilant for use against a wide range of microorganisms including bacterial spores (**1**). Antimicrobial activity is achieved at ambient temperatures in the presence of humidity using low sterilant concentrations. The method does not require lengthy aeration, and after the sterilization cycle completes, the low-level residual sterilant does not result in environmental or human toxicity (1). For these reasons, NO_2 sterilization is an ideal alternative to ethylene oxide (EO) on an industrial scale for sterilizing prepackaged heat, radiation, and/or moisture-sensitive medical devices and prefilled syringes.

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The process variables for NO_2 sterilization are similar to those for EO and include sterilant concentration, humidity, temperature, exposure time, and pressure (2). Control over any or all process variables is easily achieved using a modern NO_2 sterilizer with multiple sensors. A complete description of the mechanics of an NO_2 sterilizer as well as its mechanism of action is available in the literature (1).

For NO_2 to be a viable industrial sterilization method, it must achieve a sterility assurance level (SAL) of 10^{-6}, which is equivalent to the probability of one in one million nonsterile units after exposure to the sterilization process. This in turn requires the demonstration of a 12 $\log_{10}$ microbial kill (i.e., microbial inactivation from 6 $\log_{10}$ to −6 $\log_{10}$,).

As with EO, when using the overkill method (half-cycle), the objective is to demonstrate a 6 $\log_{10}$ reduction in the specified minimum NO_2 exposure time (half-cycle) using a reference microorganism, usually a spore former. The

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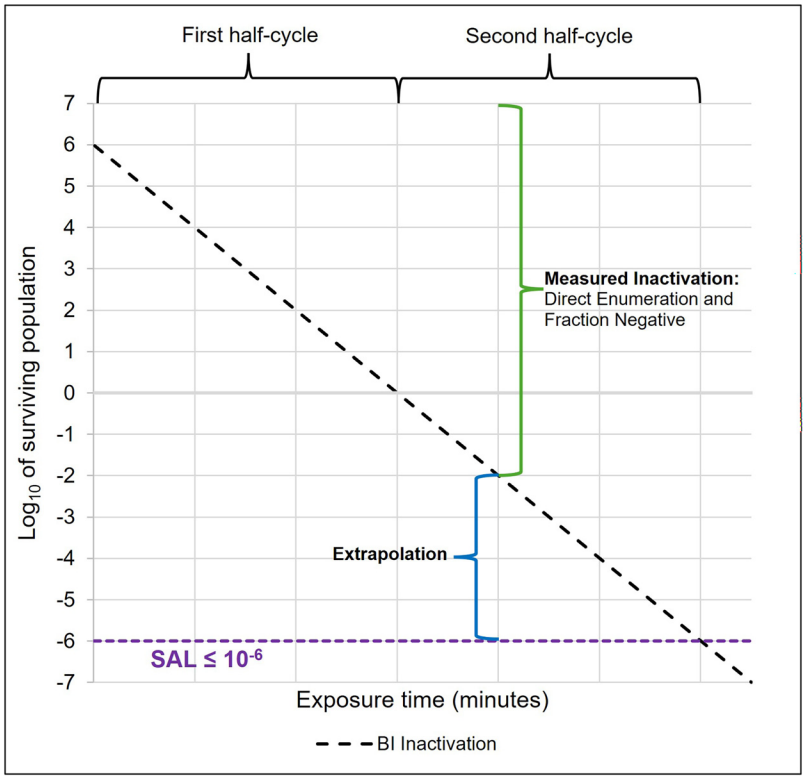


Figure 1

Schematic of an overkill sterilization cycle requiring two half-cycles. Empirical data are generated to demonstrate an 8 $\log_{10}$ reduction using a combination of Direct Enumeration and Fraction Negative methods. If the resulting curve is linear (i.e., the coefficient of determination, r^2, is not less than 0.8), the curve is extrapolated to -6 $\log_{10}$, consistent with an SAL of 10^{-6}.

specified NO_2 exposure time for the sterilization process shall be at least double of this minimum time. Thus, in the overkill method, a 12 spore $\log_{10}$ reduction (SLR) is calculated (i.e., microbial inactivation from 6 $\log_{10}$ to -6 $\log_{10}$).

Since it is impractical to validate a full 12 $\log_{10}$ inactivation curve using only microbiology test data, ISO 14937 (3) recommends that a mathematical relationship characterizing the microbial inactivation with the sterilizing agent exposure be established. To define the upper section of the microbial survivor curve, direct enumeration methods are used. For the section of the curve where there is a low number of survivors occurring (quantal region), fraction negative methods are employed. In the construction of such survivor curves, the practical lower limit of estimation of average numbers of surviving microorganisms is 0.01 (or approximately -2 $\log_{10}$).

