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Result: COMPLETE

Report Date: 02-Oct-2015

Customer Name: Float On
Location of Testing: NSF Ann Arbor
Description: Organism Viability Testing in Epsom Salt and Control Solution
Test Type: Test only
Job Number: J-00183734
Project Number: W0216797
NSF Corporate: C0262109
Project Manager: David Nance

Executive Summary:

The antimicrobial efficacy of high concentration Epsom salt water was tested against two challenge organisms, *Pseudomonas aeruginosa* and *Enterococcus faecium*, over a 24 hour exposure period. Three time points were examined, 0 hour, 1 hour, and 24 hours, in both salt challenge water and control challenge water. *E. faecium* did not exhibit any significant reduction after 24 hours of salt water exposure when comparing to the control water exposure. In contrast to the *E. faecium*, *P. aeruginosa* was undetectable after 24 hours in the saltwater. Interestingly, *P. aeruginosa* grew in the control water after the same time period. The result was a 6.42 log₁₀ reduction.

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: _____
Robin Bechanko/Bryan Schindler – Microbiology Lab

This report replaces the report with serial number FI20151002000011. It is being reissued to correct typographical errors. This does not change the overall status of the report.

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Scope of Test

The antimicrobial efficacy of Epsom salt in constructed challenge water was analyzed against two challenge organisms: *Pseudomonas aeruginosa* ATCC #27313 (*Pa* 27313) and *Enterococcus faecium* ATCC #6569 (*Ef* 6569). Challenge water was prepared according to the NSF 50 Annex H formulation, and the Epsom salt product was added to the challenge water to achieve a density of 1.3 g/mL. Sterile challenge water was analyzed in parallel as a control.

The challenge organisms were prepared as a concentrated suspension and spiked into pre-warmed salt water for a target concentration of 1×10^8 CFU/mL per organism. The spiked salt and control water samples were then dispensed into individual 25 mL aliquots. Time points were processed via membrane filtration at 0, 1, and 24 hours. Samples for the 0 hour time point were immediately processed. The remaining samples for 1 hour and 24 hours were periodically shaken for 15 minutes at 150 rpm on an orbital shaker in an incubator set at $35 \pm 1^\circ\text{C}$. The organisms were then enumerated on respective selective media and log reductions were calculated comparing the Epsom salt water to the control water.

Media and Materials

NSF 50 Annex H formulated challenge water: 500 mL volumes sterilized via autoclave at $121 \pm 1^\circ\text{C}$ for 15 minutes.

Epsom salt: MgSO_4 septahydrate made by San Francisco Salt Company and supplied to NSF by Float On.

Salt Challenge Water: Pre-warm 500mL of sterile challenge water in a water bath to $44 \pm 1^\circ\text{C}$. Dissolve 650g of Epsom salt into 500 mL of pre-warmed challenge water to achieve water density of 1.3g/mL. Verify density via gravimetric analysis.

Cetrimide Agar (CA): selective-differential media for enumeration of *P. aeruginosa*. Prepare and sterilize according to manufacturer's instructions.

Enterococcosel Agar (EA): selective-differential media for enumeration of *E. faecium*. Prepare and sterilize according to manufacturer's instructions.

Sterile Buffered Dilution Water (SBDW): Buffered water used for rinsing, elution, and dilutions of samples.

Methodology

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Organism Preparation:

Fresh cultures of *Pseudomonas aeruginosa* ATCC #27313 and *Enterococcus faecium* ATCC #6569 were prepared by inoculating 350 mL of sterile TSB with 0.1 mL of freezer stock culture. The cultures were incubated overnight at 35±1°C, then centrifuged for 15 minutes at 4,000 x g. The concentrated pellets were then re-suspended in 10 mL of SBDW. The suspension density was then determined via pour plate using serial dilutions with a non-selective media.

Challenge Preparation:

Stock challenge water was prepared by the NSF Pools and Spas lab according to NSF 50 Annex H formulation. See Appendix A, Table 1, for water chemistry criteria. For test purposes, 500 mL aliquots were sterilized via autoclave. On test day, a bottle of sterile challenge water was pre-warmed in a water bath at 44.5±1°C for approximately 3 hours. Epsom salt was added to the challenge water to achieve a density of 1.30 g/mL, approximately 650 g of salt into 500 mL of water. The density was verified using a calibrated scale for gravimetric analysis. An additional sterile 500 mL volume was used as the control challenge water. Sterile aliquots of challenge water were sampled before and after the salt addition and analyzed for water chemistry characteristics by the NSF Pools/Spa lab. See Appendix A, Table 2., for results.

