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A FOOD TESTING LABORATORY SINCE 1967

December 21, 2005

Mr. Harley Pattee
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Dear Harley,

Please find attached a report for the project "The Disinfection Efficacy of Gaseous Ozone Treatments against Selected Microorganisms Inoculated onto Dry and Wet Surfaces."

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

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 Selected Microorganisms Inoculated onto Dry and Wet Surfaces.

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. ABC Research Corp. provided the materials upon which to inoculate the microorganisms and perform the efficacy testing.

B. TEST MICROORGANISM

Six representative microorganisms of public health concern were used to test the efficacy of ozone treatments as follows: *E. coli* (ATCC #11229), *Salmonella typhimurium* (ATCC #14028), *Listeria monocytogenes* (ATCC #19115), *Staphylococcus aureus* (ATCC #6538), *Streptococcus pyogenes* (ATCC 19615), and *Stachybotrys chartarum* (ATCC #9182). *E. coli* is a common bacterial indicator of human/animal feces. *Salmonella* is a common Gram-negative enteric bacterial pathogen associated with various foods and foodborne disease. *Listeria monocytogenes* is a common Gram-positive bacterial pathogen associated with various foods and foodborne disease. *Staphylococcus aureus* is a common Gram-positive bacterial pathogen associated with skin infections as well as with foods and foodborne disease. *Streptococcus pyogenes* is a common Gram-positive bacterial pathogen associated with upper respiratory tract and skin infections. *Stachybotrys chartarum* is a toxic mold often associated with contamination of wet indoor environments.

Each bacterial strain (i.e., *E. coli*, *Salmonella*, *Listeria*, *Staphylococcus*, and *Streptococcus*) was individually propagated via at least two serial transfers in Tryptic Soy (TSB) broth and incubated at 35°C for 24 h before the experiment. For each culture, cells were centrifuged at 10,000 x g for 10 min. and concentrated/resuspended in sterile Butterfield's phosphate buffer (BPB) with 5% HBS (horse blood serum) to obtain at least 10 ml of a suspension with an approximate cell density of approx. 5×10^9 CFU/mL. Horse blood serum (5% HBS) is

commonly used for the inocula suspensions in disinfection efficacy studies with food surfaces to simulate a natural soil matrix (e.g., food residue or animal wastes). The final working suspensions were enumerated on appropriate media (see section E).

Stachybotrys chartarum was grown on Potato Dextrose Agar (PDA) to promote the formation of spores. Fungal spores (and some mycelia) were harvested from the agar surfaces and suspended in BPB. The initial suspension was centrifuged at 10,000 x g for 10 min. and concentrated/resuspended in BPB with 5% HBS to obtain a suspension having approx. 5×10^9 CFU/ml. The inoculum suspension was enumerated prior to the experiment to verify the target concentration and stored at 35°F until used in the experiment. The actual inoculum count was less than target (i.e., 9×10^4 CFU/ml)

C. TEST PROCEDURES

Dry Surface.

For each culture, a plastic surface (i.e., polystyrene petri dish) was inoculated and evaluated for disinfection efficacy using the following procedures.

- 1) Inoculation sites (i.e., 10 cm²) were marked on the bottom (outside surface) of sterile petri dishes.
- 2) On the same day as the treatments, the designated sites on the inside/bottom of petri dishes were each inoculated by adding 0.1 ml of the designated inoculum suspension in 0.01 ml droplets across the site and then distributing it as a thin film over the site.
- 3) After inoculation, the inocula were allowed to dry completely at room temperature in a biohood prior to treatment and/or microbial analysis.
- 4) For each inoculum, inoculated petri dishes were analyzed without any ozone treatment (i.e., pre-treatment or untreated) after complete drying.
- 5) For each inoculum, two samples were analyzed after a 15, 30, and 60 min. as well as an 8 hour ozone treatment.

Wet Surface.

For each culture, a plastic surface (i.e., polystyrene petri dish) was inoculated and evaluated for disinfection efficacy using the following procedures.