An extrapolation of the inactivation kinetics to reach an SLR of 12 $\log_{10}$ or a SAL of 10^{-6} is readily performed when the survivor curve is log-linear (first-order kinetics for microbial kill versus exposure time, see Figure 1). According to ISO 11138-1:2017 (4) clause C.3.8, a survivor curve is considered linear when the determination for the regression coefficient (r^2) is not less than 0.8.

This is the first published research study establishing that NO_2 sterilization follows first-order log-linear microbial inactivation kinetics. This is also one of the few published studies demonstrating that for any "nontraditional" sterilization method, biological indicators (BIs)exposed to the process follow a log-linear survivor curve from 6 $\log_{10}$ to -2 $\log_{10}$. In accordance with ISO 11138-1:2017 (4) clause 6.4.3, *D* values obtained using the direct enumeration and the fraction negative methods are compared to establish that there are no serious deviations from a linear survivor curve. Finally, the study demonstrates that NO_2 process variables are easily controlled over multiple test cycles, which is a necessary precursor to characterization of the manufactured BIs and for routine use of NO_2 sterilization in an industrial process.

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TABLE I
Process Variables and Parameters for All Cycles Maintained at a Constant Value, with the Exception of Exposure Time

Cycle Process Variables	Process Parameters & Tolerance	Primary Measurement Method: Range of Values for All Cycles; Mean Value	Secondary Measurement Method: Range of Values for All Cycles; Mean Value
NO_2 Concentration [NO_2]	1.8 ± 0.1 mg/L	1.83–1.89; Mean = 1.86	1.82–1.88; Mean = 1.86
Percent Relative Humidity (% RH)	80 ± 10%	78.8–83.8 Mean = 81.1	83.1–83.1; Mean = 83.1
Initial Vacuum Pressure	7.0 ± 0.7 mmHg	6.5–7.4; Mean = 7.0	Not Applicable
Absolute Pressure during Exposure	584 ± 25 mmHg	592– 598; Mean = 595	Not Applicable
ΔP (Change in Pressure) during Exposure	577 ± 25 mmHg	585–591; Mean = 588	Not Applicable
Chamber Temperature during Exposure	23 ± 2.0 ˚C	23.2–24.7; Mean = 23.9	Not Applicable

Materials/Methods

Process and Study Parameters

All testing was performed at Noxilizer, Inc. laboratories (Hanover, MD).

Microbial inactivation kinetics were measured using a resistometer, which is a 42 L test chamber having similar functionality to commercial NO_2 Sterilizers (Noxilizer Inc., Hanover, MD). Since the sterilization process requires the addition of both humidity and sterilant in no specific order (1), humidity was added before NO_2 gas to precisely control sterilant exposure time.

For all tests, process parameters were maintained constant within an error margin that would not influence inactivation kinetics, with only exposure time being varied. Although NO_2 can sterilize at a wide range of different pressures, a conventional cycle was selected requiring an initial vacuum of 7.0 ± 0.7 mmHg, after which time humidity, sterilant, and dry air were added until the chamber pressure increased to 584 ± 25 mmHg (see Table I). Sterilant concentration was kept constant at 1.8 ± 0.1 mg/L, while relative humidity (RH) was 80 ± 10% (absolute humidity of 16.5 ± 2 mg/L H_2O). All tests were run at room temperature (23 ± 2˚C). The chamber pressure versus time curve during a cycle is provided in Figure 2.

Two separate methods were used to measure both sterilant concentration and percent relative humidity (%RH), and the results of the two methods were required to be in agreement.

To have confidence that all process parameters were maintained constant throughout the study, the resistometer was equipped with multiple process monitors for both NO_2 concentration and %RH, which was measured by both differential pressure and Fourier transform infrared (FTIR). In routine use, an NO_2 optical sensor, which measures gas concentration by light absorption, and an RH sensor, which measures relative humidity without the presence of sterilant in a connected but separate Relative Humidity Chamber, are also employed.

The primary method for measuring the amount of NO_2 delivered to the resistometer chamber was differential pressure (ΔP) using a small secondary chamber connected to the resistometer. The differential pressure was then used to calculate the NO_2 concentration using a series of equations described in the literature (5). The secondary method for NO_2 measurement used FTIR spectroscopy without the presence of humidity.

