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FINAL REPORT

In-Plant Validation of Ozone Treatment for The Reduction of *Staphylococcus aureus* on Stainless Steel Surfaces

PREPARED BY:

Rhaisa A. Crespo, Ph.D.
Research Scientist
Food Safety Net Services, Ltd.

PREPARED FOR:

BioSecurity Technology, Inc.

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1.0 STUDY DATES

Study Initiation Date: 02/03/2022
Date Testing Began: 02/22/2022
Date Testing Ended: 02/24/2022
Final Report Date: 03/07/2022

2.0 PARTIES

Study Sponsor: BioSecurity Technology, Inc., Omaha, NE (BIOSEC)
Study Contractor: Food Safety Net Services, Ltd., San Antonio, TX (FSNS)

3.0 OBJECTIVES

The objective of this project was to provide sufficient evidence to validate the ozone treatment that BioSecurity Technology (BIOSEC) has developed as a hard surface sanitizer, using stainless steel surfaces. Stainless steel coupons were inoculated with *Staphylococcus aureus*, and allowed to attach for one hour. The inoculated stainless-steel coupons were subjected to ozone treatment and analysis was performed to determine the concentrations of *Staphylococcus aureus* that remained viable on the coupons. A reduction of ≥ 3.0 $\log_{10}$ CFU/cm^2 for *Staphylococcus aureus* under the ozone treatment was targeted.

4.0 MATERIALS AND METHODS

The overall goal of this study was to validate ozone as a hard surface sanitizer by providing a ≥ 3.0 log CFU/cm^2 lethality for *Staphylococcus aureus* contamination. The ozone treatment followed BIOSEC instructions exactly as mentioned below. A total of 25 pre-treatment and 25 post-treatment stainless-steel coupons were analyzed. *Staphylococcus aureus* was inoculated onto the surface of the stainless-steel coupons. The coupons were treated with ozone in groups of five. An atomizer provided by BIOSEC was used for the treatment application. All stainless-steel coupons were inoculated with *S. aureus* in order to obtain the microbiological lethality data needed to validate the ozone treatment.

The inoculum used consisted of a single strain of *Staphylococcus aureus* ATCC 6538. A culture of *Staphylococcus aureus* ATCC 6538 was prepared by streaking a loopful of culture from a -80˚C freezer stock onto plates of Tryptic Soy Agar (TSA) and incubating at 35 ± 2°C for 21 ± 3 h. One isolated colony from each TSA plate was transferred into Tryptic Soy Broth (TSB), and incubated at 35 ± 2°C for 21 ± 3 h. A 100 µL aliquot of each TSB culture was plated to one plate of TSA for each tube. These plates were incubated at 35 ± 2°C for 21 ± 3 h. After incubation, 10 ml of Butterfield's Phosphate Buffer (BPB) was added to each TSA plate, and growth from the plate was re-suspended with a spreader. A serological pipette was used to collect the re-suspended cells, and the cells were collected in 50 ml centrifuge tubes. This suspension of cells had an estimated concentration of ~10.0 $\log_{10}$ CFU/ml. The liquid culture was vortexed to distribute cells equally throughout the entire volume. This suspension served as the inoculum. The concentration of *S. aureus* cells in the suspension was confirmed by plating the culture onto Staph Express Petrifilm™

(STX; 3M™ Company). After incubation at 35 ± 2°C for 24 ± 2 h, colonies were counted to determine the starting concentration.

The stainless-steel coupons were inoculated with *S. aureus*. A 500 µl aliquot of the inoculum was evenly spread onto the coupons. Once this was performed, a stabilization period of 1 hour occurred allowing for appropriate matrix absorption of the inoculum. A starting concentration of ≥ 6.0 $\log_{10}$ CFU/cm^2 was targeted.

Nine different treatments were performed, with each treatment having a total of 5 samples pre- and 5 samples post-treatment. Thus, a total of 50 stainless-steel coupons were required for enumeration (25 pre- and 25 post-treatment). The treatment performed consisted of three different components. First, the spray time – samples were sprayed for 30 seconds or for 1 minute. Second, the contact time – samples had either 10 or 30 minutes of contact time. And third, wiping technique – samples were wiped with a microfiber towel. One additional set was carried out, where samples were sprayed for around 2 seconds and wiped immediately (no contact time). The combination of treatments is listed in Table 1.

