AirROS by SAGE Industrial ROS Technology, Creating a Safe Food Environment by Eliminating *Influenza A*, *mRSA*, *Norovirus*, and *Rhinovirus* on Various Inoculated Surfaces.

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ABSTRACT:
The effects of reactive oxygen species (ROS) concentration, at 75.0°F at a relative humidity (RH) 40% and treatment time of 30 minutes, 1, 2, 4, 8, 12 and 24 hours on the inactivation of *Influenza A*, *mRSA*, *Norovirus* and *Rhinovirus* on a selection of surfaces have been studied using response surface methodology (RSM). In these tests the log reduction of the above mentioned problematic bacteria and viruses show the destructive effects delivered with the AirROS ROS treatment. The statistical analysis of developed predictive model suggested that ROS concentration, RH and treatment time all significantly ($P<0.01$) increased the rate of log reduction. Among the three factors, the effect of ROS concentration on bacterial inactivation was the greatest, while effect of RH was the least. The interaction between ROS concentration and RH exhibited a significant and synergistic effect ($P<0.05$).

A. Current Trial

This trial was designed to demonstrate the benefits of using AirROS ROS technology to reduce/ preclude one bacteria and three specific viruses on various surfaces; plastic, stainless steel and tile flooring coupons (similar to commercial/hospital use).

For this trial *Influenza A "bird flu"* (ATCC # VR-1284), *mRSA "*Methicillin-resistant *Staphylococcus Aureus"* (ATCC # BAA-38), *Norovirus* (ATCC # VR-782) and *Rhinovirus* (ATCC # VR-1121), were studied.

Previously, AirROS ROS treatment has been demonstrated to have a highly beneficial effect on the reduction of bacteria, mold and food pathogens in refrigerated and non-refrigerated environments. In a growing number of commercial applications, these benefits have enabled growers/shippers, wholesalers and retailers of perishable commodities to significantly expand their marketing window, reduce losses due to decay and disease and reduce operational risk and costs.

To date, specific testing of the impact of ROS on viruses has been limited. This study will significantly add the impact of ROS on viruses to this developing body of knowledge.

B. Bacterial and Viral cultures

Influenza A, H1N1 (ATCC # VR-1284), *mRSA* (ATCC # BAA-38), *Norovirus* (ATCC # VR-782) and *Rhinovirus* (ATCC # VR-1121), were acquired from ATCC, Manassas, VA., USA.

1. **Bacteria:** *mRSA* was maintained at 8.0°C on slants of agar Trypticase Soy Agar (TSA, Hardy Diagnostics, Santa Maria, CA., USA) and cultured in Trypticase Soy Agar with defibrinated sheep blood (TSA w/ SB, Hardy Diagnostics, Santa Maria) in aerobic growth conditions at 37.0°C.

2. **Viruses:** *Influenza A*, *Norovirus* and *Rhinovirus* were maintained on ATCC complete growth medium and minimum essential medium (ATCC, Manassas, VA., USA) with 2 µM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 µM non-essential amino acids, and

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1.0 µM sodium pyruvate, 90%; fetal bovine serum, 10% and cultured in Trypticase Soy Agar with added; sodium bicarbonate, non-essential amino acids, and combination of sodium pyruvate and fetal bovine serum, in aerobic growth conditions at 37.0°C and *Influenza A* at 33-35° C.[1]

Cells from both of the above (approx. $1x10^7$ CFU/ml) from a 24 hour static culture incubated at 37.0°C and *Influenza A* at 33-35°C were used to inoculate various 5 cm x 5 cm plastic, stainless steel and tile flooring coupons. The inoculum suspensions were enumerated by surface plating in duplicate samples on TSA after serial dilution in 0.1% peptone solution. The plates were incubated for 24 hour at 37.0°C.