The primary method for RH measurement was FTIR without NO_2 present. The secondary method for RH measurement used the change in pressure (ΔP) of the humidified air added to the resistometer chamber. The

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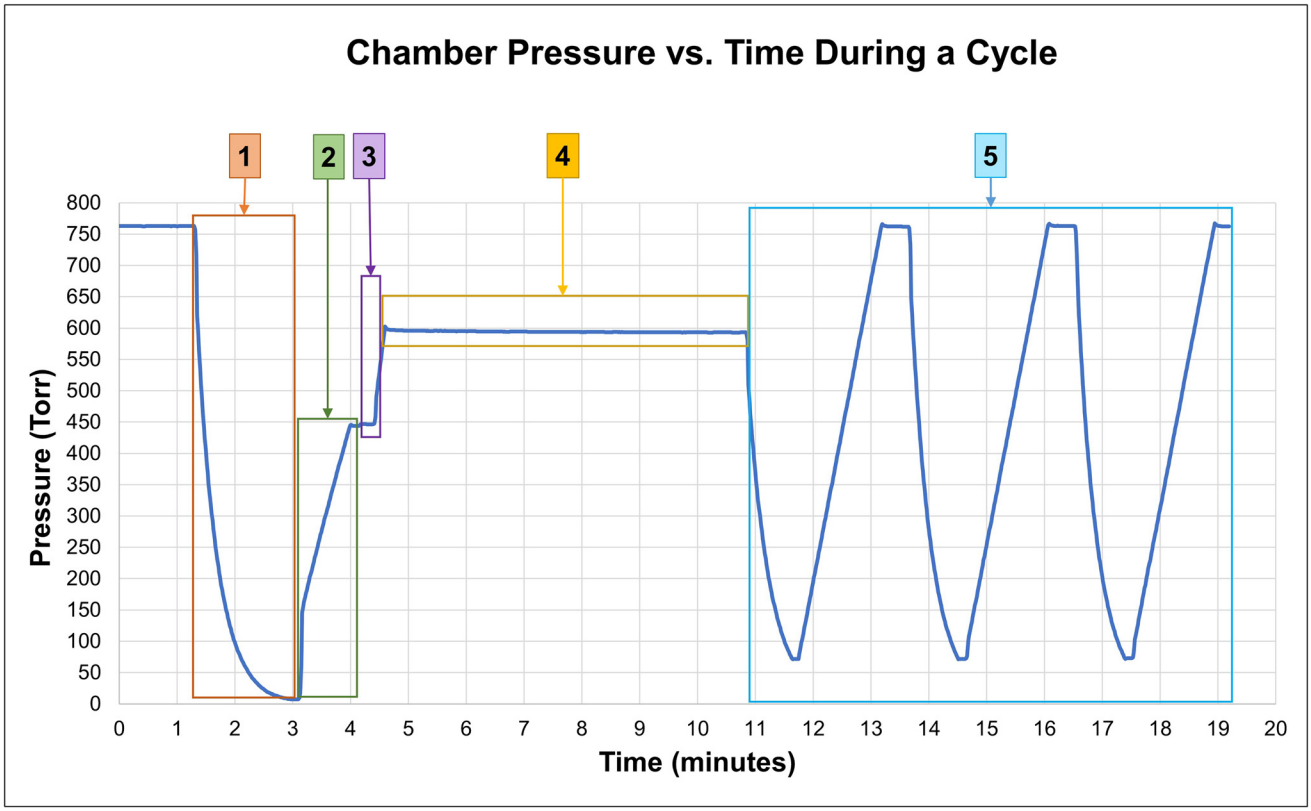


Figure 2

Chamber Pressure (mmHg) versus Time (minutes) for all cycles using a resistometer. Step 1 is a preconditioning phase with a vacuum being drawn to 7 mmHg. Step 2 is injection of humidity. Step 3 is injection of NO_2 gas. Step 4 is an exposure phase (in which exposure time is varied). Step 5 is an aeration phase.

resulting humidity delivered for a specified ΔP of humidified air was established before using the resistometer for BI exposure.

Exposure time, which was purposely varied, was measured in accordance with ISO 18472:2018 (6), Section 4.5, which is applicable to EO resistometers but is equally relevant for any gas sterilant requiring the addition of RH. Specifically, exposure time began after a minimum pressure was achieved in the resistometer chamber reflecting the addition of RH, NO_2, and dry air (in that order). Exposure time ended when the pressure was reduced by a predetermined level during the aeration phase consistent with quenching antimicrobial activity (Step 5, Figure 2).

Finally, temperature was measured after the exposure phase was initiated (Step 4, Figure 2) following stabilization after the introduction of NO_2 and RH. Although exposure temperature was maintained within ± 2°C, in practice the kinetics of NO_2 sterilization are very stable between 20°C and 30°C.

Biological Indicators

BIs are used to demonstrate microbicidal efficacy in gaseous and vapor sterilization methods including NO_2.

