Overview of the Nitrogen Dioxide Sterilization Process

General Description of the Process

Nitrogen Dioxide (NO_2) gas is a rapid and effective sterilant for use against a wide range of microorganisms. The unique physical properties of NO_2 gas allow for sterilant dispersion in an enclosed environment at room temperature and ambient pressure. These same properties also allow for quicker removal of the sterilant and residuals through aeration of the enclosed environment. The combination of rapid lethality and easy removal of the gas allows for shorter overall cycle times during the sterilization (or decontamination) process and a lower level of sterilant residuals than are found with other sterilization methods.

NO_2 has a boiling point of 21°C at sea level, which results in a relatively high saturated vapor pressure at ambient temperature. Because of this, liquid NO_2 may be used as a convenient source for the sterilant gas. Liquid NO_2 is often referred to by the name of its dimer, dinitrogen tetroxide (N_2O_4). In the gaseous state, NO_2 and N_2O_4 are in equilibrium, with the former being heavily favored at the concentrations used during the sterilization process. In order to deliver sterilant to an enclosed environment that is at either ambient pressure or under a vacuum, a pre-chamber and vacuum pump system is used to meter the prescribed dose of NO_2. The pre-chamber, which is connected by solenoid valves to a cylinder of liquid NO_2, is first evacuated. The valve between the prechamber and cylinder is then opened, which causes the liquid NO_2 to evaporate into the evacuated pre-chamber until a pre-set pressure is reached in the pre-chamber. This pre-set pressure corresponds to the prescribed dose of NO_2 gas in the enclosure to be sterilized, while accounting for the NO_2 and N_2O_4 equilibrium.

Once the pre-chamber has been filled to the appropriate pressure of NO_2 and N_2O_4 gas, it is opened to the evacuated sterilization chamber, or to the air stream that is circulating within the isolator generator system. This introduces the sterilant gas to the volume to be exposed to the NO_2. At this point, humid air may be added to the sterilization chamber. However, the isolator environment will have already been humidified to a recommended level by using some means of water vaporization. Since the saturated vapor pressure of NO_2 gas at 21°C is 1 atm and the concentration used are typically around 1% of the saturated vapor pressure, there is no need to heat the chamber in order to support the desired effective dose.

Sterilant removal from an exposed volume is readily accomplished by air exchanges with fresh air that has been passed through a HEPA filter. The sterilant in the exhausted air can be scrubbed from the air stream by chemical means. The chemical removal of sterilant from the air stream uses a solid chemical scrubber material. The scrubber material neutralizes and captures the NO_2 in a non-hazardous, solid material. The spent scrubber material may be disposed of as non-hazardous solid waste with no special handling measures required. The scrubber also allows the scrubbed air to be vented directly to the room.

Microbiology

The most-resistant organism (MRO) to sterilization with NO_2 gas has been proven to be the spore of *Geobacillus stearothermophilus*, which happens to be the same MRO for both steam and hydrogen

peroxide sterilization processes. The spore form of *G. stearothermophilus* has been well-characterized over the years as a biological indicator in sterilization applications.

Microbial inactivation of *G. stearothermophilus* with NO_2 gas proceeds rapidly in a log-linear fashion, as is typical of other sterilization processes. Figure 1 shows the log-linear progression of population reduction as exposure time to NO_2 gas is increased. The log-linear response to exposure time provides a predictable model for inactivation upon which appropriate cycles can be based.

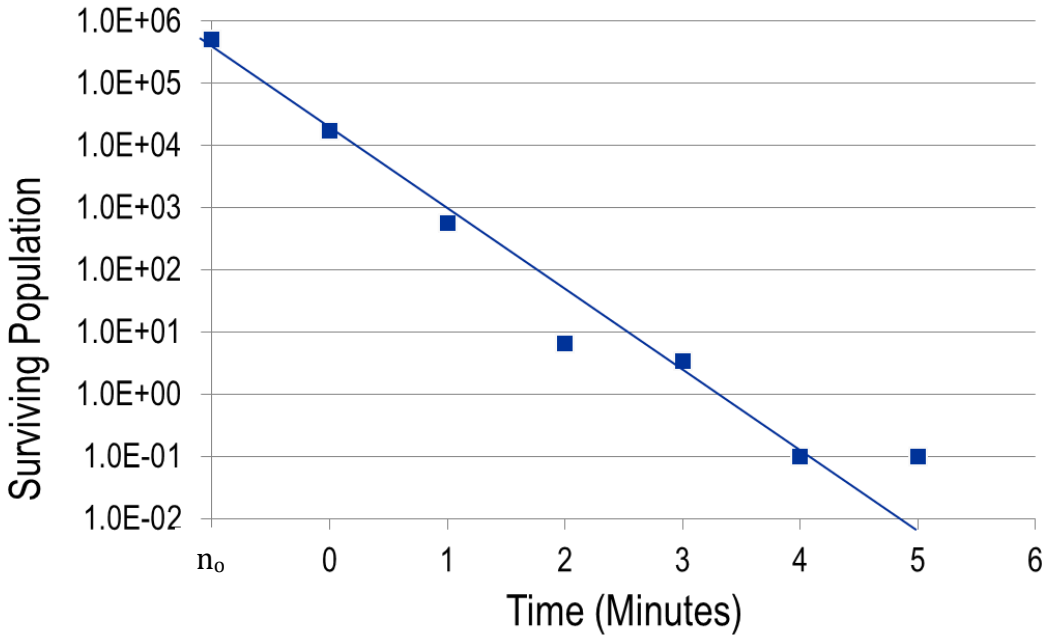

Figure 1: The inactivation of the exposed organisms follows a log-linear response.

