Comparison of Nitrogen Dioxide and Hydrogen Peroxide Biodecontamination Processes

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Introduction to NO_2 Technology

Nitrogen dioxide (NO_2) is an effective sterilant and can rapidly kill microorganisms and has advantages over the use of hydrogen peroxide for biodecontamination. The NO_2 technology can reduce the biodecontamination time by more than 50%, reduce the time needed to aerate to ppb levels, and generate the NO_2 sterilant gas using electricity so that shipping and management of sterilant is not necessary. NO_2 is a rapid and effective sterilant, successful in a wide range of applications (1). A growing number of pharmaceutical and medical device manufacturers are adopting NO_2 technology for a variety of terminal sterilization and decontamination applications. These applications include terminal sterilization of medical devices and drug delivery systems, as well as the decontamination of aseptic filling lines and isolators.

NO_2 Sterilant Properties

At ambient conditions, NO_2 is a true gas and not a vapor. The gaseous nature of NO_2 results from the low boiling point of NO_2, which is 21°C at sea level, and corresponds to a relatively high (1 atm) saturation concentration at room temperature. This gaseous nature of NO_2 at ambient conditions allows for uniform sterilant gas dispersion throughout the isolator volume. Also, the gaseous and non-condensing nature of NO_2 allows for quicker removal of the sterilant gas and sterilant residuals during aeration of the isolator and sterilization load. This is in contrast to the distribution of vaporous sterilants, like hydrogen peroxide vapor (HPV), which are sensitive to temperature gradients and can condense on surfaces (2). Such condensation produces a non-uniform distribution of sterilant and increases aeration time. Therefore, all other factors being equal, the NO_2 sterilization/decontamination process requires less time than other sterilization methods.

The biological indicators (BIs) used for the validation and monitoring of the NO_2 process consist of *Geobacillus stearothermophilus* as the indicator organism on a suitable carrier. The inoculated BI carrier is placed in a Tyvek®/Mylar® pouch. The BIs can be made with a variety of carrier materials, with the most common form being *G. stearothermophilus* on stainless steel in a Tyvek/Mylar pouch. Other carrier materials include small discs of common isolator materials and glass filter media. Standard procedures are used with these BIs for handling, incubation, D-value measurements and process validation.

Biological indicators, consisting of spores of *G. stearothermophilus*, exhibit a log-linear inactivation kinetics with increasing duration of NO_2 gas exposure. Figure 1 illustrates the log-linear progression of population reduction as exposure time to NO_2 gas is increased, evaluated with direct enumeration of the surviving viable spore population. The BIs used to measure these data were comprised of *G. stearothermophilus* spores inoculated onto 7.2 mm diameter stainless steel discs. Once dried, the BI discs were packaged in Tyvek®/Mylar® pouches, placed into a test chamber and exposed to 2.75 mg/L of NO_2 with 70% to 80% relative humidity. The D-value for the inactivation shown in Figure 1 is calculated to be 1.77 minutes. This D-value characterization of the BIs was conducted in a small volume chamber used for BI characterization.

D-values Measure with NO_2 and H_2O_2

For the comparison of the HPV and NO_2 processes, the D-values of BIs were measured for both the HPV and NO_2 processes using the transfer airlock of a 4-glove stainless steel and glass isolator (the Getinge ISOCYT FREJA aseptic processing isolator). A fraction-negative technique was used for the measurement of D-values, which consists of simply placing the BI in incubation tubes with TSB, incubated for 7 days, and inspecting the growth medium for evidence of microbial growth. The same lot of BIs was used for both HPV and NO_2 processes, with each BI consisted of a 7.2 mm stainless steel discs, inoculated with spores of *G. stearothermophilus.* The BIs were individually packaged in Tyvek/Mylar pouches. The population on each BI carrier was found to be 1.9×10^6 spores. For the D-value test, the BIs were placed on the shelf in the transfer airlock, near the outlet filter. At this location, the sterilant is expected to be well mixed, providing consistent lethality.