For all tests, BIs were used as test substrates (Noxilizer Inc., Hanover, MD), consisting of a 6 mm diameter quartz filter media disk (Cytiva, Marlborough, MA) inoculated with 6 $\log_{10}$ *Geobacillus stearothermophilus* spores (ATCC 7953 type, True Indicating, Toledo, OH). *G. stearothermophilus* was previously found to be the most resistant organism to the NO_2 sterilization process (1).

Each lot of BIs was characterized for spore population, *D* value (i.e., time at a specified dose required to inactivate 90% of test microorganisms), and survival/kill window in accordance with ISO 11138-1:2017 (4). Each BI was packaged in a heat-sealed Tyvek/Mylar pouch before being placed into inventory. Packaging was removed before use of BIs in this study.

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The carrier material and packaging materials in the presence of the sterilization exposure conditions were previously found to not exhibit growth inhibition per Annex B of ISO 11138–1:2017 (4).

Prior to use during this study, the spore population was confirmed for every BI lot, per Annex A of ISO 11138–1:2017 (4).

For all tests (direct enumeration and fraction negative, as described following), three BI lots from each of three different BI spore crops were evaluated.

Direct Enumeration Tests

Direct counting of surviving spores, or colony forming units (CFU), recovered from a BI was done in accordance with ISO 11138–1:2017 (4), Annex C and ISO 11138–7:2019 (7), Annex F. For each exposure time, four (4) BIs were placed in a Petri dish with a wire mesh covering to allow for gas exposure, then placed in the resistometer. Within 2 hours or less of cycle completion, the BIs were aseptically removed from the Petri dish and transferred into individual sterile conical tubes containing 10 mL of sterile phosphate-buffered saline (PBS) with glass beads and mixed using a vortex mixer for 2 minutes (i.e., the time required to macerate the quartz carrier). After mixing, 1:10 serial dilutions were prepared as required using sterile water. Duplicate 1 mL samples were pour plated on Tryptic Soy Agar (TSA, General Laboratory Products, Yorkville, IL) plates and incubated at 55°C–60°C for 40–72 hours, at which point the plates were counted.

For long sterilant exposure times, where fewer than 50 CFU were expected to be recovered from the serial dilution, the entire volume of PBS buffer was inoculated directly onto multiple TSA plates using an exhaustive plating method. Each lot of TSA was previously validated to demonstrate growth promotion with spore populations fewer than 100 CFU, as required by ISO standards (4, 7).

Direct enumeration was used to establish the resistance of spores for surviving populations greater than approximately 50 CFU (which roughly represents the practical lower limit for enumeration tests). The recovery efficiency of the enumeration method was previously measured and found to be in close agreement with the inoculum count; therefore, no correction factor was used.

A minimum of five exposure times were used for each direct enumeration test including:

- One exposure in which the BIs were not subjected to the sterilant (e.g., "0" time exposure)
- At least one exposure in which the viable population was reduced to 0.01% of the original inoculum (4 $\log_{10}$ reduction)
- A minimum of three exposures covering the interval between the "0" time exposure and the 4 $\log_{10}$ reduction exposure.

In total, data from a minimum of 60 BI carriers were evaluated for each spore crop (i.e., a minimum of four BIs per exposure time and five exposure times per lot, which was repeated three times for a single spore crop). For all three spore crops under evaluation, a minimum of 180 BI carriers were evaluated. In practice, a higher number of cycles were added beyond the minimum required by ISO standards (4, 7), thus increasing the total number of BIs used in the study.

Survival data in which no growth was observed for one or more of the four BIs were not included in the analysis, since any value below the lower limit of 50 CFU falls into the fraction negative area, per ISO 11138–1:2017 (4) Annex C.

The *D* value and coefficient of determination for the regression (r^2) was calculated for each of the nine (9) direct enumeration survivor curves as a function of the exposure time.

Fraction Negative Tests

Fraction negative tests were completed in accordance with ISO 11138–1:2017 (4), Annex D and ISO 11138–7:2019 (7), Annex C. A total of 20 BIs were used for each exposure time. The Holcomb-Spearman-Karber procedure (HSKP) was used to estimate the *D* value.

For each exposure time, 20 BIs were placed in a Petri dish with a wire mesh covering to allow for gas exposure, then placed in the resistometer. Within 2 hours or less of cycle completion, the BIs were aseptically removed from the Petri dish and transferred into individual sterile conical tubes containing 5 mL of Tryptic Soy Broth (TSB) growth medium. The tubes were incubated at between 55°C and 60°C for 14 days. The

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growth media was previously validated to demonstrate growth of spore populations fewer than 100 CFU as required by ISO standards (4, 7).