- 1) For each culture, a second inoculum suspension was prepared by mixing the initial inoculum with sterile BPB containing 5% HBS.
- 2) For each culture, petri dishes were inoculated with the second inoculum suspension just prior to the ozone treatment. An inoculum suspension of 5 ml was used for the 15, 30 and 60 min. treatments and a suspension of 10 ml was used for the 8 hour treatment.
- 3) For each inoculum, two inoculated petri dishes were analyzed without any ozone treatment (pre-treatment or untreated).
- 4) For each inoculum, two samples were analyzed after a 15, 30, and 60 min. as well as an 8 hour ozone treatment.

D. MICROBIAL ANALYSES

For the dry inoculum samples, the designated inoculation sites (10-cm²) were sampled using the swab technique. The sites were thoroughly swabbed and the swabs placed in 10 mL of sterile neutralizing buffer.

For the wet inoculum samples, aliquots of the suspension were collected with a pipet for microbial analysis. The post-treatment suspension volumes were measured and corrections made in counts for any evaporation occurring during treatment.

For bacteria inocula, the swab rinsates or inoculum suspensions were analyzed via surface plating aliquots of appropriate serial dilutions onto pre-poured plates of Trypticase Soy Agar (TSA) with incubation at 35°C for 24-36 h. For the *Stachybotrys*, the swab rinsates or inoculum suspensions were analyzed via surface plating aliquots of appropriate serial dilutions onto pre-poured plates of Potato Dextrose Agar (PDA) with incubation at 25°C for 5 days.

Results were expressed as colony forming units (CFU's) per sq. cm. or per ml 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 sample counts.

RESULTS:

Dry Surface/Inoculum.

The results of disinfection efficacy of gaseous ozone against selected microorganisms dried onto a plastic surface are presented in Tables 1 and 2. For all bacteria there was generally a progressive inactivation efficacy with increasing exposure times and a decrease of at least one log₁₀-unit (or 90%) was observed for all ozone exposure times. The mold, *Stachybotrys chartarum*, results are not presented because the inoculum level was insufficient following drying.

For dry *E. coli*, the 15 min., 30 min., 60 min., and 8 hr. ozone treatments resulted in mean reductions of 2.44, 2.35, 2.71, and greater than 4.49 log₁₀ CFU/cm², respectively. Correspondingly, the 15 min., 30 min., 60 min. and 8 hr. treatments resulted in 99.6, 99.5, 99.8, and >99.997% reductions, respectively. There was no surviving *E. coli* detected after the 8 hr ozone treatment.

For dry *Salmonella typhimurium*, the 15 min., 30 min., 60 min., and 8 hr. ozone treatments resulted in mean reductions of 1.25, 1.94, 2.29, and greater than 6.15 log₁₀ CFU/cm², respectively. Correspondingly, the 15 min., 30 min., 60 min., and 8 hr. treatments resulted in 94.4, 98.9, 99.5, and >99.99993% reductions, respectively. There were no surviving *Salmonella typhimurium* detected after the 8 hr ozone treatment.

For dry *Listeria monocytogenes*, the 15 min., 30 min., 60 min., and 8 hr. ozone treatments resulted in mean reductions of 1.94, 3.19, 4.12, and greater than 5.25 log₁₀ CFU/cm², respectively. Correspondingly, the 15 min., 30 min., 60 min., and 8 hr. treatments resulted in 98.9, 99.9, 99.99, and >99.9994% reductions, respectively. There was no surviving *Listeria monocytogenes* detected after the 8 hr ozone treatment.

For dry *Staphylococcus aureus*, the 15 min., 30 min., 60 min., and 8 hr. ozone treatments resulted in mean reductions of 1.63, 1.73, 3.33, and greater than 7.41 log₁₀ CFU/cm², respectively. Correspondingly, the 15 min., 30 min., 60 min., and 8 hr. treatments resulted in 97.8, 98.1, 99.95, and >99.999996% reductions, respectively. There was no surviving *Staphylococcus aureus* detected after the 8 hr ozone treatment.

