



3437 SW 24th Avenue Fax: 352-378-6483
Gainesville, FL 32607 E-mail: info@abcr.com
Tel: 352-372-0436 Web: www.abcr.com

A FOOD TESTING LABORATORY SINCE 1967

December 21, 2005

Mr. Harley Pattee
A to Z Ozone, Inc.
1909 NE 25th Ave.
Ocala, FL 34470
Tel: 352-401-0183
Fax: 352-401-0868
E-mail: harleypattee@aol.com

Dear Harley,

Please find attached a report for the project entitled "The Disinfection Efficacy of Gaseous Ozone Treatments against *Bacillus atrophaeus* Spores (a surrogate of *Bacillus anthracis*) Inoculated onto Office Materials."

Please let me know if you have any questions. We at ABC Research appreciate the opportunity to work with you and A to Z Ozone, Inc.

Best Regards,

James E. (Ken) Kennedy, Ph.D.
Vice President, Research Microbiology
ABC Research Corp.

Enclosure:

A FOOD TESTING LABORATORY SINCE 1967

RESEARCH PROJECT REPORT
RESEARCH MICROBIOLOGY DEPARTMENT

DATE: December 21, 2005

PREPARED FOR: A to Z Ozone, Inc.

CLIENT CONTACTS: Mr. Harley Pattee

TITLE: The Disinfection Efficacy of Gaseous Ozone Treatments against *Bacillus atrophaeus* Spores (a surrogate of *Bacillus anthracis*) Inoculated onto Office Materials.

EXPERIMENTAL APPROACH:

A. MATERIALS

The client provided and operated a portable ozone treatment chamber and ozone generating equipment at the ABC Research Corp. facility to conduct this experiment. The client also provided representative office materials (e.g., paper, file folders, pictures) for the experiment.

B. TEST MICROORGANISM

A spore suspension inoculum of *Bacillus atrophaeus* (ATCC 9372) was prepared in sterile distilled water using appropriate microbiological methods. This organism is commonly used for sterilization assurance, ethylene oxide sterilization control, dry heat sterilization control, and recommended by American Type Culture Collection (ATCC) for tests described in USP and military specification "MIL S-36586A". It has also been used in sterilization/disinfection efficacy studies as a surrogate for *Bacillus anthracis* (JAMA. 2003;289:1274-1277).

The culture was grown on solid media (i.e., Nutrient agar with manganese) to promote the formation of spores (3-5 days at 35°F). Spores were harvested from the agar surface once 90% of cells had sporulated (as determined by phase/contrast microscopic examination). Spores were harvested from the solid media surface via scraping/rinsing and then washed two times by centrifugation at 10,000 x g for 10 min with sterile distilled water. The initial spore suspension was enumerated via a spore plate count using Tryptic Soy agar. The final spore suspension was adjusted to achieve approx. 10⁹ CFU/ml for this study. The final spore suspension was heated at 70°C for 10 min. to eliminate vegetative cells and stored in sterile distilled water at 35°F until used in the study.

C. TEST PROCEDURES

The following representative office materials were used in the experiment: paper, file folder, photograph (glossy side), and photograph (back side). Each office material was inoculated and evaluated for disinfection efficacy using the following procedures.

- 1) Using sanitary technique, materials were cut into 5 x 5 cm. pieces (i.e., 25 cm²).
- 2) Each piece was inoculated by adding 0.1 ml of the designated spore suspension in 0.01 ml droplets across the piece and then distributing it as a thin film over the site. This resulted in an inoculum level of 10⁷ - 10⁸ spores per piece.
- 3) After inoculation, the pieces were allowed to dry completely at room temperature for 16-24 h in a biohood prior to treatment or microbial analysis.
- 4) Two inoculated samples of each material were left untreated (i.e., pre-treatment) to determine the initial *Bacillus atrophaeus* spore counts on the samples.
- 5) One set of inoculated samples were fully exposed (i.e., open to the air in the treatment chamber) during the ozone treatment whereas another set was placed inside of envelopes within file folders contained in a file box (placed in the treatment chamber) during the ozone treatment.
- 6) Two samples of each material (exposed or occluded) were analyzed after a 24-hour ozone treatment. The level of ozone during the 24-hour treatment ranged from 110 to 138 ppm.

D. MICROBIAL ANALYSES

Each designated material piece was sampled by aseptically placing it in a stomacher bag with 20 mL of sterile BPB diluent. Samples were stomached for 1 min. to release the spores from the material and the rinsate serially diluted in sterile BPB diluent as required. The *Bacillus atrophaeus* was enumerated in sample rinsates via pour plating technique with Tryptic Soy Agar (TSA). TSA plates were incubated at 35°C for 24 h.