A minimum of five exposure times were used for each HSKP test including:

- One exposure in which all BIs demonstrate growth
- At least two exposures in which a fraction of the BIs demonstrate growth
- At least two sequential exposures in which no growth is observed.

For a given BI lot, at least 100 BIs were required (excluding controls), reflecting the minimum of five required exposure conditions with 20 BIs per exposure. Since the study was repeated for each of three separate lots and for each of three separate spore crops, a total of at least 900 BIs were required. In practice, a higher number of cycles were added beyond the minimum required by ISO standards, thus increasing the total number of BIs used in the study.

The Halvorson-Ziegler Equation was used for estimating the probable number of surviving spores per carrier in the quantal region (i.e., most probable number or MPN). Specifically, fractional BI data were used to determine the MPN of surviving spores at a given exposure time, using the following formula, where n is the total number of BIs tested at a given exposure time, and r is the number of negative BIs recorded at that exposure time:

$$\log_{10} N = \log_{10}\left(\ln\frac{n}{r}\right)$$

The D value and coefficient of determination (r^2) were calculated for each of the nine (9) fraction negative survivor curves as a function of the exposure time.

Combining Direct Enumeration with Fraction Negative Curves

Lethality data from both the direct enumeration and fraction negative tests were combined and plotted on the same curve (i.e., $\log_{10}$ of the surviving population against exposure time in minutes). The D value and r^2 for the regression were calculated for each of the nine (9) combined survivor curves. Linearity was demonstrated by the coefficient of determination being not less than 0.8 (i.e., $r^2 \geq 0.8$), consistent with ISO 11138–1:2017 (4) Section 6.4.4.

Results

Process Variables

A summary of process variables (including NO_2 concentration, RH, pressure, temperature, and exposure time) for all cycles including the range and mean values is provided in Table I. In total, 111 cycles were completed for this study, including on average of 6 direct enumeration cycles and 7 fraction negative cycles per BI lot. In no instance was an individual cycle parameter outside of the required range.

Direct Enumeration Test Data

On average, six exposure cycles were completed for each BI lot using direct enumeration, which is one more than required by ISO standards (4, 7). D value and regression analysis for each of three separate BI lots (for three separate spore crops) are summarized in Table II.

The D values are consistent from lot to lot. The D value for each of the three BI lots made from a single spore crop were within $\pm$ 20% of the spore crop average D value (Table II). The coefficient of determination values (r^2) were always greater than 0.8 and thus consistent with first-order kinetics.

Fraction Negative Test Data

On average, seven exposure cycles were completed for each BI lot. Each BI lot D value is calculated using the HSK fraction negative formula, meaning that at least two additional fractional-growth cycles were added beyond the minimum required by ISO standards. In a typical example, three cycles were run at exposure times providing data points in the quantal region (after completion of MPN analysis), with one additional cycle showing all growth and three cycles showing no growth. D value and regression analysis for each of three separate BI lots (for three separate spore crops) are summarized in Table II.

The D value demonstrates excellent consistency from lot to lot. Specifically, the D value for each of the three (3) BI lots made from a single spore suspension were

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TABLE II
Individual Direct Enumeration and Fraction Negative *D* Values and r^2 for Each BI Lot and Spore Crop, as Well as the Combined Survivor Curve r^2 Value. *D* Value is Expressed in Minutes

Lot #		Direct Enumeration		Fraction Negative		Combined Survivor Curve
		D Value	r^2	*D* Value	r^2	r^2
Spore Crop 1						
Lot 1	CNS2105120	0.42	0.88	0.37	0.95	0.94
Lot 2	CNS2106170	0.40	0.95	0.37	0.88	0.90
Lot 3	CNS2108100	0.33	0.94	0.46	0.90	0.87
Average *D* value ±20% (Range)		0.36 ± 0.07 (0.29–0.43)		0.40 ± 0.08 (0.32–0.48)		
Spore Crop 2						
Lot 1	CNS2108170	0.32	0.92	0.40	0.99	0.95
Lot 2	CNS2109020	0.43	0.97	0.33	0.82	0.82
Lot 3	CNS2110260	0.32	0.98	0.43	0.99	0.95
Average *D* value ±20% (Range)		0.40 ± 0.08 (0.32–0.48)		0.39 ± 0.08 (0.31–0.47)		
Spore Crop 3						
Lot 1	CNS2201060	0.33	0.88	0.33	0.88	0.86
Lot 2	CNS2203020	0.32	0.97	0.48	0.92	0.90
Lot 3	CNS2203080	0.36	0.93	0.42	0.94	0.93
Average *D* value ±20% (Range)		0.34 ± 0.07 (0.27–0.41)		0.41 ± 0.08 (0.33–0.49)		

within ± 20% of the spore crop average *D* value, which is a requirement of ISO 11138–1 (4) Section 6.4.3 (see Table II). As with the direct enumeration data, the r^2 values for the survivor curve using the fraction negative data were always greater than 0.8 and thus are consistent with first-order kinetics.