For dry *Streptococcus pyogenes*, the 15 min., 30 min., 60 min., and 8 hr. ozone treatments resulted in mean reductions of 4.79, >4.79, >4.79, and >5.44 log₁₀ CFU/cm², respectively. Correspondingly, the 15 min., 30 min., 60 min., and 8 hr. treatments resulted in 99.998, >99.998, >99.998%, and >99.9996% reductions, respectively. There was no surviving *Streptococcus pyogenes* detected after the 8 hr ozone treatment.

Wet Surface/Inoculum.

The results of disinfection efficacy of gaseous ozone against selected microorganisms suspended in a wet environment (i.e., 5-10 ml of 5% HBS in BPB) on a plastic surface are presented in Tables 3 and 4. For all microorganisms there was generally a progressive inactivation efficacy with increasing exposure times. For the bacteria in a wet environment, the 15 and 30 min. ozone treatments did not result in a significant reduction (i.e., at least 90% or one log-unit).

For *E. coli* in a wet environment, the 60 min. and 8 hr. ozone treatments resulted in mean reductions of 1.43 and greater than 8.72 log₁₀ CFU/ml, respectively. Correspondingly, the 60 min. and 8 hr. treatments resulted in 96.3 and >99.9999998% reductions, respectively. There was no surviving *E. coli* detected after the 8 hr ozone treatment.

For *Salmonella typhimurium* in a wet environment, the 60 min. and 8 hr. ozone treatments resulted in mean reductions of 2.76 and greater than 8.94 log₁₀ CFU/ml, respectively. Correspondingly, the 60 min. and 8 hr. treatments resulted in 99.8 and >99.9999999% reductions, respectively. There was no surviving *Salmonella typhimurium* detected after the 8 hr ozone treatment.

For *Listeria monocytogenes* in a wet environment, the 60 min. and 8 hr. ozone treatments resulted in mean reductions of 1.92 and greater than 8.22 log₁₀ CFU/ml, respectively. Correspondingly, the 60 min. and 8 hr. treatments resulted in 98.8 and >99.9999994% reductions, respectively. There was no surviving *Listeria monocytogenes* detected after the 8 hr ozone treatment.

For *Staphylococcus aureus* in a wet environment, the 60 min. and 8 hr. ozone treatments resulted in mean reductions of 3.58 and greater than 8.84 log₁₀ CFU/ml, respectively. Correspondingly, the 60 min. and 8 hr. treatments resulted in 99.97 and >99.9999999% reductions, respectively. There was no surviving *Staphylococcus aureus* detected after the 8 hr ozone treatment.

For *Streptococcus pyogenes* in a wet environment, the 60 min. and 8 hr. ozone treatments resulted in mean reductions of 4.66 and greater than 7.74 log₁₀ CFU/ml, respectively. Correspondingly, the 60 min. and 8 hr. treatments resulted in 99.998 and >99.999998% reductions, respectively. There was no surviving *Streptococcus pyogenes* detected after the 8 hr ozone treatment.

For *Stachybotrys chartarum* in a wet environment, the 15, 30 and 60 min. treatments resulted in mean reductions of 3.00, >4.45 and >4.45 log₁₀ CFU/ml, respectively. Correspondingly, the 15, 30 and 60 min. treatments resulted in 99.9, >99.996, and >99.996% reductions, respectively. There was no surviving *Stachybotrys chartarum* detected after the 30 and 60 min. ozone treatments.

Summary.

There was progressive inactivation efficacy with increasing exposure times to ozone. For dry bacteria there was at least a one log unit (or >90%) reduction evidenced for all treatment times. For bacteria in a wet environment, there at least a one log unit (or >90%) reduction observed after 60 min. or more of treatment. The 8 hour ozone treatment was extremely effective for all bacteria in a dry or wet environment. There were no surviving bacteria of any type detected after the 8 hr ozone treatment in dry or wet environments with reductions ranging from >7.74 to >8.94 log-units in the wet environment. There was no surviving *Stachybotrys chartarum* detected in a wet environment after the 30 and 60 min. ozone treatments with a >4.45 log unit reduction observed.