C. Inoculation of various media surface areas

A 100 µl droplet from the initial inoculum suspension of each of the bacteria/viruses was used to inoculate the external surface (5 cm x 5 cm) on plastic, stainless steel and tile flooring coupons, with the final inoculum level to be approximately 7.0-log CFU/5 g sample. The inoculated samples were dried by air-blowing for 1 hour at 22.0°C prior to AOC treatment being initiated. The 1 hour drying allows the inoculated cells to attach to the surface host and minimize the growth of inoculated cells during drying.

D. AirROS Reactive Oxygen Species (ROS) treatment

ROS treatment was carried out using the AirROS ROS unit installed in a refrigerated (refrigeration not used for these tests) testing chamber. The chamber was monitored by a Programmable Logic Controller (PLC, Unitronics) with a R-10 Aeroqual sensor (Aeroqual Limited) monitoring O_3 (an indicator of ROS production) as well as temperature and relative humidity.

The plastic, stainless steel and tile flooring (5 cm x 5 cm) coupon surfaces were inoculated with the mentioned bacteria and viruses and were treated with 0.04 ppm ROS for 30 minutes, 1, 2, 4, 8, 12 and 24 hour increments at 75°F at 40% RH. After the treatment, the samples were subjected to enumeration by surface plating. The log reduction of the bacteria and viruses was evaluated with and without the consideration of resuscitation of injured cells after ROS treatment.

Three different controls were prepared in each ROS treatment. For a positive control, a 5 cm x 5 cm area of the three coupons were inoculated with bacteria and virus cells and dried for 1 hour but not exposed to the ROS treatment. There were three negative controls, in which the 5 cm x 5 cm coupons were inoculated with 100 µl droplet of sterile water and dried for 1 hour. One negative control was treated with ROS and the other was not subjected to the ROS treatment. Each treatment sample and the 3 controls were prepared in triplicate.

E. Recovery of *pathogens* from the surface samples

After ROS treatment, each of the 5 cm x 5 cm coupons were transferred into a 400 ml stomacher bag (Fisher Scientific Inc., PA., USA) combined with 50 ml sterile 0.1% peptone solution, and then blended with a AES Easy Mix Stomacher (AES Laboratories, Princeton, NJ., USA) for 2 min at normal speed. Wash fluid was serially diluted, followed by surface plating for enumeration.

[1] Cells expressing heteroresistance grow more slowly than the oxacillin-susceptible population and may be missed at temperatures above 35°C. This is why CLSI recommends incubating isolates being tested against oxacillin, methicillin, or nafcillin at 33-35° C (maximum of 35°C) for a full 24 hours before reading.

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A centrifugation method was used to recover low populations of ROS injured bacteria and viruses. The centrifugation method (Mossel and others 1991) was modified and used to concentrate the bacterial and virus populations in the wash fluid so that less than 250 CFU/ml of bacteria can be enumerated by the surface plating.

F. Study Results and Discussion

The effects of ROS concentration, 0.04 ppm, at 75.0°F at a 40% RH with treatment times of 30 minutes, 1, 2, 4, 6, 8, 12 and 24 hours on the inactivation of four (one bacteria and 3 viruses) problematic organisms of public health concern on a selection of surface samples is obvious from the Tables 1 - 9 attached.

1. **Overall log reduction**
   - 99.5 percent reduction was seen after 8 hour exposure.
   - The largest reduction, (3.3-log) was seen after the first 30 minute exposure.

2. **Impact on organisms**
   - The 30 minute ROS exposure results show a slightly greater average log reduction on *mRSA* (the bacteria) 3.55-log vs. 3.15-log, 3.18-log and 3.21-log on *Influenza A*, *Norovirus* and *Rhinovirus* (the viruses), respectively.
   - One hour ROS exposure again show a greater average log reduction on *mRSA* (the bacteria) 5.16-log vs. 4.46-log, 4.70-log and 4.80-log on *Influenza A*, *Norovirus* and *Rhinovirus* (the viruses), respectively.