Sample Preparation:

Both challenge waters were aseptically adjusted to an equal volume of 300 mL per bottle. The entire volumes of challenge organism suspensions were equally divided between the challenge water bottles; 4.5 mL of *Pa* 27313 and 5.0 mL of *Ef* 6569 were spiked together into each 300 mL challenge volume to create a two-organism suspension with a theoretical concentration of approximately 2×10^8 CFU/mL of each organism. The spiked challenge waters were then aseptically dispensed into 25 mL aliquots in 50 mL centrifuge tubes for each time point replicate.

Time Kill Set Up:

Pictures of set up and processing can be seen in Appendix B.

Three time points were sampled; 0 hour (T0), 1 hour (T1), and 24 hours (T24). Each time point was tested in triplicate samples, and each replicate tube was plated and enumerated in triplicate. A T0 and T24 sterility control was processed in duplicate for the sterile challenge salt and control waters.

T0 samples were immediately vortexed for homogenization and processed. T1 and T24 samples were secured to an orbital horizontal platform shaker in an incubator set at 35 ± 1 °C and shaken periodically at 150 rpm for 15 minutes. All T1 and T24 samples were shaken immediately, and then again just before the 1 hour time point. The remaining T24 samples were shaken at every 60 ± 10 minute mark for the first 12 hours. The T24 samples were shaken once more before being sampled at the 24 hour time point.

Time Point Sampling and Processing:

All time points were sampled and processed with uniform procedure. Samples were taken by diluting 1 mL of the 25 mL sample into 99 mL of Sterile Buffered Dilution Water (SBDW).

Next, the full 100 mL sample dilution is filtered via sterile membrane filtrations using a 47 mm membrane filter with 0.45 µm pores. Following complete filtration, the samples were rinsed with two 250 mL SBDW washes (pre-warmed to 35 ± 1 °C to facilitate salt removal).

Finally, the filters were eluted in 10 mL of SBDW in 50 mL centrifuge tubes. Each tube was vortexed on the highest setting with six 5-second pulses for a total of 30 seconds. The samples were then diluted to the respective concentration ranges and spread plated in triplicate on both cetrimide agar and enterococcosel agar. All plates were inverted and incubated at 35 ± 1 °C for 24 ± 2 °C hours.

By sampling 1 mL of the original sample, eluting into 10 mL represents a 1:10 dilution. Furthermore, the least diluted spread plate processed was 0.1 mL of the 10 mL elution (i.e., another 1:10 dilution). Therefore, the detection limit of the study is 100 CFU/mL.

Results and Discussion

Table 3 in Appendix A shows average log₁₀ counts and reductions of each organism over the three time points for the control and salt water samples. Table 4 offers more detailed average CFU/mL counts for each time point replicates. Additionally, Figure 1 in Appendix A illustrates the average log CFU/mL counts for each challenge over the three time points.

Media and Challenge Water Controls:

Respective sterility, selective organism, and positive organism controls performed as expected for all lots of cetrimide and enterococcosel agar used in the study. Additionally, unspiked salt and control challenge waters taken at 0 and 24 hours showed no growth of either target organism, indicating no background contamination.

T0 densities:

T0 counts averaged $1.67 \pm 0.38 \times 10^8$ CFU/mL for *Ef* 6569 in the control water and $1.53 \pm 0.32 \times 10^8$ CFU/mL in the salt water. T0 counts for *Pa* 27313 averaged $2.90 \pm 1.31 \times 10^7$ and $3.43 \pm 3.16 \times 10^7$ CFU/mL for control and challenge waters, respectively.

E. faecium Performance:

In the control water, *Ef* 6569 showed marginal change after 1 hour, and only a 0.30 log reduction after 24 hours. In the salt water, *Ef* 6569 decreased from an average CFU/mL of $1.53 \pm 0.32 \times 10^8$ at T0 to $8.17 \pm 3.90 \times 10^7$ at T24, a 0.27 log reduction. When normalized to the control water at T24, *Ef* 6569 exhibited a negligible 0.01 log reduction in the salt water over 24 hours. This result suggests *E. faecium* is not susceptible to high concentrations of Epsom salt water.