PREPARED BY: _____

James E. (Ken) Kennedy, Ph.D.
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Table 1. Disinfection Efficacy of Gaseous Ozone against Selected Microorganisms on a Dry Surface over 60 min.

Exposure Time (min.)	<i>E. coli</i>		<i>Salmonella typhimurium</i>		<i>Listeria monocytogenes</i>		<i>Staphylococcus aureus</i>		<i>Streptococcus pyogenes</i>		
	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	
0	rep. 1	71,000	4.85	650,000	5.81	66,000	4.82	18,000,000	7.26	50,000	4.70
	rep. 2	110,000	5.04	860,000	5.93	91,000	4.96	50,000,000	7.70	76,000	4.88
	Mean		4.95		5.87		4.89		7.48		4.79
15	rep. 1	260	2.41	36,000	4.56	920	2.96	670,000	5.83	1	0.00
	rep. 2	400	2.60	49,000	4.69	870	2.94	750,000	5.88	<1	<0.00
	Mean		2.51		4.62		2.95		5.85		0.00
	Reduction		2.44		1.25		1.94		1.63		4.79
	% Reduction		99.6		94.4		98.9		97.8		99.998
30	rep. 1	440	2.64	9,100	3.96	70	1.85	430,000	5.63	<1	<0.00
	rep. 2	360	2.56	8,100	3.91	35	1.54	740,000	5.87	<1	<0.00
	Mean		2.60		3.93		1.69		5.75		<0.00
	Reduction		2.35		1.94		3.19		1.73		>4.79
	% Reduction		99.5		98.9		99.9		98.1		>99.998
60	rep. 1	120	2.08	250	2.40	7	0.85	26,000	4.41	<1	<0.00
	rep. 2	250	2.40	59,000	4.77	5	0.70	7,500	3.88	<1	<0.00
	Mean		2.24		3.58		0.77		4.15		<0.00
	Reduction		2.71		2.29		4.12		3.33		>4.79
	% Reduction		99.8		99.5		99.99		99.95		>99.998

Notes: 1) Reduction = (Mean Log₁₀ count of untreated "0" min. samples) - (Mean Log₁₀ count of subject time variable).

2) % Reduction based upon mean Log₁₀ reduction.

3) Ozone levels for the 15, 30, and 60 min. treatments were 120, 133, and 131 ppm, respectively.

Table 2. Disinfection Efficacy of Gaseous Ozone against Selected Microorganisms on a Dry Surface over 8 hours.

Exposure Time (hours)	<i>E. coli</i>		<i>Salmonella typhimurium</i>		<i>Listeria monocytogenes</i>		<i>Staphylococcus aureus</i>		<i>Streptococcus pyogenes</i>		
	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	
0	rep. 1	35,000	4.54	7,800,000	6.89	150,000	5.18	41,000,000	7.61	310,000	5.49
	rep. 2	27,000	4.43	250,000	5.40	210,000	5.32	16,000,000	7.20	240,000	5.38
	Mean		4.49		6.15		5.25		7.41		5.44
8	rep. 1	<1	<0.00	<1	<0.00	<1	<0.00	<1	<0.00	<1	<0.00
	rep. 2	<1	<0.00	<1	<0.00	<1	<0.00	<1	<0.00	<1	<0.00
	Mean		<0.00		<0.00		<0.00		<0.00		<0.00
	Reduction		>4.49		>6.15		>5.25		>7.41		>5.44
	% Reduction	>99.997		>99.99993		>99.9994		>99.999996		>99.9996	

Notes: 1) Reduction = (Mean Log₁₀ count of untreated "0" min. samples) - (Mean Log₁₀ count of subject time variable).

2) % Reduction based upon mean Log₁₀ reduction.

3) Ozone levels for the 8 hour treatment was ca. 158 ppm.

Table 3. Disinfection Efficacy of Gaseous Ozone against Selected Microorganisms on a Wet Surface over 60 min.