3. **Impact on surfaces**

   After 4 hour ROS exposure the stainless steel coupon showed the greatest log reduction of 6.64 followed by the plastic tile floor and the plastic coupons at 6.50 and 6.45 log reductions, respectively.

The results are unambiguous and indicate a clear correlation between AirROS ROS ROS treatment at the indicated concentration and the stated log reductions on the bacteria and viruses on all surfaces tested.

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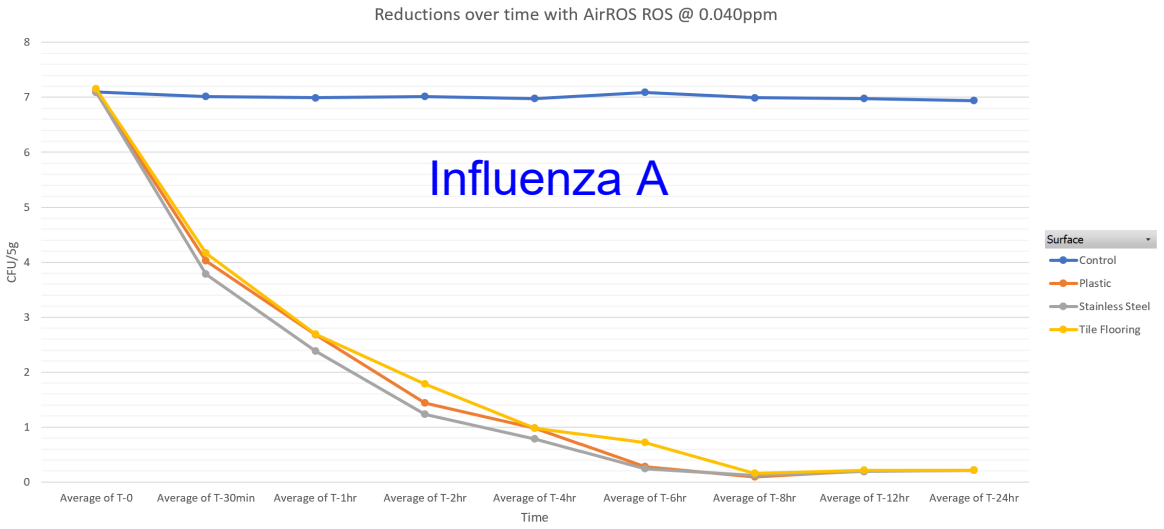
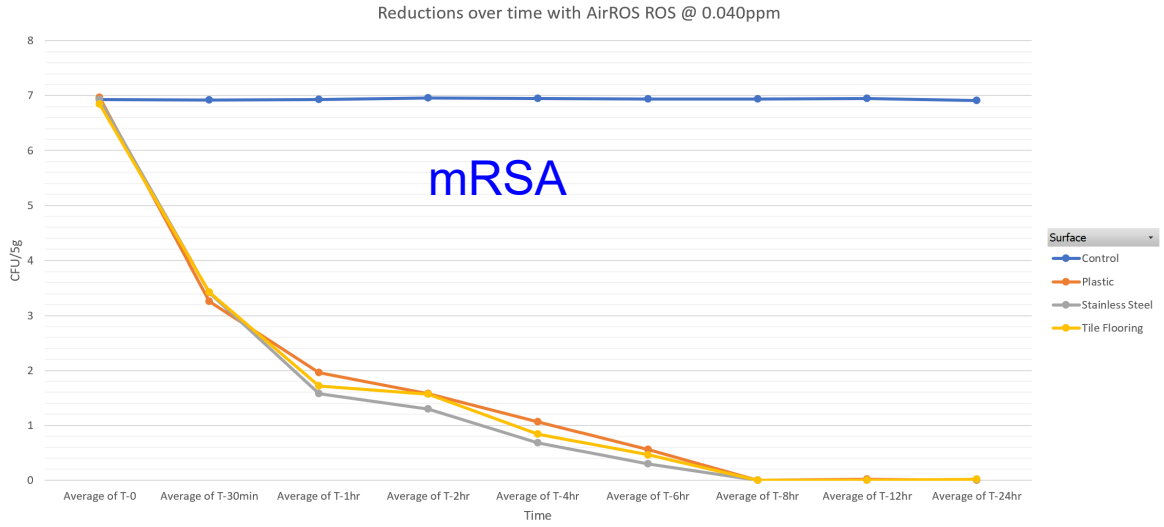
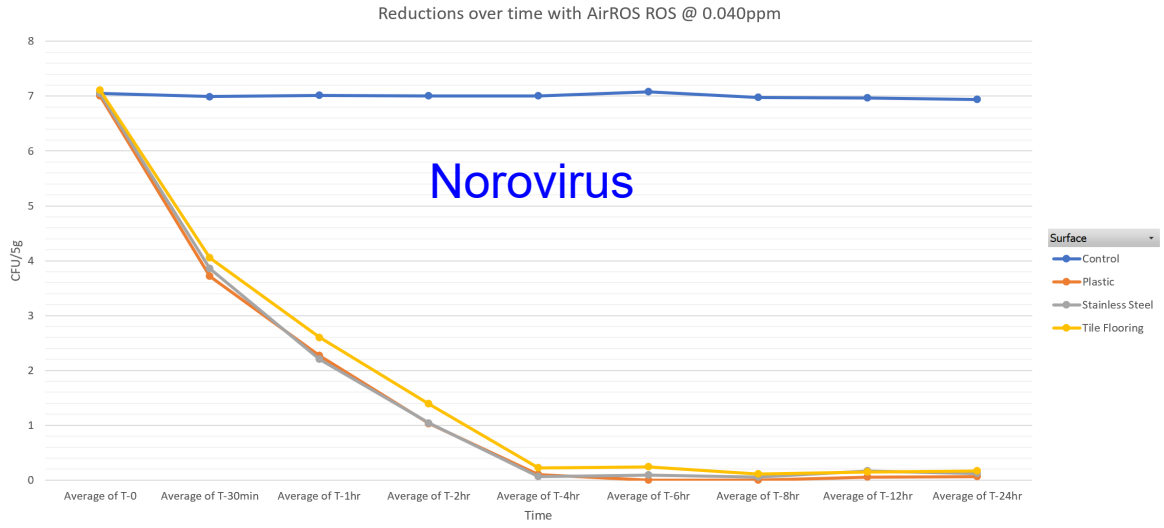
Graphic representation of reductions