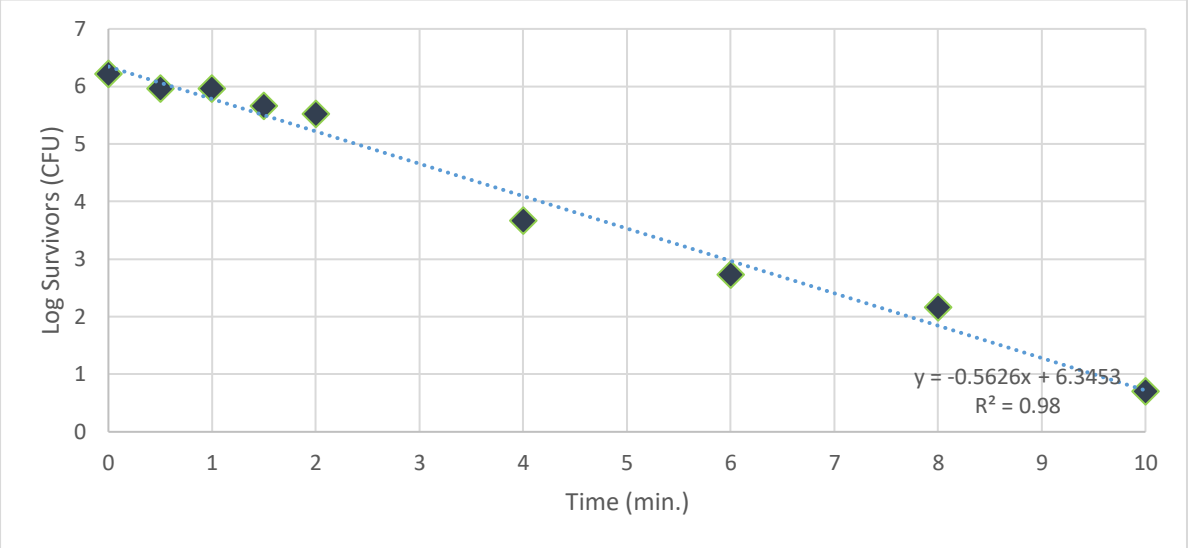


Figure 1. The log of the number of spores surviving the NO_2 exposure process is shown to decrease with increasing exposure time. The amount of sterilant and relative humidity added to the sterilization for these cycles were the same for all exposure cycles.

For measuring the D-value with the NO_2 process the sterilant concentration was 5 mg/L during the exposure phase. The relative humidity was between 70% RH and 75% RH. Ten BIs were exposed to each exposure time. The results of these exposure cycles are shown in Table 1. The D-value for the data in Table 1 was calculated using the Holcomb Spearman Karber (HSK) method (3), and the D-value was found to be 1.4 minutes.

It is important to note that the sterilant concentration used for the NO_2 D-value testing was significantly below the normal process concentration. For the D-value results shown in Table 1, the NO_2 concentration was 5 mg/L. However, the concentration during a normal process is more than 10 mg/L. The lower concentration is used for the D-value testing to provide a wider range of exposure dwell times over which the fractional BI response is observed. When the normal process concentration of NO_2 is used, then the BIs may be negative after less than 4 minute exposure dwell. This flexibility of varying the gaseous sterilant concentration (without causing condensation of the sterilant) is not available to the HPV process.

Table 1. Data used for determining the D-value of BIs consisting of *G. stearothermophilus* spores on stainless steel discs, in a Tyvek/Mylar pouch and exposed to 5 mg/L NO_2 and 70% RH to 75 % RH. The average starting population of spores on the BI carriers was 1.9 x 10^6 CFU.

Exposure Time (min.)	Number of BIs Exposed	Fraction of BIs Negative (r/n)
4	10	0/10
7	10	2/10
10	10	8/10
13	10	8/10
16	10	9/10
20	10	10/10
30	10	10/10

The D-value was measured with the HPV process using process parameters that consisted of dehumidification to 10% RH, injection of H_2O_2 at 4.5 g/minute for one minute, continued injection of the H_2O_2 (stabilization phase) at 1.5 g/minute for the remaining exposure time, followed by aeration. The air flow during the injection and stabilization phases was 10 m^3/hr. The microbiological data from these HPV processes is shown in Table 2. The D-value from the HPV process is found to be 0.6 minutes using the HSK method of calculation.

Table 2. Data used for determining the H_2O_2 D-value of BIs consisting of *G. stearothermophilus* spores on stainless steel discs, in a Tyvek/Mylar pouch. The average starting population of spores on the BI carriers was 1.9 x 10^6 CFU.

Exposure Time (sec)	Number of BIs Exposed	Fraction of BIs Negative (r/n)
0.5	10	0/10
1	10	1/10
3	10	5/10
6	10	6/10
8	10	10/10
9	10	10/10

Validation of Decontamination Process

In order to compare the performance of the HPV biodecontamination process with the NO_2 biodecontamination process, a single isolator was configured for both processes. Using the same test system is critical for the comparison of lethality and aeration rates, as both gas processes need to use the same isolator design, materials of construction and the same air flow rates. Therefore, process time comparisons were completed using the Getinge ISOCYT FREJA aseptic processing isolator, having a volume of 1000 L.

To determine the time required to inactivate the BIs, biodecontamination cycles were completed over a range of dwell times. The shortest dwell time that resulted in all BIs being killed was defined as the Total Kill Time, or TKT. The exposure dwell used for the biodecontamination process for both HPV and NO_2 was at least 20% longer than the TKT. These cycles were repeated three times in the full isolator as a validation of the biodecontamination process. The process parameters used for the HPV and NO2 biodecontamination are shown in Table 3.