Exposure Time (min.)	<i>E. coli</i>		<i>Salmonella typhimurium</i>		<i>Listeria monocytogenes</i>		<i>Staphylococcus aureus</i>		<i>Streptococcus pyogenes</i>		<i>Stachybotrys chartarum</i>		
	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	
0	rep. 1	460,000,000	8.66	200,000,000	8.30	34,000,000	7.53	800,000,000	8.90	140,000,000	8.15	35,000	4.54
	rep. 2	320,000,000	8.51	390,000,000	8.59	29,000,000	7.46	790,000,000	8.90	110,000,000	8.04	23,000	4.36
	Mean		8.58		8.45		7.50		8.90		8.09		4.45
15	rep. 1	280,000,000	8.45	220,000,000	8.34	13,000,000	7.11	340,000,000	8.53	34,000,000	7.53	29	1.46
	rep. 2	270,000,000	8.43	160,000,000	8.20	19,000,000	7.28	140,000,000	8.15	37,000,000	7.57	28	1.45
	Mean		8.44		8.27		7.20		8.34		7.55		1.45
	Reduction		0.14		0.17		0.30		0.56		0.54		3.00
	% Reduction		--		--		--		--		--		99.9
30	rep. 1	410,000,000	8.61	120,000,000	8.08	6,600,000	6.82	75,000,000	7.88	16,000,000	7.20	<1	<0.00
	rep. 2	320,000,000	8.51	170,000,000	8.23	11,000,000	7.04	190,000,000	8.28	15,000,000	7.18	<1	<0.00
	Mean		8.56		8.15		6.93		8.08		7.19		0.00
	Reduction		0.02		0.29		0.57		0.82		0.90		>4.45
	% Reduction		--		--		--		--		--		>99.996
60	rep. 1	7,100,000	6.85	2,000	3.30	440,000	5.64	110,000	5.04	3,900	3.59	<1	<0.00
	rep. 2	28,000,000	7.45	120,000,000	8.08	330,000	5.52	390,000	5.59	1,900	3.28	<1	<0.00
	Mean		7.15		5.69		5.58		5.32		3.43		0.00
	Reduction		1.43		2.76		1.92		3.58		4.66		>4.45
	% Reduction		96.3		99.8		98.8		99.97		99.998		>99.996

Notes: 1) Reduction = (Mean Log₁₀ count of untreated "0" min. samples) - (Mean Log₁₀ count of subject time variable).

2) % Reduction based upon mean Log₁₀ reduction. Values of less than 90% are not presented.

3) Ozone levels for the 15, 30, and 60 min. treatments were 108, 115 and 103 ppm, respectively.

Table 4. Disinfection Efficacy of Gaseous Ozone against Selected Microorganisms on a Wet Surface over 8 hours.

Exposure Time (hours)	<i>E. coli</i>		<i>Salmonella typhimurium</i>		<i>Listeria monocytogenes</i>		<i>Staphylococcus aureus</i>		<i>Streptococcus pyogenes</i>		
	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	CFU/cm ²	Log CFU/cm ²	
0	rep. 1	500,000,000	8.70	760,000,000	8.88	93,000,000	7.97	910,000,000	8.96	59,000,000	7.77
	rep. 2	550,000,000	8.74	990,000,000	9.00	290,000,000	8.46	530,000,000	8.72	51,000,000	7.71
	Mean		8.72		8.94		8.22		8.84		7.74
8	rep. 1	<1	<0.00	<1	<0.00	<1	<0.00	<1	<0.00	<1	<0.00
	rep. 2	<1	<0.00	<1	<0.00	<1	<0.00	<1	<0.00	<1	<0.00
	Mean		<0.00		<0.00		<0.00		<0.00		<0.00
	Reduction		>8.72		>8.94		>8.22		>8.84		>7.74
	% Reduction	>99.9999998		>99.9999999		>99.9999994		>99.9999999		>99.9999998	

Notes: 1) Reduction = (Mean Log₁₀ count of untreated "0" min. samples) - (Mean Log₁₀ count of subject time variable).

2) % Reduction based upon mean Log₁₀ reduction.

3) Ozone levels for the 8 hour treatment was ca. 158 ppm.