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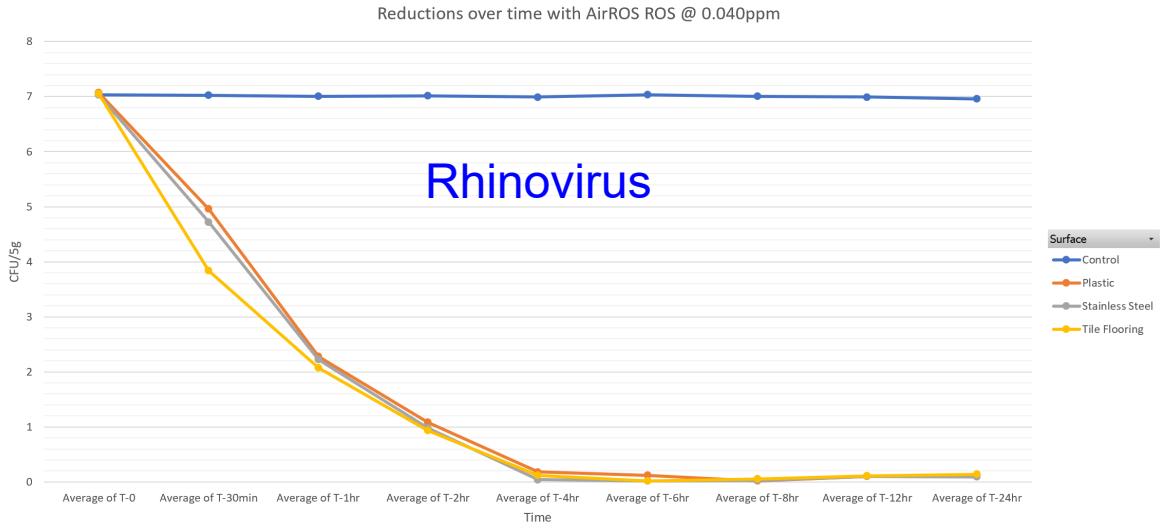