Table 1. Ozone treatment characteristics; spray time, contact time, and wiping technique.

Sample Number	Spray Time	Contact Time	Wipe
1 – 5	30 sec	10 min	Yes
6 – 10	1 min	30 min	Yes
11 – 15	30 sec	30 min	Yes
16 – 20	1 min	10 min	Yes
21 – 25	2 - 3 sec	None	Yes

The 25 pre-treatment samples were not subjected to the ozone treatment. The post-treatment samples were subjected to the ozone treatment in groups of five as described in Table 1.

To determine the *S. aureus* concentration in the pre- and post-treatment samples, each individual sample was placed into a sterile Whirl-Pak bag along with 50 ml of BPB, and was homogenized manually. The homogenate was serially-diluted in BPB, plated to STX, and the Petrifilm plates were incubated at 35 ± 2°C for 24 $\pm$ 2 h. Typical colonies were counted from each of the countable plates for each sample to determine the CFU/cm^2 value for the sample, and these CFU/cm^2 values were converted to their corresponding $\log_{10}$ CFU/cm^2 values.

After the $\log_{10}$ CFU/cm^2 value was obtained for each sample, all pre-treatment sample $\log_{10}$ CFU/cm^2 values (n = 5) and all post-treatment sample $\log_{10}$ CFU/cm^2 values (n = 5) per treatment combination were entered into Excel to calculate the log CFU/cm^2 reduction obtained for each of the nine treatment combinations.

5.0 RESULTS

The overall goal of this study was to investigate the efficacy and resultant lethality of the ozone treatment that BIOSEC recommends as a hard surface sanitizer.

Table 2 presents the *Staphylococcus aureus* cell counts in CFU/cm^2 and $\log_{10}$ CFU/cm^2 transformed cell counts for the pre- and post-ozone treatment samples using stainless-steel coupons.

Table 3 presents the *Staphylococcus aureus* mean cell count values in CFU/cm^2 and the mean $\log_{10}$ CFU/cm^2 transformed cell count values for the pre- and post-ozone treatment samples using stainless-steel coupons. The reduction between pre- and post-ozone treatment mean values was calculated to provide quantitative evidence for the efficacy of the ozone treatment on reducing *S. aureus* contamination on stainless-steel coupons. The mean $\log_{10}$ CFU/cm^2. **A reduction of 5.46 $\log_{10}$ CFU/cm^2 was observed between the starting *S. aureus* levels and the levels following the ozone treatment when subjecting the samples to a 1-minute spray time with a 10-minute contact time followed by wiping the surface with a microfiber towel. A reduction of 5.13 $\log_{10}$ CFU/cm^2 was observed between the starting *S. aureus* levels and the levels following the ozone treatment when subjecting the samples to a 1-minute spray time with a 30-minute contact time followed by wiping the surface with a microfiber towel.** A reduction of 3.61 $\log_{10}$ CFU/cm^2 was observed with the Spray and Wipe protocol. A reduction of 3.53 $\log_{10}$ CFU/cm^2 was observed with the 30-seconds spray time with a 30-minute contact time followed by wiping the surface with a microfiber towel protocol. A reduction of 3.22 $\log_{10}$ CFU/cm^2 was observed with the 30-seconds spray time with a 10-minute contact time followed by wiping the surface with a microfiber towel protocol.

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Table 2. *Staphylococcus aureus* Cell Counts in CFU/cm^2 and Log$_{10}$ CFU/cm^2 Transformed Cell Counts for the Pre- and Post-Ozone Treatment Samples

