Biological Consulting Services
of North Florida, Inc.

February 09, 2012

Patrick Lucci
Lynnfield Green Technologies
5 Longbow Road
Lynnfield, Massachusetts 01940

RE: Toucan-Eco Spray Disinfection Efficacy Study Report.

Dear Mr. Lucci,

We have conducted the antimicrobial efficacy testing on the liquid produced by the provided Toucan-Eco system. The testing was conducted as per AOAC Method 961.02 (AOAC Official Methods of Analysis; 2005), ASTM E1053 "Standard Test Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces", and ASTM E2111-00 "Standard Quantitative Carrier Test Method to Evaluate the Bactericidal, Fungicidal, Mycobactericidal and Sporicidal Potencies of Liquid Chemical Germicides". Based on the observed results, the generated disinfectant liquid exhibited excellent antibacterial, sporocidal, and antiviral efficacy.

Additionally, the disinfestation efficacy rate of the generated disinfectant was tested using ASTM 2315-08 "Standard Guide for Assessment of Antimicrobial Activity Using a Time-Kill Procedure". The generated disinfectant inactivated *E. coli* O157:H7 and *Listeria monocytogenes* by >99.999% within 10 seconds of contact.

In the following pages, you will find a summary of the methodology used and the results of our analysis. Should you have any questions or concerns please do not hesitate to contact me.

Best Regards,

George Lukasik, Ph.D.
Laboratory Director

BCS Laboratories, Inc. -Gainesville
4609-A NW 6th Street, Gainesville, Florida 32609
Tel. (352) 377-9272, Fax. (352) 377-5630
www.microbioservices.com
FL DOH Laboratory #E82924, EPA# FL01147

Methicillin Resistant *Staphylococcus aureus* (MRSA, BAA-41), *Staphylococcus aureus* (ATCC 6538), *Salmonella enterica* (ATCC 9712)*, Listeria monocytogenes* (ATCC 4428), *Pseudomonas aeruginosa* (ATCC 15442), *Bacillus subtilis* (19659) and *Escherichia coli* O157:H7 (ATCC 35150) stock cultures were obtained from American Type Culture Collection (ATCC) or Microbiologics Inc. and maintained as per the suppliers recommendations. The cultures were propagated as per AOAC 961.02 and the related referenced AOAC and ASTM protocols. For challenge experiments, cultures were grown and purified as per the protocols as well as incubated at temperature and times set-forth in the specified method. Purified spore suspension of *Bacillus subtilis* was produced as per ASTM E2111 on the day of study.

Poliovirus Lsc1 Chat strain (ATCC VR-1562) was propagated and enumerated as plaque forming units (pfu) using EPA ICR Methodology (EPA 600/R-95/178). For enumeration, aliquots containing poliovirus were inoculated on freshly prepared monolayers of Buffalo Green Monkey (BGM) kidney cells. Infectious assays were conducted using a MPN based method. Infected flasks were examined for infectious

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foci and cytopathic effects (CPE) as per methodology outlined in EPA 600/R9-95/178. Cell flasks were incubated at 36.5°C and in 5% CO2 for 5 days.

Murine Norovirus (MNV-1) was used as a model for human norovirus. MNV-1 was propagated on RAW 264.7 cells (ATCC TIB-71). Viruses were harvested by infecting cell monolayers and incubating at 37°C and 5% CO2 until 70–95% of the cells showed a cytopathic effect. The cells were frozen and thawed twice, followed by high speed centrifugation and filtration through a 0.1 µm filter. The supernatant was aliquotted as test virus suspension and stored at -80°C. Viral enumeration was performed using infection of cell monolayers and observation for cytopathic effect development. Cell flasks were incubated at 36.5°C and in 5% CO2 for 5 days. A most probable number (MPN) calculation (EPA 600/R-95/178) was used to calculate the number of infectious viral units.

For challenge experiments, frozen viral stock (typically 1 x 10^8 iu/ml) was thawed rapidly in a 35°C water bath on the day of the experiment. The virus stock was tittered by performing serial ten-fold dilutions in PBS and was inoculated onto the respective cells as described above.

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On January 04, 2012, a Toucan-Eco unit was delivered to BCS Laboratories from Falcon Pro Solutions, Inc. The unit was issued BCS identifier 1201002. The unit's directions were followed based on the client's recommendations. City of Gainesville tap water was used in the unit. One cycle and 3.5 scoops of active substrate were used for the generation of the disinfectant used in each study. The total chlorine residual was measured by the use of commercial colometric test. Total chlorine concentration was measured to be in the 250-300 ppm range (Series 942 Mini-Analyst, Orbeco-Hellige Inc., USA). The solution was used within 60 minutes of preparation for the microbial spray disinfection studies. The temperature of the liquid prior to application and during disinfection efficacy testing was maintained at 18-22°C. The liquid was generated and applied using the provided handheld spray bottle. All tests were conducted in a biological laminar flow hood.