Table 1
Results of ROS Treatment on Surface Inoculation
Time Zero, Before Treatment

Organism	Surface	Initial Population[2] Log (cfu/5g)
Influenza A	plastic	7.09 ± 0.13
	stainless steel	7.10 ± 0.12
	tile flooring	7.15 ± 0.15
mRSA	plastic	6.97 ± 0.10
	stainless steel	6.93 ± 0.14
	tile flooring	6.85 ± 0.12
Norovirus	plastic	7.00 ± 0.18
	stainless steel	7.05 ± 0.13
	tile flooring	7.11 ± 0.19
Rhinovirus	plastic	7.07 ± 0.09
	stainless steel	7.03 ± 0.11
	tile flooring	7.05 ± 0.12

[2] Values are means ± standard deviations (n=4).

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Table 2
Results of ROS Treatment on Surface Inoculation
30 Minutes into Treatment

Organism	Surface	Log Destruction[3] (cfu/5g)	Control (cfu/5g)
Influenza A	plastic	3.07 ± 0.19	7.01 ± 0.15
	stainless steel	3.32 ± 0.20	
	tile flooring	2.99 ± 0.18	
mRSA	plastic	3.71 ± 0.20	6.92 ± 0.13
	stainless steel	3.51 ± 0.21	
	tile flooring	3.42 ± 0.23	
Norovirus	plastic	3.28 ± 0.24	6.99 ± 0.14
	stainless steel	3.19 ± 0.22	
	tile flooring	3.06 ± 0.19	
Rhinovirus	plastic	2.11 ± 0.24	7.02 ± 0.12
	stainless steel	2.31 ± 0.20	
	tile flooring	3.21 ± 0.18	

Table 3
Results of ROS Treatment on Surface Inoculation
1 Hour into Treatment

Organism	Surface	Log Destruction[3] (cfu/5g)	Control (cfu/5g)
Influenza A	plastic	4.41 ± 0.23	6.99 ± 0.09
	stainless steel	4.72 ± 0.21	
	tile flooring	4.46 ± 0.24	
mRSA	plastic	5.01 ± 0.21	6.93 ± 0.12
	stainless steel	5.35 ± 0.19	
	tile flooring	5.13 ± 0.20	
Norovirus	Plastic	4.73 ± 0.22	7.01 ± 0.11
	stainless steel	4.85 ± 0.26	
	tile flooring	4.51 ± 0.18	
Rhinovirus	Plastic	4.79 ± 0.21	7.00 ± 0.08
	stainless steel	4.81 ± 0.22	
	tile flooring	4.98 ± 0.23	

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[3] Log destruction = $\log_{10}$ cfu/5g count - control
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Table 4
Results of ROS Treatment on Surface Inoculation
2 Hours into Treatment

