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TEST REPORT

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Result: **COMPLETE** Report Date: 30-Nov-2012

Customer Name: [redacted]
Location of Testing: NSF International, 789 N. Dixboro Rd., Ann Arbor, MI 48105
Description: Organism Time Kill in Float[redacted] Water
Test Type: Test Only
Job Number: J-00114729
Project Number: 9130808 (ML01)
Project Manager: Sung Choe

Executive Summary:
A time kill test on stagnant water sampled from the Client's product was conducted using 5 organisms: MS2 coliphage ATCC 15597-B1, *Enterococcus faecium* ATCC 6569, *Pseudomonas aeruginosa* ATCC 27313, *Aspergillus niger* ATCC 6275 and *Candida albicans* ATCC 10231. The study was designed to evaluate the growth or decline of each of the organisms in the water over multiple sampling points when held at a temperature of 35 ± 1 °C. At each of the designated time points (0, 1, 4, 8 and 24 hours post organism inoculation) a sample was taken from the inoculated water and plated on selective media to determine the concentration of each organism. Log reductions were calculated for each time point using the 0 hour time point as the baseline. The results from this study are represented in Appendix A.

Thank you for having your product tested by NSF International.

Please contact your Project Manager if you have any questions or concerns pertaining to this report.

Report Authorization:_____

Dr. Robert Donofrio – Director, Microbiology Laboratory

Scope of Test Report

A time kill test on stagnant water sampled from the Client's product was conducted using 5 organisms: MS2 coliphage ATCC 15597-B1, *Enterococcus faecium* ATCC 6569, *Pseudomonas aeruginosa* ATCC 27313, *Aspergillus niger* ATCC 6275 and *Candida albicans* ATCC 10231. The study was designed to evaluate the growth or decline of each of the organisms in the water over multiple sampling points when held at a temperature of 35 ± 1 °C. At each of the designated time points (0, 1, 4, 8 and 24 hours post organism inoculation) a sample was taken from the inoculated water and plated on selective media to determine the concentration of each organism. Log reductions were calculated for each time point using the 0 hour time point as the baseline. The results from this study are represented in Appendix A.

Methodology

Culture Preparation

Each organism was individually cultured using the following methodology:

- MS2 coliphage ATCC 15597-B1: A freezer stock was diluted and combined with the host organism (*E.coli* ATCC 15597) and plated onto Tryptic Soy agar using top agar (TSB +1 % agar) methodology. The resulting stock was harvested by removing the top agar layer (containing the propagated phage and host) and dissolving it in Tryptic Soy Broth containing EDTA and lysozyme. The suspension was centrifuged to isolate the broth containing the phage. Following harvest, the phage suspension's density was determined by top agar overlay methodology and refrigerated until use.
- *Enterococcus faecium* ATCC 6569: A freezer stock was cultured in Brain Heart Infusion Broth (BHIB) for 24 hours and was passed consecutively for 2 days. Following incubation, the cell suspension density was determined using pour plate method with Standard Plate Count Agar (SPC) and refrigerated until use.
- *Pseudomonas aeruginosa* ATCC 27313: A freezer stock was cultured in Tryptic Soy Broth (TSB) for 24 hours and was passed consecutively for 2 days. Following incubation, the cell suspension density was determined using pour plate method with Standard Plate Count Agar (SPC) and refrigerated until use.
- *Aspergillus niger* ATCC 6275: A freezer stock was cultured on Sabouraud Dextrose agar at a temperature of 25 ± 1 °C for 7 days. Following incubation, the spores were harvested using Sterile Buffered Deinoized Water (SBDW) and filtered through glass wool. The resulting spore suspension density was enumerated using a hemocytometer and refrigerated until use.
- *Candida albicans* ATCC 10231: A freezer stock was culture in Yeast and Mold Broth at a temperature of 25 ± 1 °C for 2 days. After incubation, the cell suspension density was determined by using a hemocytometer and refrigerated until use.

Water Sampling and Organism Spike

A one liter volume of water was sampled from the Client's product using a 1L sterile Nalgene bottle.

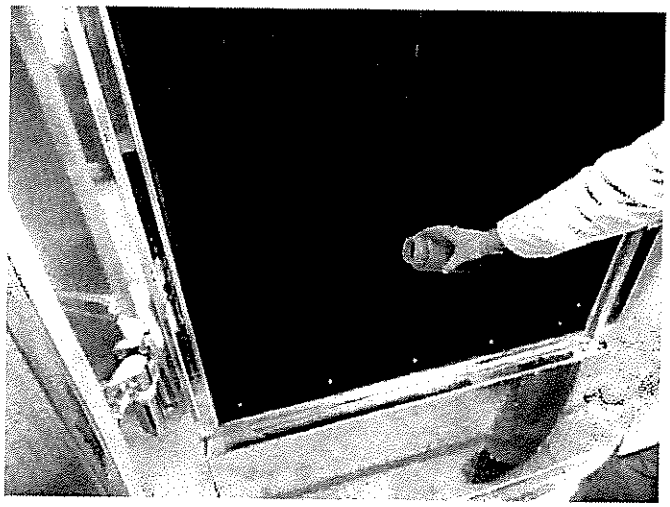


Figure 1. Float Lab Water Sampling

The one liter volume of water was separated into nine individual 100 mL aliquots in 125ml sterile Nalgene bottles. Three 100 mL allquots were individually spiked with the following organisms to a final target concentration of $1x10^5$ CFU/mL per organism:

