Center for Biofilm Engineering Proposal

Attachment A

September, 2007

Special Water Testing Project

Submitted to:	Special Water B.V. Email: mail@aquafinesse.com
Contact:	Jan de Rijk or Eric de Jong
Address:	425 Church Street, Suite 1600 Nashville, TN 37219-2308
Phone:	(615) 423-5775
Email:	eric@aquafinesse.com

Submitted by:	Center for Biofilm Engineering
Contact:	Diane K. Walker
Address:	Montana State University-Bozeman 366 EPS Building P.O. Box 173980 Bozeman, MT 59717-3980
Phone:	(406) 994-6059 Fax: (406) 994-6098
Email:	dianew@erc.montana.edu

INTRODUCTION

The CBE was contacted to perform testing to determine the mitigating effects of AquaFinesse™ treatment against a *Pseudomonas aeruginosa* biofilm grown in the CDC biofilm growth reactor. Two parallel CDC reactors were utilized with one acting as an untreated control and the second as the reactor that received treatment. ASTM Standard Test Method E2562-07[1] was modified as outlined in the materials and methods section below at the request of Special Water to simulate nutrient concentrations and bacterial densities representative of those found in hot tubs.

MATERIALS AND METHODS

Biofilm reactor system. This project used an *in vitro* model system for biofilm growth called the CDC reactor. The CDC biofilm growth reactor[2] was developed by the Centers for Disease Control and modified by the Center for Biofilm Engineering and was recently accepted as a standard method by the American Society for Testing and Materials for developing repeatable, pure culture *Pseudomonas aeruginosa* biofilms. The CDC reactor consists of a one-liter glass beaker fitted with a drain spout such that the fluid volume is ~400 mL. The vessel contains a magnetically driven stir baffle and has eight removable rods, each holding three 1.27 cm diameter polycarbonate coupons.

Bacterial nutrients. The nutrient concentrations in the reactor were modified from the standard method from 300 mg/L Tryptic Soy Broth (TSB) in batch and 100 mg/L TSB during continuous flow, to 10 mg/L TSB under both batch and continuous flow conditions.

Inoculum and batch phase. The CDC reactor was injected with 1 ml of a 10^6 *Pseudomonas aeruginosa* inoculum (modified from a 10^8 cell density used in the standard method) and stirred in batch mode for 24 hours.

Treatment preparation. The treatment feed was prepared by vigorously shaking product and adding it to sterile water for a final concentration equivalent to 4ml/10L.

System operation. Two CDC biofilm growth reactors were operated simultaneously. Upon startup of continuous flow to the reactors, both received 10 mg/L TSB media feed. One did not receive treatment and served as control. The second reactor received the treatment to determine its efficacy after 6 hr, 24 hr and 48 hr of continuous flow.

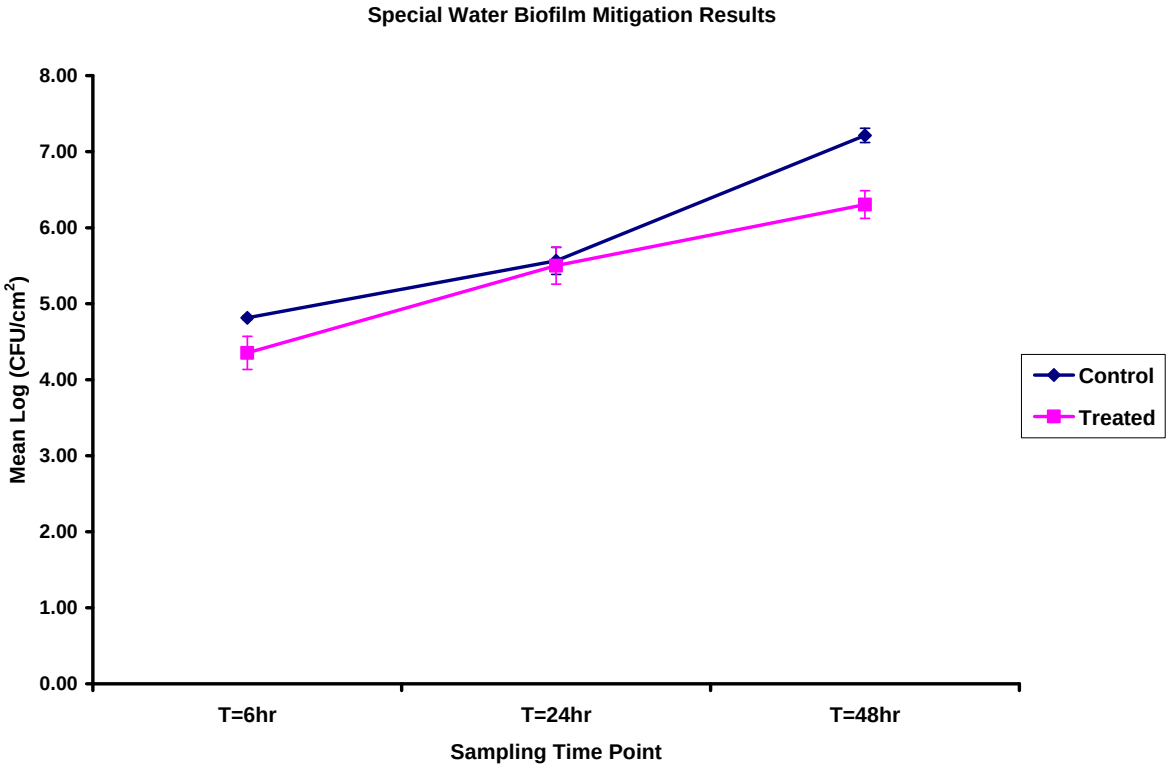
Sampling procedures. After 6 hours, coupons were sampled in triplicate from each reactor. Each coupon was placed in 10 ml sterile dilution water[3] and sonicated for 1 minute to remove and disaggregate biofilm. A dilution series was performed and each dilution was plated in duplicate on R2A agar using the drop plate method[4] to determine viable cell counts.