Table 3. Comparison of Process Steps for the 1000 L ISOCYT FREJA Isolator

NO_2 Process	**Time (Min.)**	**H_2O_2 Process**	**Time (Min.)**
Humidification to 60% RH	1	Dehumidification to 20% RH	8
Dosing (10 mg/L of NO_2 added)	1	Dosing (Injection Phase at 4.5 g/min.)	1
Dwell (NO_2 circulated)	28	Dwell (Stabilization Phase at 1.5 g/min.)	36
Aeration to 1 ppm	6	Aeration to 1 ppm	40
Total Process Time	**36 Minutes**	**Total Process Time**	**85 Minutes**

The validation process for the full 1000 L ISOCYT FREJA included placing 32 BI throughout the isolator. The BIs were widely distributed in the volume being decontaminated. After exposure to the biodecontamination process, the BIs were placed in incubation tubes with Tryptic Soy Broth (soybean-casein digest media, or TSB) and incubated for 7 days. Biodecontamination was considered successful when the BIs are sterile, meaning that none of the tubes containing exposed BIs exhibited growth (turbidity). The BIs exposed in all cycles using the process parameters shown in Table 3 were sterile.

The time required to complete a biodecontamination process with the NO_2 process is faster than a comparable process completed with HPV. A review of the time required for each process step, listed in Table 3, shows that the dehumidification, dwell and aeration phases for the H_2O_2 process takes more time than the corresponding time with the NO_2 process. The dehumidification of an isolator is analogous to aeration of an isolator. This is due to the similar interactions that humidity and vapor phase H_2O_2 have with the surfaces of the isolator. Conversely, adding humidity can be quite rapid. With the equipment used for the NO_2 process, an isolator can be humidified at a rate of 1000 L per minute. This humidification system is scalable to larger isolator volumes.

Adding sterilant to the isolator requires about 1 minute for both the HPV and the NO_2 processes. One difference between these HPV and NO_2 process steps is that the injection of the H_2O_2 is followed by a stabilization phase that includes a continuous addition of the H_2O_2. Conversely, the NO_2 sterilant gas is recirculated throughout the dwell phase without adding additional NO_2 sterilant.

The biodecontamination process ends with the aeration step. Aeration air flow through the isolator is 60 m^3/hr (or about 75 air exchanges per hour). With testing completed in the 1000 liter Getinge ISOCYT FREJA isolator, the time required for the HPV concentration to reach 1 ppm was 40 minutes, as measured with gas sampling tubes (a.k.a., Draeger tubes). The time required for the HPV to reach 0.1 ppm was 140 minutes. The NO_2 aeration process required 6 minutes to reach 1 ppm of residual sterilant and 30 minutes of aeration to 0.1 ppm (100 ppb). The HPV and NO_2 aeration process is shown in Figure 2.

Aeration of Sterilant to ppb-levels

For many applications, aeration of the isolator to 1 ppm is sufficient. In certain applications, pharmaceutical manufacturers will require that residual sterilant is reduced to a level that will not damage the pharmaceutical products. For example, to avoid degradation of biologics, the safe residual sterilant level may be as low as 30 ppb (4, 5). Aeration of hydrogen peroxide to 30 ppb can take several hours. Conversely, NO_2 aeration to a level that is safe for drug products in the filling line is faster than aeration of H_2O_2, when the same air exchange rates are used for the comparison. There are two reasons for faster NO_2 aeration. The first reason is that H_2O_2 sterilant

is applied to the isolator at concentration levels that exceed the saturation concentration of the H_2O_2, causing condensation of the H_2O_2. This condensation requires long aeration time to evaporate from surfaces. The second reason is that the environmental background of NO_2 is much higher than the environmental levels of H_2O_2. Because NO_2 is present in the ambient environment, at levels that often exceed 30 ppb, aeration to 30 ppb is not necessary.