P. aeruginosa Performance:

Pa 27313 showed meaningful growth over time in the control water; a 0.43 log increase after 1 hour, and 0.96 log increase after 24 hours. In contrast to *Ef* 6569, *Pa* 27313 exhibited reduction from $3.43 \pm 3.16 \times 10^7$ average CFU/mL at T0 to undetectable levels (<100CFU/mL), a 5.54 log reduction. When normalized to the increase in the organism control, the salt water samples averaged a 6.42 log reduction. Given the *Pa* 27313 control sample grew over time, the results suggests *P. aeruginosa* can thrive in the challenge water conditions but is very susceptible to the high concentration of Epsom salt water.

CONCLUSION:

The high density Epsom salt water has evident potential for greater than 5 log reduction of *P. aeruginosa* over 24 hours, and greater than 6 log reduction when compared to the possible growth of the organism in control water over the same period of time. By contrast, *E. faecium* displayed no evidence of significant reduction over 24 hours when comparing to both the T0 of salt and the T24 of the control water.

References

NSF/ANSI 50-2015: Equipment for Swimming Pools, Spas, Hot Tubs and Other Recreational Water Facilities, Annex H

Appendix A

Table 1. Challenge Water Chemistry Before and After Salt Addition

Parameter	Control Water	Saltwater	Acceptable Criteria
pH	8.33	6.45	7.2-7.6
Alkalinity	82 mg/L	Unable to measure*	60-100 ppm (CaCO ₃)
Hardness	225 mg/L	Unable to measure*	200-400 ppm (CaCO ₃)
Turbidity	0.65 NTU	1.73 NTU	<2.0 NTU
Temperature	73.3°F	90.2°F	Modified for test plan
Total/Free Available Chlorine	0.00 mg/L	Unable to measure*	0 ppm

*Instruments unable to measure parameter due to amount of salt added

Table 2. Average Organism Counts per Time Point and Calculated Log₁₀ Reductions of Epsom Salt Water