Viable cell enumeration. Bacteria were enumerated and log density per surface area (CFU/cm^2) was calculated (see Results).

RESULTS

The goal of the Special Water testing project was to determine the mitigating effects of AquaFinesse™ against a *Pseudomonas aeruginosa* biofilm grown in the CDC reactor. The data compiled from triplicate samples indicate that bacterial growth was consistent in both the control and treated reactors through the 24 hr test period. At the 48 hr sample time point, however, the mean log density of bacteria in the treated reactor is lower suggesting a mitigating effect by the presence of AquaFinesse™ product (see table and chart below).

Sample Time Point	Control		Treated	
	Mean Log (CFU/cm^2)	Standard Deviation	Mean Log (CFU/cm^2)	Standard Deviation
T=6hr	4.82	0.023	4.35	0.218
T=24hr	5.56	0.178	5.50	0.244
T=48hr	7.21	0.093	6.30	0.183



1 ASTM Designation: E2562-07 Standard Test Method for Quantification of Pseudomonas Aeruginosa Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor
2 Goeres, D.M., Loetterle, L.R., Hamilton, M.A., Murga, R., Kirby, D.W., and Donlan, R.M. 2005. *Statistical assessment of a laboratory method for growing biofilms*. Microbiology. 151: 757-762.
3 AHPA, Standard Methods for the Examination of Water and Waste Water (1995) 19th Ed., 9-17.
4 Herigstad, B., M. Hamilton, and J. Heersink, *How to optimize the drop plate method for enumerating bacteria*. J. Microbiol. Methods, 44(2): 121-129 (2001).

Extra information Special Water Holding B.V.

Results from the Center for Biofilm Engineering show a mitigating effect of AquaFinesse after 48 hours. This is the main conclusion however the following remarks have to be made to the results. The test were done by their CDC Biofilm reactors. This device is a small 400ml grow chamber with small coupons for bacterial growth. The CDC reactor is developed for medical toxicity studies and to test biocidal effects. Therefore a rather “fat” biofilm is grown (high nutrition load) on the coupons. So results show the effect in a worst case situation which hardly occur in the normal way of hot tub maintenance i.e. we never leave the biofilm to growth in hot tubs to the extent found in the reactors. Still a considerable decrease (10 times) in colony forming units is found after 48 hours so that is a good result. Keep in mind that AquaFinesse is NOT a biocide it only interferes with the biofilm to get it off from the walls of hot tubs and their piping equipment.
The used concentration of AquaFinesse were resembling the fouling situation with a rather fouled hot tub and does not exaggerate in applied concentrations. So to summarize we believe that the results are in good accordance with the normal daily procedures for cleaning up a severely fouled hot tubs and getting, and keeping the hot tub clean.

Kind Regards,

Special Water Europe B.V.

Jan de Rijk