Mechanism of Action

The mechanism for lethality has been investigated, and it is found that degradation of DNA in the spore core by absorbed NO_2 kills the exposed organism. This has been demonstrated in the Noxilizer's lab in multiple studies and is supported by published reports from other labs.

In one study, purified DNA that was extracted from *Bacillus subtilis* was exposed to the NO_2 sterilization process. The DNA was dried in microcentrifuge tubes, and then exposed to 3 mg/L NO_2 in a dry atmosphere in the sterilization chamber for 2, 8, or 16 minutes. After exposure, the DNA was analyzed via agarose gel electrophoresis using a 0.7% agarose-TBE gel. The DNA samples were analyzed under both non-denaturing and denaturing (4M Urea) conditions. The results are shown in Figure 2.

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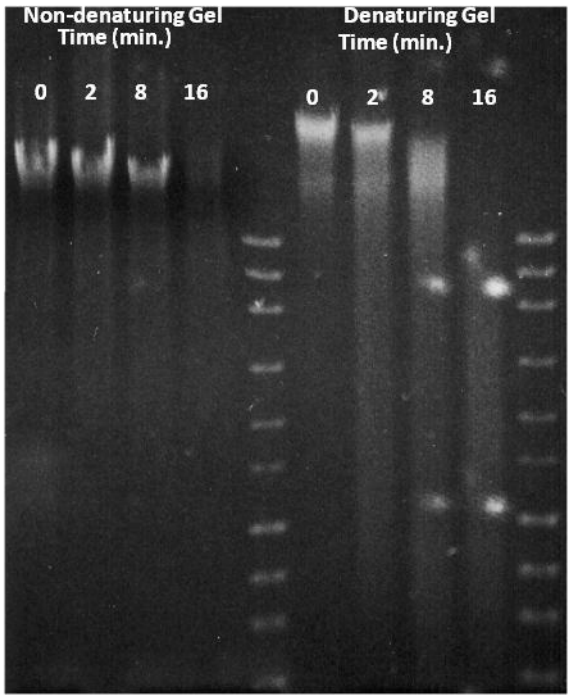

Figure 2. The integrity of DNA exposed to the NO_2 is shown in this image with double strand DNA (non-denaturing gel) and single strand DNA (denaturing gel). Single strand breaks accumulate after short NO_2 exposure times, whereas double strand breaks accumulate more slowly.

As the DNA is degraded through strand breaks, the shortened molecules can travel farther down the gel in a given period of time. Thus, the stain applied to the DNA is spread further down the column. The length of the smear is an indication that there is a wide dispersion of molecular weights of fragmented DNA. In the denaturing gel columns on the right side of Figure 2, one can see that the DNA begins to show degradation after the 2-minute exposure time. This decrease in molecular mass continues through to the 16-minute exposure column where signs of intact DNA have virtually disappeared.

Comparing the denaturing gel behavior to the non-denaturing gel columns on the left side of Figure 2, it can be seen that the degradation of DNA occurred primarily due to single-strand breaks. The positions of the stain markers due to the intact DNA remained essentially unmoved from zero to 8 minutes of exposure time. At 16 minutes of exposure time, the stain had moved almost entirely down the column. This indicated that the breakages occurred independently on single strands of the double-stranded helix, rather than across both sides of the helix simultaneously. The helical structure of the DNA was able to remain intact despite the single-strand breaks (SSB's) until the number of accumulated SSB's resulted in defects that were close enough on opposing strands to result in a double-strand break. This critical point was reached somewhere between the 8-minute and 16-minute exposure conditions. The dye marker at 8 minutes indicated intact DNA, while at 16 minutes enough breakages had occurred to cause fragments of DNA that were short enough to traverse the length of the gel column during the analytical time.

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Similar DNA fragmentation results were observed in DNA that was extracted from whole spores of *B. subtilis* that had been exposed to the humidified NO_2 sterilization process (data not shown). The fact that the DNA single strand breaks occur in purified state DNA on a substrate or contained within a whole spore indicates support for the conclusion that SSB's are the mechanism of lethality for spores during sterilization with NO_2 gas. While *G. stearothermophilus* is the MRO for NO_2, the data obtained with *B. subtilis* is relevant as both organisms are from the *Bacilli* family, and both have similar spore structures.

These conclusions are further supported from a review of the literature. Two such examples are listed below:

- Nitrogen dioxide induces DNA single-strand breaks in cultured Chinese hamster cells; Goersdorf, et al.; Carcinogenesis (1990) 11(1): 37-41.
- DNA strand breaks caused by exposure to ozone and nitrogen dioxide; Bermúdez, et al.; Environ Res. (1999) 81(1): 72-80.

Conclusion

Given these results, NO_2 can be effectively used as a sterilizing agent. NO_2 exposure provides a rapid inactivation process and can be quickly aerated from exposed materials. The mechanism of action is found to be single strand breaks of the organism DNA.

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