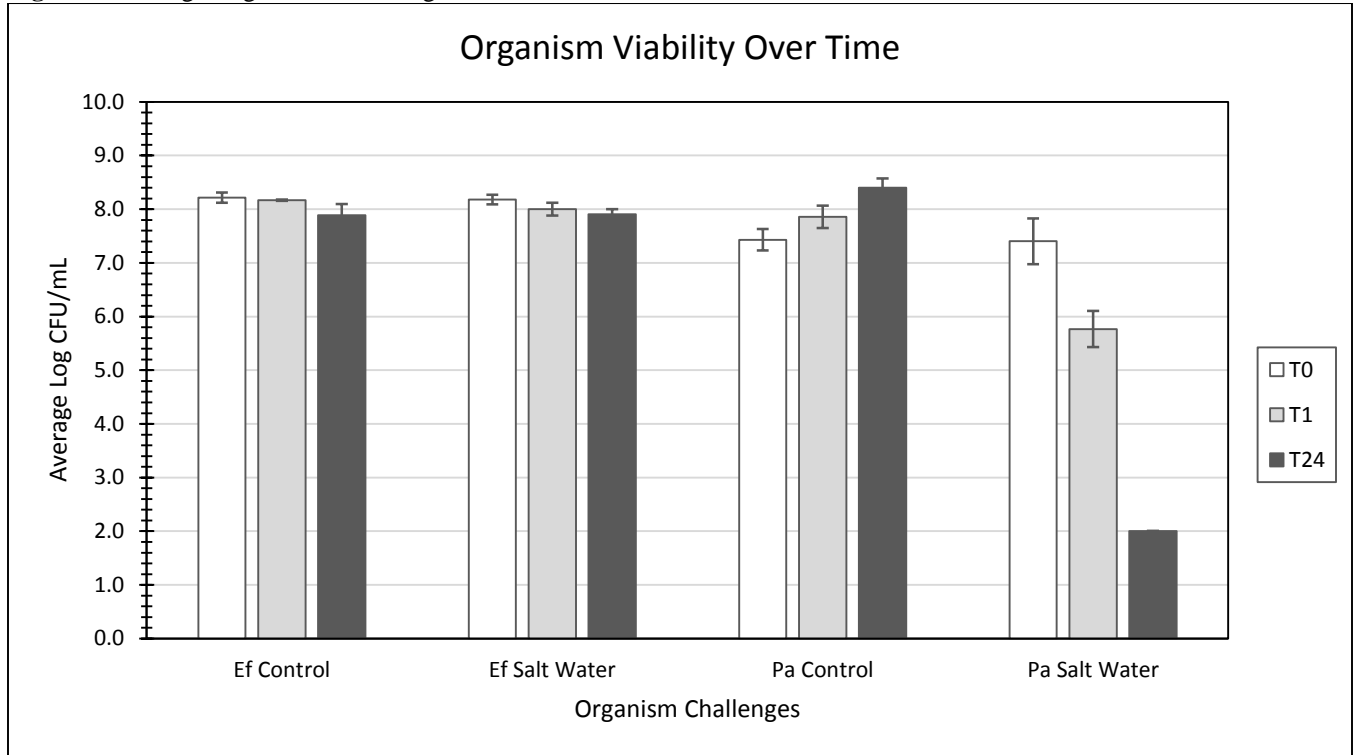
Organism	Challenge Water	Time Point Average Counts (Log ₁₀ CFU/mL)			Log ₁₀ Reduction*	
		T0	T1	T24	Control T1 - Salt T1	Control T24 - Salt T24
<i>Ef 6569</i>	Control	8.22 ± 0.09	8.17 ± 0.01	7.92 ± 0.21		
	Salt	8.19 ± 0.09	8.01 ± 0.12	7.91 ± 0.09	0.16	0.01
<i>Pa 27313</i>	Control	7.46 ± 0.20	7.89 ± 0.21	8.42 ± 0.17		
	Salt	7.54 ± 0.42	5.84 ± 0.34	2.00 ± 0.00	2.05	6.42

*Normalized salt reduction compared to control

Table 3. Average CFU/mL Organism Counts for All Time Point Replicates

Organism	Challenge Water	Time Point Counts (CFU/mL)					
		T0	T0 Avg	T1	T1 Avg	T24	T24 Avg
<i>Ef 6569</i>	Control	1.50 x 10 ⁸	1.67 ± 0.38 x 10 ⁸	1.52 x 10 ⁸	1.47 ± 0.05 x 10 ⁸	7.60 x 10 ⁷	8.37 ± 3.90 x 10 ⁷
		2.10 x 10 ⁸		1.47 x 10 ⁸		4.90 x 10 ⁷	
		1.40 x 10 ⁸		1.43 x 10 ⁸		1.26 x 10 ⁸	
	Salt	1.40 x 10 ⁸	1.53 ± 0.32 x 10 ⁸	9.60 x 10 ⁷	1.03 ± 0.29 x 10 ⁸	6.50 x 10 ⁷	8.17 ± 1.76 x 10 ⁷
		1.90 x 10 ⁸		7.80 x 10 ⁷		1.00 x 10 ⁸	
		1.30 x 10 ⁸		1.35 x 10 ⁸		8.00 x 10 ⁷	
<i>Pa 27313</i>	Control	2.70 x 10 ⁷	2.90 ± 1.31 x 10 ⁷	1.20 x 10 ⁸	7.83 ± 3.79 x 10 ⁷	3.07 x 10 ⁸	2.65 ± 0.91 x 10 ⁸
		4.30 x 10 ⁷		6.90 x 10 ⁷		1.61 x 10 ⁸	
		1.70 x 10 ⁷		4.60 x 10 ⁷		3.28 x 10 ⁸	
	Salt	2.30 x 10 ⁷	3.43 ± 3.16 x 10 ⁷	2.40 x 10 ⁵	6.93 ± 4.01 x 10 ⁵	1.00 x 10 ²	1.00 ± 0.00 x 10 ²
		7.00 x 10 ⁷		1.00 x 10 ⁶		1.00 x 10 ²	
		1.00 x 10 ⁷		8.40 x 10 ⁵		1.00 x 10 ²	

Figure 1. Average Log CFU/mL of Organisms Over Time Points



Appendix B.