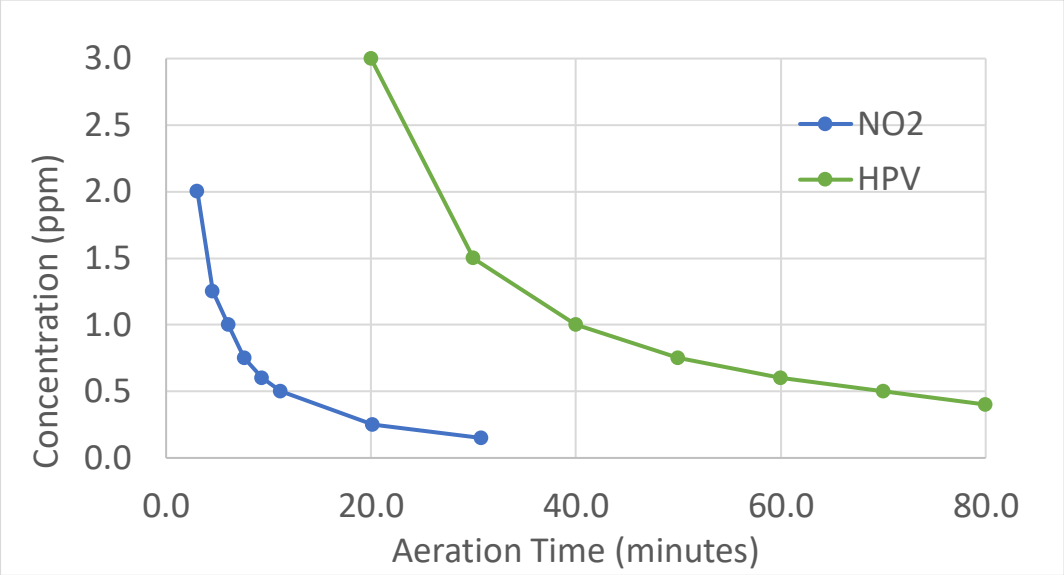


Figure 2. This graph shows the concentration of HPV and NO_2 in the isolator during aeration.

The concentration of hydrogen peroxide during a biodecontamination process is determined by the amount injected. The air flowing through the isolator which carries the H_2O_2 has a limited capacity to hold H_2O_2 in gaseous form. The maximum amount of H_2O_2 that air will hold is referred to as the saturation concentration. Concentration levels above the saturation concentration will cause condensation of the H_2O_2 onto the surfaces in the isolator.

During the stabilization phase, H_2O_2 is added at a rate of 1.5 g per minute (1500 mg/min.). The air flow rate that carries the hydrogen peroxide into the isolator is 10 m^3/hr (167 L/min.). This stabilization rate of HPV addition corresponds to delivering 9 mg/L (6472 ppm) to the isolator. The saturation concentration of H_2O_2 at 25 °C and 10 % relative humidity is 1.71 mg/L (1230 ppm) (6). Therefore, the HPV is injected into the isolator at a rate that is more than 500 % of the saturation concentration. Injecting this amount of hydrogen peroxide creates a supersaturated vapor and contributes to sterilant condensation on the isolator surfaces (7).

The condensation of the H_2O_2 sterilant can lead to material damage, degradation of the products being filled, and lengthens the time needed for aeration. Some biotech drugs are based on proteins and large molecules that are highly sensitive to residual H_2O_2. Residual H_2O_2 sterilant levels as low as 100 ppb can degrade pharmaceuticals (4). Therefore, aeration down to 30 ppb is considered safe, and can be required for some filling line applications. Current specifications for aeration of H_2O_2 to 30 ppb is as long as 9 hours (4).

An additional complication is that the saturation concentration of any gas (and H_2O_2) is very temperature dependent, with about 6% change in H_2O_2 saturation concentration with every degree Celsius change in temperature. This means that the observed lethality could also be

changing with temperature (8). When a process is validated at 20 °C, and then retested at 23 °C, then there could be an 18 % change in the saturation concentration of the hydrogen peroxide.

The NO_2 dose used for biodecontamination is at concentration levels far below the saturation concentration for NO_2, and typically about 1 % of the NO_2 saturation concentration. Therefore, the temperature dependence of the NO_2 saturation concentration does not affect the delivery of NO_2 sterilant. Furthermore, with the typical dose so far from the saturation concentration of NO_2, the NO_2 will not condense onto surfaces and aeration is much faster. Aeration of NO_2 shown in Figure 2 demonstrates that 100 ppb (0.1 ppm) of NO_2 is achieved after 30 minutes. This measurement was done in the Getinge La Calhene ISOCYT FREJA isolator. During aeration, the (turbulent) air flow rate was 60 m^3/hr., equating to 75 air exchanges per hour.

The ambient environment concentration of H_2O_2 is found to be 0.001 ppb to 5.0 ppb (9). Therefore, the target aeration level of 30 ppb is approximately six times greater than the maximum observed environmental level. The annual mean concentrations of NO_2 in urban areas are generally in the range 10-45 ppb (10). Before filling biologics or other compounds that might be sensitive to residual sterilant, and just as with HPV, the target aeration level of NO_2 in the isolator should be some multiple of the ambient environment concentration. Given this high ambient background level of NO_2, and using a target aeration level that is only three times the maximum observed environmental level, the residual NO_2 sterilant level is 0.1 ppm (100 ppb).

Conclusion

The NO_2 biodecontamination process requires less than half of the time needed to complete an H_2O_2 biodecontamination process. Using the NO_2 process, the time required for conditioning the isolator (dehumidification or humidification), dwell and aeration are all reduced. When the same air flow rates are used for aeration of both the H_2O_2 and NO_2, the rate of NO_2 aeration is much faster than the aeration observed with HPV.

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