Organism	Surface	Log Destruction[3] (cfu/5g)	Control (cfu/5g)
Influenza A	Plastic	5.65 ± 0.18	7.01 ± 0.11
	stainless steel	5.87 ± 0.20	
	tile flooring	5.37 ± 0.22	
mRSA	Plastic	5.39 ± 0.24	6.96 ± 0.07
	stainless steel	5.63 ± 0.21	
	tile flooring	5.28 ± 0.22	
Norovirus	Plastic	5.97 ± 0.25	7.00 ± 0.10
	stainless steel	6.01 ± 0.20	
	tile flooring	5.72 ± 0.23	
Rhinovirus	Plastic	5.99 ± 0.21	7.01 ± 0.09
	stainless steel	6.05 ± 0.23	
	tile flooring	6.12 ± 0.20	

Table 5
Results of ROS Treatment on Surface Inoculation
4 Hours into Treatment

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Organism	Surface	Log Destruction[3] (cfu/5g)	Control (cfu/5g)
Influenza A	Plastic	6.11 ± 0.25	6.98 ± 0.08
	stainless steel	6.32 ± 0.23	
	tile flooring	6.17 ± 0.21	
mRSA	Plastic	5.91 ± 0.22	6.95 ± 0.11
	stainless steel	6.25 ± 0.20	
	tile flooring	6.01 ± 0.25	
Norovirus	Plastic	6.90 ± 0.22	7.00 ± 0.10
	stainless steel	6.99 ± 0.23	
	tile flooring	6.89 ± 0.19	
Rhinovirus	Plastic	6.89 ± 0.24	6.99 ± 0.07
	stainless steel	6.99 ± 0.25	
	tile flooring	6.93 ± 0.21	

Table 6
Results of ROS Treatment on Surface Inoculation
6 Hours into Treatment

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Organism	Surface	Log Destruction[3] (cfu/5g)	Control (cfu/5g)
Influenza A	Plastic	6.81 ± 0.21	7.09 ± 0.07
	stainless steel	6.86 ± 0.22	
	tile flooring	6.43 ± 0.24	
mRSA	Plastic	6.41 ± 0.20	6.94 ± 0.10
	stainless steel	6.63 ± 0.25	
	tile flooring	6.39 ± 0.22	
Norovirus	Plastic	7.01 ± 0.24	7.08 ± 0.10
	stainless steel	6.96 ± 0.21	
	tile flooring	6.87 ± 0.24	
Rhinovirus	Plastic	6.95 ± 0.21	7.03 ± 0.11
	stainless steel	7.01 ± 0.20	
	tile flooring	7.03 ± 0.23	

[3] Log destruction = $\log_{10}$ cfu/5g count - control

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Table 7
Results of ROS Treatment on Surface Inoculation
8 Hours into Treatment

Organism	Surface	Log Destruction[3] (cfu/5g)	Control (cfu/5g)
Influenza A	plastic	7.00 ± 0.24	6.99 ± 0.10
	stainless steel	6.98 ± 0.20	
	tile flooring	6.99 ± 0.22	
mRSA	plastic	6.99 ± 0.23	6.94 ± 0.07
	stainless steel	6.97 ± 0.21	
	tile flooring	6.87 ± 0.19	
Norovirus	plastic	7.01 ± 0.22	6.98 ± 0.09
	stainless steel	7.00 ± 0.25	
	tile flooring	7.00 ± 0.21	
Rhinovirus	plastic	7.05 ± 0.20	7.00 ± 0.10
	stainless steel	7.00 ± 0.26	
	tile flooring	7.00 ± 0.24	

Table 8
Results of ROS Treatment on Surface Inoculation
12 Hours into Treatment

Organism	Surface	Log Destruction[3] (cfu/5g)	Control (cfu/5g)
Influenza A	plastic	6.89 ± 0.20	6.98 ± 0.10
	stainless steel	6.91 ± 0.17	
	tile flooring	6.94 ± 0.21	
mRSA	plastic	6.95 ± 0.18	6.95 ± 0.07
	stainless steel	6.93 ± 0.19	
	tile flooring	6.86 ± 0.21	
Norovirus	plastic	6.95 ± 0.16	6.97 ± 0.08
	stainless steel	6.88 ± 0.19	
	tile flooring	6.96 ± 0.23	
Rhinovirus	plastic	6.97 ± 0.23	6.99 ± 0.09
	stainless steel	6.93 ± 0.20	
	tile flooring	6.94 ± 0.21	