Figure 2. Plates and Dilutions Organized Before Processing

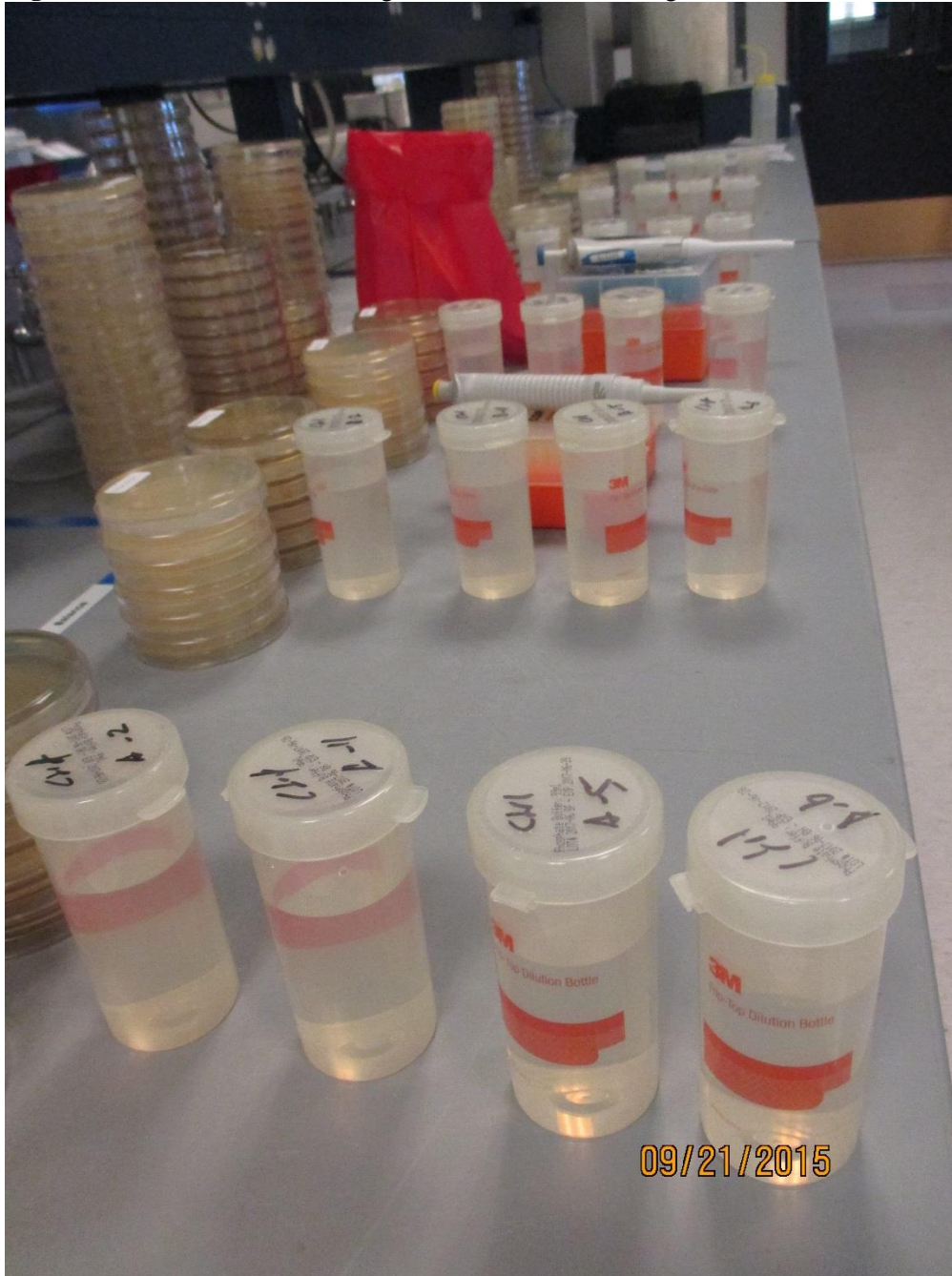


Figure 3. Membrane Filtration Sample Processing



Figure 4. Spread Plate Processing of Samples