Cultures of the bacterial species were grown as specified in Method 961.02. The bacterial population in each of the cultures was determined to be greater than 10^8 cfu/ml. On day of study, bacterial cultures were agitated until evenly dispersed prior to use. Viral stock concentrate was removed from -80º C, and thawed rapidly on the day of the study. Viral concentrates typically contained approximately 10^8 infectious units/ ml.

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Ten-microliters of one of the homogenized microbial suspension was placed and spread onto sterile 24x26 mm glass slides (Propper Scientific, NY). Eleven slides of each bacterial species were prepared and used to test the efficacy of the provided disinfectant. Six slides were used for the viral study. The inoculum was allowed to semi-dry in a covered chamber at 37°C for 30-40 minutes. Ten of the 11 inoculated slides (5 of 6 for the virus) were then sprayed for 10 seconds from a distance of approximately 12" with the provided solution. The glass slides were completely saturated with sprayed solution. They were then allowed to incubate at 20-22°C for 10 minutes. Following, each of the slides was picked up with sterile forceps, the excess liquid was allowed to run off, and the slide was placed into sterile glass tubes containing
20 ml of appropriate growth media. The tubes were then incubated as per method requirements. Additionally, un-inoculated slides were used as negative controls and the eleventh remaining inoculated slide that was not exposed to the spray disinfectant was used as a positive growth control. Following incubation, the tubes were examined for microbial growth at 48 and 72 hour intervals. Sub cultures were also removed and examined for growth as described in the method. Selected tubes demonstrating "no growth" following the incubation period were inoculated with 10-100 cfu of the respective microorganisms and were observed for growth after 24 hours; this was done to ensure the absence of antimicrobial residual effect.

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For the viral study, each of the glass slides was placed into a tube containing 10 ml Dulbecco's Modification of Eagles Medium (DMEM, Mediatech, USA) supplemented with 10% Fetal Bovine Serum (Invitrogen, USA). The tubes were placed onto a rotary shaker for 15 minutes to elute the viral particles. Following, the eluates were assayed for the presence of infectious virus particles using a MPN based assay onto the RAW 264.7 and BGM cell monolayers. Positive, negative, cytotoxicity, and neutralization controls were performed as per ASTM recommendations and validated the test results.

Microbial Inactivation Efficacy Study - Time-Kill Challenge Study

A Time kill study based on ASTM 2315-08 was conducted to evaluate the inactivation efficacy rate of the disinfectant liquid generated by the Toucan-Eco unit. One sterile plastic flask containing 200 ml of sterile Class I ASTM water (Remel Inc, USA) and one containing 200 ml disinfectant freshly prepared by the Toucan-Eco system were used in the testing of the disinfection efficacy rate. A 1/100 dilution of an overnight culture (TSB) of each *E. coli* O157:H7 (ATCC 35150) and *Listeria monocytogenes* (ATCC 4428) was prepared in PBS. To each of two flasks, 5.0 ml of the PBS dilution was added. A calibrated traceable laboratory timer was started to track time from start of study. The temperature of the suspensions prior to application and during the efficacy

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testing was maintained at 20-22°C. The flasks were placed onto the orbital shaker and agitated at a medium speed. Ten milliliter samples from each of the flasks were removed at the following time points: 0 minutes, 30, 60, 90, 120, 180 seconds, and 5 minutes following the start of the study. The samples were added to a sterile tube containing 0.2 ml 10% sodium thiosulfate. The number of viable bacterial species in the neutralized samples was enumerated by spread plating onto TSA. Plates were incubated anaerobically at 36.5º C for 7 days. The resulting colonies were then counted and cfu/ml was determined. Prior to the study initiation it was determined that the amount of added sodium thiosulfate was sufficient to neutralize the chlorine in the samples.

All data are summarized in the following Tables. The results presented pertain only to the samples analyzed and identifier number(s) indicated. They are not representative nor are they indicative of a process. Positive and negative controls were performed as outlined in the method and as per Good Laboratory Practices. All analyses were performed in accordance to laboratory practices and procedures set-forth by our NELAC accreditation standards (ISO 17025) unless otherwise noted.

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Table 1. The bacterial disinfection efficacy of the disinfectant liquid generated by the provided Toucan-Eco unit. Test was conducted as per AOAC Official Method 961.02; Germicidal Spray Products as Disinfectants (2005)

Microorganism	Number of Sprayed Inoculated Slides	Number of Tubes Demonstrating Growth	Positive Control (un-sprayed slide)	Negative Control (un-inoculated slide)
Staphylococcus aureus (MRSA)	**10**	**None**	**Growth**	**No-Growth**
Salmonella enterica	**10**	**None**	**Growth**	**No-Growth**
Listeria monocytogenes	**10**	**None**	**Growth**	**No-Growth**
Pseudomonas aeruginosa	**10**	**None**	**Growth**	**No-Growth**
***E. coli* O157:H7**	**10**	**None**	**Growth**	**No-Growth**

* Glass slides were inoculated with the indicated microorganisms and allowed to dry. Slides were sprayed to saturation with the disinfectant and allowed to incubate at 20-22.0°C for ten minutes. Slides were eluted and examined for growth as described in the methodology section.