All results were expressed as colony forming units (CFU's) per sample piece and converted to log₁₀ transforms. The lethality for each ozone treatment/exposure was expressed as mean log₁₀ unit reduction (and/or percentage reduction). Mean reductions were calculated for treatments using respective untreated (i.e., pre-treatment) sample counts.

RESULTS:

The results of disinfection efficacy of gaseous ozone against spores of a *Bacillus anthracis* surrogate (i.e., *Bacillus atrophaeus*) inoculated and dried onto various office materials directly exposed to air are presented in Table 1. The inactivation efficacy varied with each type of material. The 24 hour ozone treatment resulted in mean reductions of 4.37, 3.66, 1.86, and 2.06 log₁₀ CFU/piece, respectively, on the paper, file folder, photograph (glossy side), and photograph (back side). Correspondingly, the 24 hr. ozone treatment resulted in 99.996, 99.98, 99.59, and 99.13% reductions, respectively, on the paper, file folder, photograph (glossy side), and photograph (back side).

The results of disinfection efficacy of gaseous ozone against spores of a *Bacillus anthracis* surrogate (i.e., *Bacillus atrophaeus*) inoculated and dried onto various office materials that were occluded within other office materials are presented in Table 2. These results indicated that there was no inactivation of the *Bacillus atrophaeus* spores on the office materials that were occluded within envelopes and file folders during the treatment.

PREPARED BY: _____
James E. (Ken) Kennedy, Ph.D.
Vice President, Research Microbiology
ABC Research Corp.

Table 1. Disinfection Efficacy of Gaseous Ozone against *Bacillus atrophaeus* Spores on Exposed Office Materials.

Sample		Pre-Treatment		Post-Treatment (24 h)			
		Bacterial Counts		Bacterial Counts		Bacterial Reduction	
		CFU	Log ₁₀ CFU	CFU	Log ₁₀ CFU	Log ₁₀ CFU	%
Paper	rep. 1	12,000,000	7.08	240	2.38		
	rep. 2	21,000,000	7.32	1,900	3.28		
	Mean		7.20		2.83	4.37	99.996
File Folder	rep. 1	15,000,000	7.18	3,400	3.53		
	rep. 2	13,000,000	7.11	2,700	3.43		
	Mean		7.15		3.48	3.66	99.980
Photograph (glossy side)	rep. 1	84,000,000	7.92	900,000	5.95		
	rep. 2	84,000,000	7.92	1,500,000	6.18		
	Mean		7.92		6.07	1.86	98.590
Photograph (back side)	rep. 1	120,000,000	8.08	2,500,000	6.40		
	rep. 2	98,000,000	7.99	360,000	5.56		
	Mean		8.04		5.98	2.06	99.130

- Notes: 1) Inoculated samples were fully exposed/open to the air containing ozone.
 2) Bacterial counts expressed as colony forming units (CFU) per sample.
 3) Reduction = (mean log₁₀ count of pre-treatment sample) - (mean log₁₀ count of respective post-treatment sample).
 4) % Reduction based upon mean Log₁₀ reduction.

Table 2. Disinfection Efficacy of Gaseous Ozone against *Bacillus atrophaeus* Spores on Occluded Office Materials.

Sample		Pre-Treatment		Post-Treatment (24 h)			
		Bacterial Counts		Bacterial Counts		Bacterial Reduction	
		CFU	Log ₁₀ CFU	CFU	Log ₁₀ CFU	Log ₁₀ CFU	%
Paper	rep. 1	12,000,000	7.08	52,000,000	7.72		
	rep. 2	21,000,000	7.32	26,000,000	7.41		
	Mean		7.20		7.57	-0.36	none
File Folder	rep. 1	15,000,000	7.18	22,000,000	7.34		
	rep. 2	13,000,000	7.11	19,000,000	7.28		
	Mean		7.15		7.31	-0.17	none
Photograph (glossy side)	rep. 1	84,000,000	7.92	200,000,000	8.30		
	rep. 2	84,000,000	7.92	190,000,000	8.28		
	Mean		7.92		8.29	-0.37	none
Photograph (back side)	rep. 1	120,000,000	8.08	78,000,000	7.89		
	rep. 2	98,000,000	7.99	92,000,000	7.96		
	Mean		8.04		7.93	0.11	none

- Notes: 1) Inoculated samples were enclosed/occluded within file folders and not directly exposed to the air containing ozone.
 2) Bacterial counts expressed as colony forming units (CFU) per sample.
 3) Reduction = (mean log₁₀ count of pre-treatment sample) - (mean log₁₀ count of respective post-treatment sample).
 4) % Reduction based upon mean Log₁₀ reduction.