Combination of Direct Enumeration and Fraction Negative Data

In accordance with ISO 11138–7 (7) Figure A.4 for determining *D* value, the number of surviving microorganisms from both the direct enumeration and the fraction negative methods were plotted on the same graph. The combination of the direct enumeration and fraction negative data shows spore inactivation from 6 $\log_{10}$ to approximately -2 $\log_{10}$. If the data is consistent with a log-linear relationship, the curve may be extrapolated to -6 $\log_{10}$, which is necessary to achieve a SAL of 10^{-6}. Establishing linearity is done by combining empirical data for each lot on a single semilogarithmic curve of the $\log_{10}$ of the surviving population against exposure time, and calculating the coefficient of determination using linear regression (r^2).

A typical example of a $\log_{10}$ of the surviving population versus exposure time is provided in Figure 3. For BI lot CNS2105120 (Spore Crop 1, Lot 1), empirical data extend from 6 $\log_{10}$ to ~ -1 $\log_{10}$ (quantal region), and the $r^2 = 0.94$.

A summary of the data used in Figure 3 is provided in Table III. Specifically, three data points were generated in the quantal region using MPN analysis, as well as one data point showing all growth and three data points showing no growth.

A summary of the linear-regression analysis for all combined survivor curves is provided in Table II. For all nine (9) plots, $r^2 \geq 0.8$, confirming a linear kill relationship.

Discussion

Starting in 2019, the United States Food and Drug Administration (FDA) began multiple initiatives to encourage the development of alternative sterilization methods to replace EO. Specifically, in July 2019, a "Public Innovation Challenge" was initiated to "encourage the development of new approaches to device sterilization ... that do not rely on ethylene oxide" (8). In addition, a challenge was initiated to develop technologies to reduce EO emissions "to as close to zero as possible from the ethylene oxide sterilization process" (9). This effort was followed in late 2019

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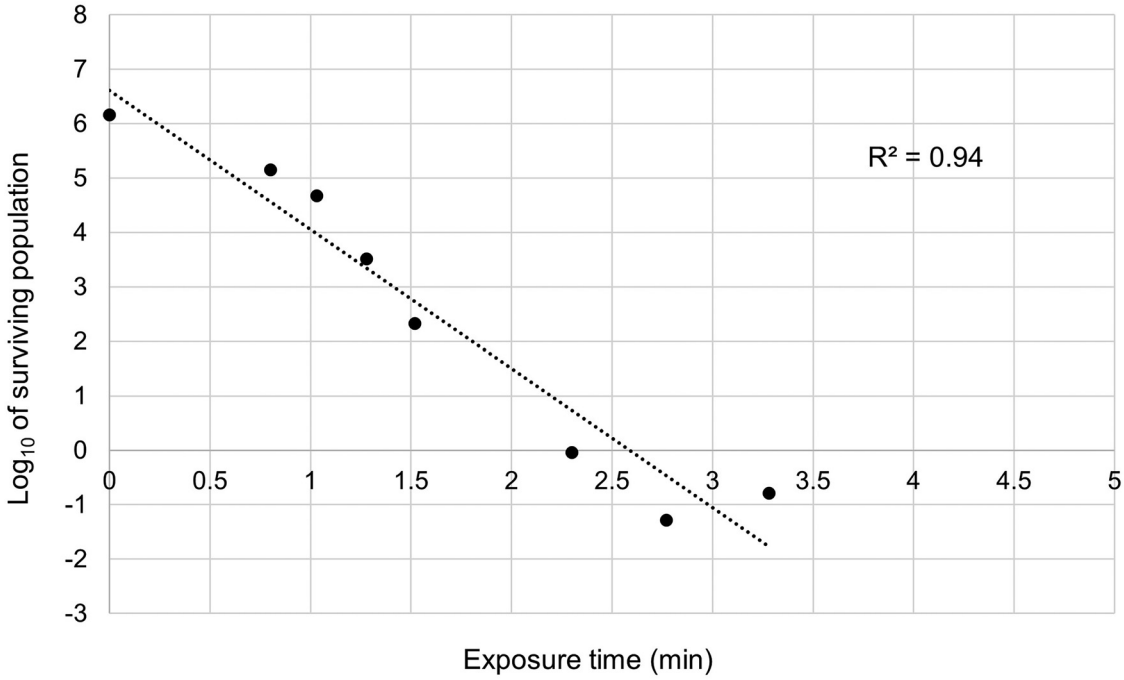