[3] Log destruction = $\log_{10}$ cfu/5g count - control

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Table 9
Results of ROS Treatment on Surface Inoculation
24 Hours into Treatment

Organism	Surface	Log Destruction[3] (cfu/5g)	Control (cfu/5g)
Influenza A	plastic	6.88 ± 0.22	6.94 ± 0.10
	stainless steel	6.89 ± 0.20	
	tile flooring	6.94 ± 0.25	
mRSA	plastic	6.97 ± 0.20	6.91 ± 0.07
	stainless steel	6.92 ± 0.19	
	tile flooring	6.83 ± 0.17	
Norovirus	plastic	6.94 ± 0.21	6.94 ± 0.08
	stainless steel	6.93 ± 0.19	
	tile flooring	6.94 ± 0.21	
Rhinovirus	plastic	6.95 ± 0.18	6.96 ± 0.09
	stainless steel	6.94 ± 0.22	
	tile flooring	6.91 ± 0.21	

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[3] Log destruction = $\log_{10}$ cfu/5g count - control

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Table 10
Results of ROS Treatment on Surface Inoculation
Positive and Negative Control Confirmation

Material	Control	ROS ppm	Time	Organism	Log CFU/5g	DNA/RNA Hybridization After 24 hours
Plastic	negative	0.04	Treated	Influenza A	7.1	< 1.0 cfu/g
	negative	0.04	Treated	mRSA	7.0	< 1.0 cfu/g
	negative	0.04	Treated	Norovirus	7.0	< 1.0 cfu/g
	negative	0.04	Treated	Rhinovirus	7.1	< 1.0 cfu/g
	negative	-	Not treated	100 µl sterile water	0.0	-
	negative	-	Treated	100 µl sterile water	0.0	-
	positive	0	Not treated	Influenza A	7.09 ± 0.13	-
	positive	0	Not treated	mRSA	6.97 ± 0.10	-
	positive	0	Not treated	Norovirus	7.00 ± 0.18	-
	positive	0	Not treated	Rhinovirus	7.07 ± 0.09	-
Stainless Steel	negative	0.04	Treated	Influenza A	7.1	< 1.0 cfu/g
	negative	0.04	Treated	mRSA	6.9	< 1.0 cfu/g
	negative	0.04	Treated	Norovirus	7.1	< 1.0 cfu/g
	negative	0.04	Treated	Rhinovirus	7.0	< 1.0 cfu/g
	negative	-	Not treated	100 µl sterile water	0.0	-
	negative	-	Treated	100 µl sterile water	0.0	-
	positive	0	Not treated	Influenza A	7.10 ± 0.12	-
	positive	0	Not treated	mRSA	6.93 ± 0.14	-
	positive	0	Not treated	Norovirus	7.05 ± 0.13	-
	positive	0	Not treated	Rhinovirus	7.03 ± 0.11	-
Linoleum Flooring	negative	0.04	Treated	Influenza A	7.2	< 1.0 cfu/g
	negative	0.04	Treated	mRSA	6.9	< 1.0 cfu/g
	negative	0.04	Treated	Norovirus	7.1	< 1.0 cfu/g
	negative	0.04	Treated	Rhinovirus	7.1	< 1.0 cfu/g
	negative	-	Not treated	100 µl sterile water	0.0	-
	negative	-	Treated	100 µl sterile water	0.0	-
	positive	0	Not treated	Influenza A	7.15 ± 0.15	-
	positive	0	Not treated	mRSA	6.85 ± 0.12	-
	positive	0	Not treated	Norovirus	7.11 ± 0.19	-
	positive	0	Not treated	Rhinovirus	7.05 ± 0.12	-

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