- Group 1: MS2 collphage ATCC 15597-B1
- Group 2: *Enterococcus faecium* ATCC 6569 and *Pseudomonas aeruginosa* ATCC 27313
- *Group 3: Aspergillus niger* ATCC 6275 and *Candida albicans* ATCC 10231

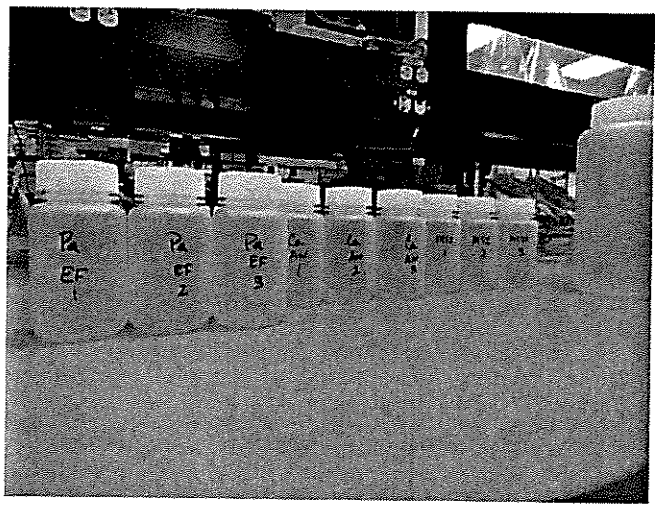


Figure 2. Water Inoculation Set Up

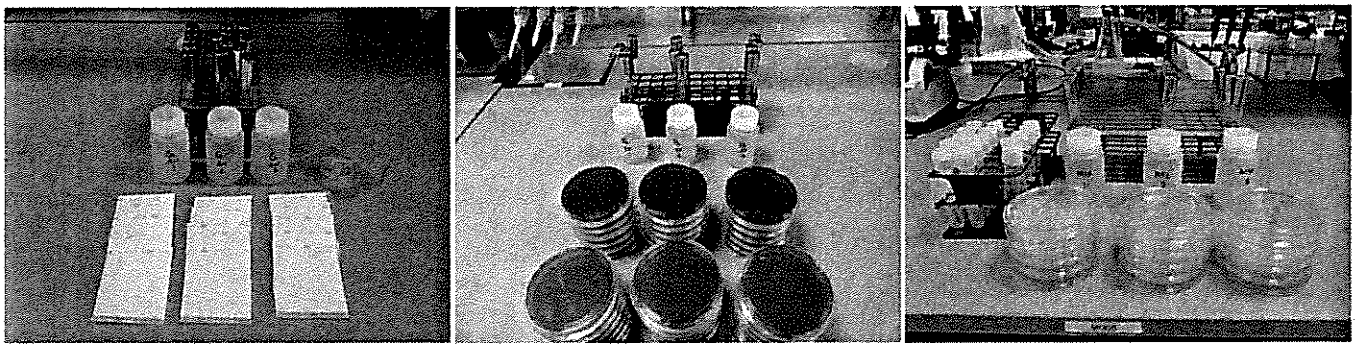
After inoculation the 100ml allquots were incubated at 35 ± 1 °C for the duration of the test and were only removed from the incubator to sample at each time point.

Sample Processing

At each of the designated time points (0, 1, 4, 8 and 24 hours post organism inoculation) a sample was taken from the inoculated water and diluted using Sterile Buffered Deionized Water (SBDW). The resulting dilutions were plated onto growth medium singly for each organism and incubated at the temperature and duration indicated in Table 1.

Table 1. Growth medium, plating method, incubation time and temperature for each organism tested

Test Organism	Growth Medium	Plating Method	Incubation Temperature (°C)	Incubation Duration
MS2 Coliphage ATCC 15597-B1	TSB + 1% agar with *E.coli* ATCC 15597 host	Top Agar Overlay	35.0 ± 1	24 ± 2 hours
Enterococcus faecium ATCC 6569	K F Streptococcus Agar	Spread Plate	35.0 ± 1	24 ± 2 hours
Pseudomonas aeruginosa ATCC 27313	M-PA-C Agar	Spread Plate	41.5 ± 0.5	72 ± 2 hours
Aspergillus niger ATCC 6275	YM Petrifilm ™	Petrifilm ™	23.0 ± 2	3-5 days
Candida albicans ATCC 10231	YM Petrifilm ™	Petrifilm ™	23.0 ± 2	3-5 days



Aspergillus niger and *Candida albicans* | *Enterococcus faecium* and *Pseudomonas aeruginosa* | MS2 coliphage