Figure 3

Combined Fraction Negative (HSKP method) and Direct Enumeration plot which extends into the quantal region of BI Lot CNS2105120 (Spore Crop 1, Lot 1). Linear Regression shows a linear relationship (r^2 = 0.94). Three data points were calculated using MPN, as detailed in Table III, with the remainder of the data points generated using Direct Enumeration.

with an FDA "EO Master File Pilot Program", which allowed manufacturers of Pre-Market Approval (PMA) devices using EO sterilization to modify the EO process to reduce emissions without needing preapproval from the FDA (10). In 2022, the FDA expanded the Master File Program to include more common 510 K-cleared devices, making it easier to reference Master Files when switching from EO to an alternative sterilization method (11). Finally, in 2023, the FDA extended the Master File Pilot Program to include gamma radiation as an alternative to EO (12).

Coinciding with the FDA's initiatives, the Environmental Protection Agency proposed a significant reduction in EO emissions by commercial sterilization facilities (13). This in turn was prompted by a prior determination that a number of sterilization facilities "pose elevated lifetime cancer risks to the surrounding communities, some of which are exceptionally high" (13). At the same time, civil litigation and regulatory actions against EO sterilization facilities rapidly increased (14).

Alternatives to EO sterilization processes include NO_2, ozone (O_3), vaporized hydrogen peroxide (VH2O2), supercritical CO_2 ($scCO_2$), and chlorine dioxide (ClO_2). However, no single method demonstrates the same level of material compatibility and predictable lethality found with EO. In addition, any alternative must be able to sterilize packaged medical products with no toxic residues and require little to no additional aeration time after removal from the sterilization chamber. In this regard, the most viable options are NO_2, VH2O2, and ClO_2, with the remainder of alternative methods having niche applications (e.g., $scCO_2$, which is primarily used for sterilizing tissue or other natural biomaterials [15]) and/or significant material-compatibility constraints (e.g., O_3, which degrades certain polymers commonly used in medical devices [16]).

VH2O2 sterilization processes have been thoroughly evaluated in large part because of its long history-of-use in small hospital-based sterilizers for processing heat-sensitive medical devices. However, VH2O2 sterilizers have complicated thermodynamics, because hydrogen peroxide is not a gas but is an aqueous liquid at ambient temperatures, and when vaporized with heat and low-pressure, will be in vapor/liquid phases equilibrium (17), ISO 22441:2022 (18), Annex I. As a result, it is

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TABLE III
A Summary of Individual Data Points, Generated from Both Direct Enumeration and Fraction Negative Analysis of BI Lot CNS2105120 (Spore Crop 1, Lot 1), Used to Plot log_{10} Population by Exposure Time as Shown in Figure 3

Test Type	Exposure Time (Min)	Surviving Population	Log_{10} of Surviving Population
Direct Enumeration	0	1.45E + 06	6.16
	0.80	1.40E + 05	5.15
	1.03	4.66E + 04	4.67
	1.28	3.22E + 03	3.51
	1.52	2.11E + 02	2.32
Fraction Negative	1.30	All growth	All growth
	2.30	9.16E-01	−0.04
	2.77	5.13E-02	−1.29
	3.28	1.63E-01	−0.79
	4.22	No growth	No growth
	5.28	No growth	No growth
	6.28	No growth	No growth
Slope		−2.56	
Combined *D* value		0.39	
r^2		0.94	

difficult for VH2O2 processes to maintain constant exposure conditions over a prolonged exposure time. This in turn can make characterization of consistent kill kinetics challenging, particularly in a large, industrial-scale sterilizer (volume greater than 1000 L in size), where small differences in chamber temperature can have a significant impact on the vapor/liquid phases equilibrium of the VH2O2 sterilizing agent (19; ISO 22441, Annex I [18]).

Among other alternative sterilization methods, a commercial white paper claimed linear kinetics for ozone sterilization combining both direct enumeration and fraction negative data, with an $r^2 = 0.97$ (20). Log kill was compared to half-cycle dose, which was reported as a percentage, presumably at a constant time. However, the curve only had one data point in the quantal region (≈ 6 log_{10} kill), with no evidence of multiple data points as required by ISO standards. Nonetheless, this is one of the few publications reporting kinetics based on the combination of direct enumeration and fraction negative data.