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Table 2. The bacterial disinfection efficacy of the disinfectant liquid generated by the provided Toucan-Eco unit; Germicidal Spray Products as Disinfectants (2005)

Microorganism	Number of Sprayed Inoculated Slides (number of replicates tested)	Average microorganism cfu/ml inoculated per slide[#]	Average cfu/ml recovered from each of slides sprayed*	Percent Reduction	Log_{10} reduction
Staphylococcus aureus (MRSA)	**10**	**>1.0 x 10^5**	**<1.0**	**>99.999%**	**>5.0**
Salmonella enterica	**10**	**>1.0 x 10^5**	**<1.0**	**>99.999%**	**>5.0**
Listeria monocytogenes	**10**	**>1.0 x 10^5**	**<1.0**	**>99.999%**	**>5.0**
Pseudomonas aeruginosa	**10**	**>1.0 x 10^5**	**<1.0**	**>99.999%**	**>5.0**
***E. coli* O157:H7**	**10**	**>1.0 x 10^5**	**<1.0**	**>99.999%**	**>5.0**

[#] This number represents the average number of microorganisms recovered from glass slides inoculated, dried, and not exposed to disinfection treatment (positive control).

* Glass slides were inoculated with the indicated microorganisms and allowed to dry. Slides were sprayed to saturation with the disinfectant and allowed to incubate at 20-22.0°C for ten minutes. Slides were eluted and examined for growth as described in the methodology section.

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Table 3. The viral disinfection efficacy of the disinfectant liquid generated by the provided Toucan-Eco unit. Test was conducted as per AOAC Official Method 961.02; Germicidal Spray Products as Disinfectants (2005) and ASTM E1053 "Standard Test Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces"

Microorganism	Number of Sprayed Inoculated Slides (number of replicates tested)	Average infectious particles (iu) /ml inoculated per slide[#]	Average iu/ml recovered from each of slides sprayed*	Percent Reduction	Log_{10} reduction
Murine Norovirus MNV-1 (Human Norovirus Surrogate)	5	4.6×10^4	<0.5	>99.999%	>5.0
Poliovirus CHAT Lsc1	5	1.3×10^5	<0.5	>99.9999%	>6.0

[#] This number represents the average number of infectious virus particles recovered from glass slides inoculated, dried, and not exposed to disinfection treatment (positive control).

* Glass slides were inoculated with the indicated microorganisms and allowed to dry. Slides were sprayed to saturation with the disinfectant and allowed to incubate at 20-22.0°C for ten minutes. Slides were eluted and enumerated for infectious viral particles on respective cell monolayers as described in the methodology section.

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Table 4. Inactivation of *E. coli* O157:H7 and *Listeria monocytogenes* at the indicated time points following introduction to the disinfectant liquid produced by the Toucan-Eco unit.

Microorganism	Bacterial cfu/ml at the indicated time points following study start[1]								
	0 (control)	10 seconds	30 seconds	60 seconds	90 seconds	120 seconds	180 seconds	5 minutes	Control (Final)
***E. coli* O157:H7**	2.9×10^6	<1.0 (>99.9999% or >6 Log_{10} reduction)	<1.0 (>99.9999% or >6 Log_{10} reduction)	<1.0 (>99.9999% or >6 Log_{10} reduction)	<1.0 (>99.9999% or >6 Log_{10} reduction)	<1.0 (>99.9999% or >6 Log_{10} reduction)	<1.0 (>99.9999% or >6 Log_{10} reduction)	<1.0 (>99.9999% or >6 Log_{10} reduction)	**2.9×10^6**
Listeria monocytogenes	1.1×10^6	<1.0 (>99.9999% or >6 Log_{10} reduction)	<1.0 (>99.9999% or >6 Log_{10} reduction)	<1.0 (>99.9999% or >6 Log_{10} reduction)	<1.0 (>99.9999% or >6 Log_{10} reduction)	<1.0 (>99.9999% or >6 Log_{10} reduction)	<1.0 (>99.9999% or >6 Log_{10} reduction)	<1.0 (>99.9999% or >6 Log_{10} reduction)	**8.9×10^5**

[1] Aliquots of the above bacteria were added to 200 ml of Class I ASTM water (Control) and 200 ml of the liquid generated by the Toucan-Eco unit. The flasks containing the liquids were agitated on an orbital shaker at a medium speed. At each of the indicated time points following the start of the study, samples were removed, neutralized and assayed for the bacterial species by spread plating onto TSA and incubation at 36.5°C for 24-36 hours.

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