Figure 3. Sample Processing Set Ups

Data Analysis

- The log change due to product water exposure was calculated as follows:
  - The geometric mean of organism concentrations present on the three Hour 0 samples were calculated (N_i).
  - The geometric mean of organism concentrations present on the three samples will be calculated separately for each sample point (N_f)
  - The calculation for to determine log change values due to exposure to product water will be as follows:
    - $LR = \log (N_i / N_f)$
    - Log change values will be calculated separately for each sampling time point

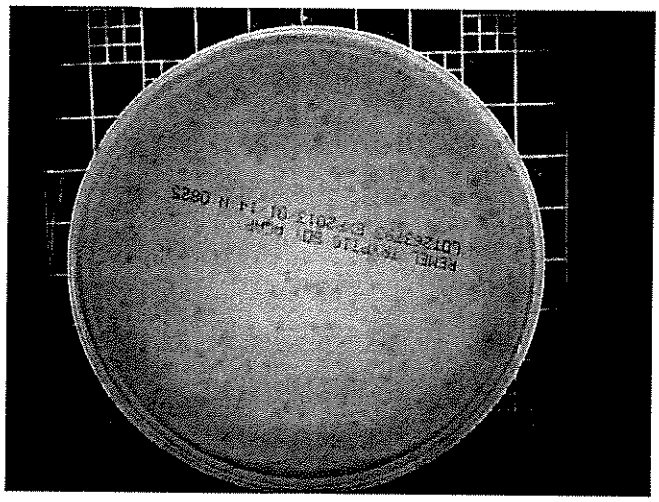


Figure. 4. Example Agar Plate Containing Coliphage MS2 ATCC 15597-B1

Results and Discussion

Log change values due to exposure to the product water were as follows at the 1, 4, 8 and 24 hour sampling time points:

MS2 coliphage ATCC 15597-B1: -0.17, -0.57, -0.55 and -1.39 respectively.

Enterococcus faecium ATCC 6569: -0.01, -0.15, -0.15, -0.76 respectively.

Pseudomonas aeruginosa ATCC 27313: -0.61, -0.85, -1.19, -2.58 respectively.

Aspergillus niger ATCC 6275: +0.13, +0.09, +0.08, +0.04 respectively.

Candida albicans ATCC 10231: +0.08, -0.12, -0.37, -1.67 respectively.

Conclusions

Pseudomonas aeruginosa appeared to be the most sensitive of all of the organisms exposed to product water, with the greatest drop in concentration over all sampling time points. *Candida albicans* ATCC 10231 was the second most sensitive with a 1.67 log reduction in concentration at the 24 hour sampling time point, but was slow to be affected at the beginning of the study with only a 0.37 log reduction at the 8 hour sampling time point. The MS2 coliphage had a similar response at the 24 hour sampling point (1.39 log reduction) but was a little quicker to die off at the 4 and 8 hour time points. With only a 0.76 log reduction after 24 hour exposure to the product water, *Enterococcus faecium* was one of the more resistant organisms in the study. Lastly, *Aspergillus niger* appeared to be the most resistant to the product water with little change in concentration over the 24 hour exposure period.

References

Standard Methods for the Examination of Water and Wastewater, 20th edition.

NSF/ANSI Standard 50-2012. Equipment for Swimming Pools, Spas, Hot Tubs and Other Recreational Water Facilities. NSF International, Ann Arbor MI.

Appendix A- Tables and Figures

Table A1. Log change values for the time kill assay. Organism concentrations presented for all sample time points are geometric means of three inoculated test water aliquots plated singly. Log change values that are negative are a reduction in growth and positive value are an increase in growth relative to Hour 0.

MS2 ATCC 15597-B1		
Time Point	Geometric Mean (CFU/mL)	Log Change
0 Hour	1.17×10^5	
1 Hour	7.95×10^4	-0.17
4 Hour	3.16×10^4	-0.57
8 Hour	3.32×10^4	-0.55
24 Hour	4.79×10^3	-1.39
***Enterococcus faecium* ATCC 6569**		
Time Point	Geometric Mean (CFU/mL)	Log Change
0 Hour	4.67×10^4	
1 Hour	4.53×10^4	-0.01
4 Hour	3.34×10^4	-0.15
8 Hour	3.30×10^4	-0.15
24 Hour	8.05×10^3	-0.76
***Pseudomonas aeruginosa* ATCC 27313**		
Time Point	Geometric Mean (CFU/mL)	Log Change
0 Hour	7.02×10^4	
1 Hour	1.73×10^4	-0.61
4 Hour	9.99×10^3	-0.85
8 Hour	4.58×10^3	-1.19
24 Hour	1.85×10^2	-2.58
***Aspergillus niger* ATCC 6275**		
Time Point	Geometric Mean (CFU/mL)	Log Change
0 Hour	5.25×10^4	
1 Hour	7.02×10^4	+0.13
4 Hour	6.41×10^4	+0.09
8 Hour	6.25×10^4	+0.08
24 Hour	5.73×10^4	+0.04
***Candida albicans* ATCC 10231**		
Time Point	Geometric Mean (CFU/mL)	Log Change
0 Hour	6.62×10^4	
1 Hour	8.00×10^4	+0.08
4 Hour	5.02×10^4	-0.12
8 Hour	2.81×10^4	-0.37
24 Hour	1.41×10^3	-1.67