Limited kinetics data have been published on ClO_2 gas sterilization by Wang et al. (21), who concluded that the method has nonlinear kill kinetics. BIs were manufactured by the author using a spore suspension of *Bacillus subtilis* var. *niger* or *S. albus* bacteria inoculated on sterile filter paper (1 cm^2) and placed in a sealed centrifuge tube. The microbial population was $\geq 10^6$ spores per carrier. In lieu of use of a resistometer, a glove box equipped with a secondary ClO_2 gas sensor was used, suggesting that studies were done at atmospheric pressure and ambient temperature, generally with 70% RH. Treatment time was varied over a wide range of values with differing sterilant concentrations, with only direct enumeration studies completed. According to the authors, kinetics was nonlinear but did fit a Weibull model; however it was acknowledged that the BI coupon "significantly affected the inactivation effect". Based on the experimental details, it would be premature to declare that alternative ClO_2 processes would not demonstrate first-order inactivation kinetics, but further study is necessary.

The current study is one of the few comprehensive evaluations of microbicidal kinetics for an alternative sterilization technology combining direct enumeration and fraction negative methods. Construction of a survivor curve by enumeration establishes the resistance for surviving populations greater than approximately 5×10^1, whereas the fraction negative method establishes a statistically based calculation of surviving test organisms below that level. Good correlation of the *D* values obtained by the two methods can therefore be used to establish that there are no serious deviations from a linear survivor curve.

This study demonstrates that NO_2 sterilization kinetics are stable and predictable using different lots of BIs

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made with different spore crops, as mandated by ISO standards and the FDA. Replication of the study using nine different BI lots provides sufficient statistical power to confirm first-order microbial inactivation. All *D* values calculated by the direct enumeration and fraction negative methods are within the normal acceptable variability for a *D* value defined at ± 20% in ISO 11138–1 (4). In addition, for all three spore crops tested, at least one lot shows a lower *D* value in the fraction negative area than by direct enumeration (Table II), which means that if there is a tailing effect, it is minimal. Finally, the linearity of the process is demonstrated by regression analysis (r^2) of combined direct enumeration and fraction negative survivor curves, which in all cases is > 0.80.

The results of Table II are enabled by the development of a well-controlled resistometer and the optimization of the BI manufacturing process for the physical and chemical nature of this sterilization process. The importance of this study is not only that it demonstrates linear kill kinetics, but also that it confirms process variables can be both measured and controlled over multiple cycles, a necessary prerequisite to eventual parametric control.

Finally, demonstrating that NO_2 has first-order inactivation kinetics on the entirety of its survivor curve (direct enumeration and fraction negative) makes the establishment of a cycle using the BI/bioburden or bioburden approaches described in Annex A of ISO 11135 (2) more predictable This is a significant advantage for an industrial sterilization process in that it allows for routine use of bioburden validation. Bioburden validation requires routine monitoring of microbial contaminants in a manufacturing facility and on finished product, both in terms of type and quantity of microbes. Since most manufacturing is done using clean rooms and other means for minimizing contamination, native bioburden is typically low (2 $\log_{10}$ to 3 $\log_{10}$ CFU), allowing for reduced sterilizing agent exposure versus the overkill method described in Annex B of ISO 11135 (2). Bioburden validation is particularly useful when a product is made of materials that are highly sensitive to the sterilizing agent exposure, including both biologics and synthetic polymers. Application of a BI/bioburden or bioburden validation approach as described in ISO 11138–7 (7) clause 7.3 and 7.4, respectively, could expand the use of alternative sterilization methods that show moderate levels of material incompatibility, thus achieving the FDA's objective to minimize the use of EO.

Conclusion

This is one of the few comprehensive studies confirming first-order inactivation kinetics for a nontraditional sterilization process in accordance with ISO 11138–1 (4) and ISO 14937 (3). Using fraction negative and direct enumeration methods, empirical data were generated that showed linear inactivation kinetics from 6 $\log_{10}$ to ≈ -2 $\log_{10}$ by varying exposure time. Regression analysis of the full survivor curve (direct enumeration and fraction negative) confirmed a linear kill relationship ($r^2 \geq 0.8$), allowing for the survivor curve to be extrapolated to a 12 $\log_{10}$ reduction, consistent with a SAL of 10^{-6}. Outcomes were replicated using multiple BI lots and spore crops. Based on this outcome, NO_2 sterilization can be considered a viable alternative to EO in industrial sterilization.

Conflict of Interest Declaration

Thomas Richards and Sylvie Dufresne received a consulting fee from Noxilizer, Inc.

David Opie, Delaney Lisco, Tiffany Bianchi, Gabriella Shahine, Huyen Nyugen, and Natalie Simmons are employees of Noxilizer, Inc.

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