		Pre		Post	
Sample	Sample Description	CFU/cm^2	Log CFU/cm^2	CFU/cm^2	Log CFU/cm^2
1	Spray 30 s - 10 min Wiped	58000000	7.76	103	2.01
2	Spray 30 s - 10 min Wiped	25000000	7.40	700	2.85
3	Spray 30 s - 10 min Wiped	24000000	7.38	59000	4.77
4	Spray 30 s - 10 min Wiped	12000000	7.08	11400	4.06
5	Spray 30 s - 10 min Wiped	680000	5.83	280	2.45
6	Spray 1 min - 30 min Wiped	5000000	6.70	5	0.70
7	Spray 1 min - 30 min Wiped	7900000	6.90	530	2.72
8	Spray 1 min - 30 min Wiped	29000000	7.46	20	1.30
9	Spray 1 min - 30 min Wiped	54000000	7.73	12	1.08
10	Spray 1 min - 30 min Wiped	16000000	7.20	260	2.41
11	Spray 30 s - 30 min Wiped	106000000	8.03	103	2.01
12	Spray 30 s - 30 min Wiped	105000000	8.02	145000	5.16
13	Spray 30 s - 30 min Wiped	104000000	8.02	186	2.27
14	Spray 30 s - 30 min Wiped	95000000	7.98	< 1	0.00
15	Spray 30 s - 30 min Wiped	82000000	7.91	78	1.89
16	Spray 1 min - 10 min Wiped	2300000	6.36	20	1.30
17	Spray 1 min - 10 min Wiped	1050000	6.02	< 1	0.00
18	Spray 1 min - 10 min Wiped	2350000	6.37	< 1	0.00
19	Spray 1 min - 10 min Wiped	860000	5.93	4	0.60
20	Spray 1 min - 10 min Wiped	390000	5.59	< 1	0.00
21	Spray and Wipe - Janitorial	680000	5.83	27000	4.43
22	Spray and Wipe - Janitorial	110000000	8.04	12600	4.10
23	Spray and Wipe - Janitorial	128000000	8.11	42000	4.62
24	Spray and Wipe - Janitorial	132000000	8.12	4800	3.68
25	Spray and Wipe - Janitorial	58500000	7.77	20000	4.30

1. The yellow highlighted cells indicate that the minimum detection limit was reached and the value was set at this minimum level of < 1 CFU/cm^2.

Table 3. *Staphylococcus aureus* Mean Value of Cell Counts in CFU/cm^2, Mean Log$_{10}$ CFU/cm^2 Transformed Cell Counts, and Reduction of Log$_{10}$ CFU/cm^2 Between Pre- and Post-Ozone Treatment Samples

Sample ID	*Treatment*	*CFU/cm^2*	*Log CFU/cm^2*	*Reduction log CFU/cm^2*
Pre	Spray 30 s - 10 min Wiped	23936000	7.38	3.22
Post	Spray 30 s - 10 min Wiped	14297	4.16	
Pre	Spray 1 min - 30 min Wiped	22380000	7.35	5.13
Post	Spray 1 min - 30 min Wiped	165	2.22	
Pre	Spray 30 s - 30 min Wiped	98400000	7.99	3.53
Post	Spray 30 s - 30 min Wiped	29073	4.46	
Pre	Spray 1 min - 10 min Wiped	1390000	6.14	5.46
Post	Spray 1 min - 10 min Wiped	5	0.68	
Pre	Spray and Wipe - Janitorial	85836000	7.93	3.61
Post	Spray and Wipe - Janitorial	21280	4.33	

1. Results in CFU/cm^2 present the average of the five samples subjected to the treatment.
2. The reduction was calculated by the following equation: Reduction = Pre Average – Post Average.

6.0 DISCUSSION

The goal of this study was to provide quantitative evidence to validate the ozone treatment provided by BioSecurity Technology applied via atomizer equipment onto stainless-steel surfaces. The process aimed at attaining a reduction of $\geq$ 3.0 log$_{10}$ CFU/cm^2 by the ozone treatment. Results showed that the ozone treatments followed by wiping the stainless-steel surface with a microfiber cloth reached a $\geq$ 3.0 log$_{10}$ CFU/cm^2 reduction. Moreover, the treatments in which the stainless steel was sprayed for 1 minute and wiped with a microfiber cloth achieved a $\geq$ 5.0 log$_{10}$ CFU/cm^2 reduction with either 10 or 30 minutes of contact time.

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7.0 REPORT APPROVAL

Prepared By:

03/09/2022

Rhaisa Crespo, Ph.D.
Research Scientist
Food Safety Net Services, Ltd.

Date

Approved By:

Dan Lynn
CEO
BioSecurity Technology

Date