Developing a Microscopy-Based Method to Quantify Biofilm Removal

Madeline Burgess*, Kelly Ferguson, Sara Mindek and Bruce Urtz

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INTRODUCTION

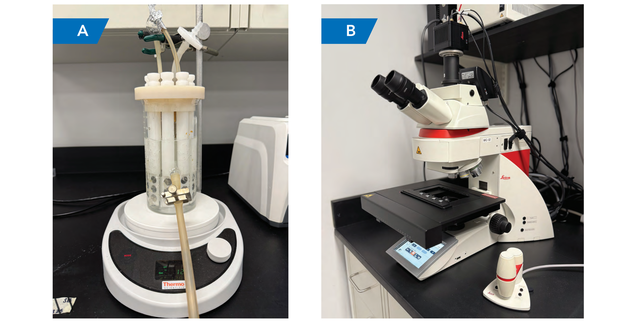
Biofilm control is critical in maintaining a safe food production environment. While EPA-approved standardized methods exist to evaluate biofilm kill and make associated claims, no such method(s) exists for biofilm removal. Traditionally, microscopy has been used to image and qualitatively analyze biofilms. In this study a method was developed to qualitatively and quantitively assess biofilm removal using a combination of fluorescent microscopy and imaging software.

METHODS

The CDC Biofilm Reactor (ASTM E3161-21) was used to grow *Pseudomonas aeruginosa* ATCC 15442 biofilm on carriers made of borosilicate glass. Rods containing carriers were removed from the reactor and treated with a) hard water (control), b) 850 ppm PAA in hard water or c) a 1:1:10 mixture of Ultra Disinfectant Cleaner Solution 1:Ultra Activator Solution:hard water (PerQuat®). The treatments were applied for 10 minutes under sheer. The carriers were then removed from the rods and stained using Invitrogen SYTO™ 9 stain for 15 minutes.

Using a Leica DM6 fluorescent microscopy system with a GFP filter cube, five image z-stacks were randomly taken at 40x per treatment group. The Leica LAS X imaging software was used to process and analyze all images taken for depth, microcolony size, biofilm structure, and biofilm removal. Removal was calculated based on the total voxel measurements in each image stack in relation to the average voxel measurement in the control. This calculation results in the percentage by which the overall biofilm quantity decreased. Results for each image were averaged to determine the percent biofilm removal per treatment group.

Figure 1 – Materials used for method development



(A) CDC biofilm reactor. **(B)** Leica DM6 fluorescent microscope. **(C)** Borosilicate glass carrier. **(D)** Image (4x magnification) carrier containing *P. aeruginosa* biofilm.

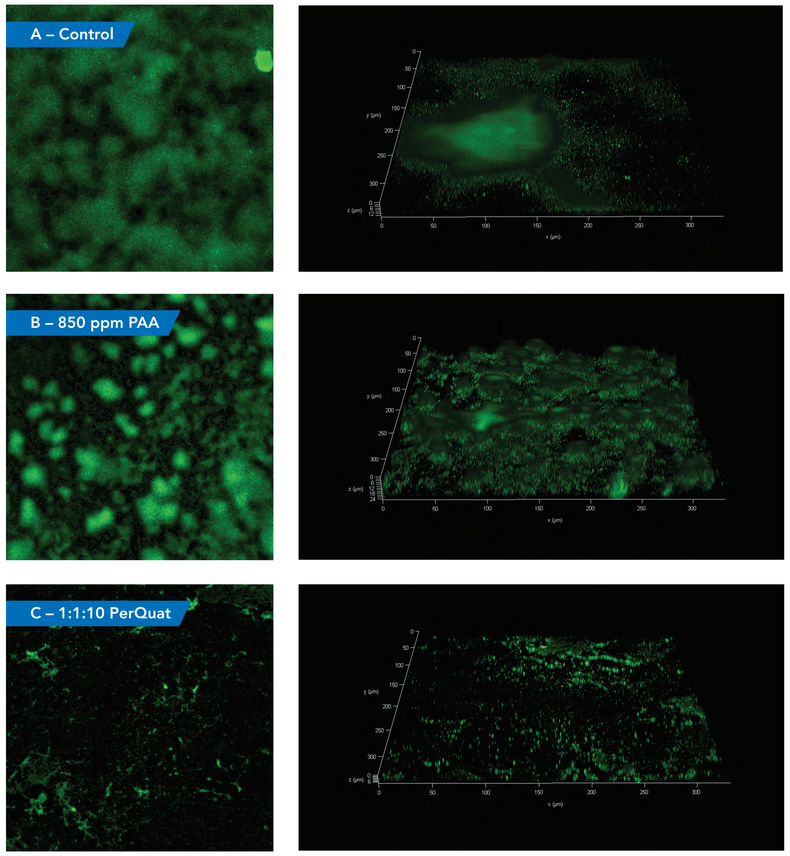
SCAN TO VIEW ONLINE

Madeline Burgess
Senior Microbiologist

www.sterilex.com
1.800.511.1659
marketing@sterilex.com

RESULTS

Figure 2 – *Pseudomonas* biofilm images at 40x

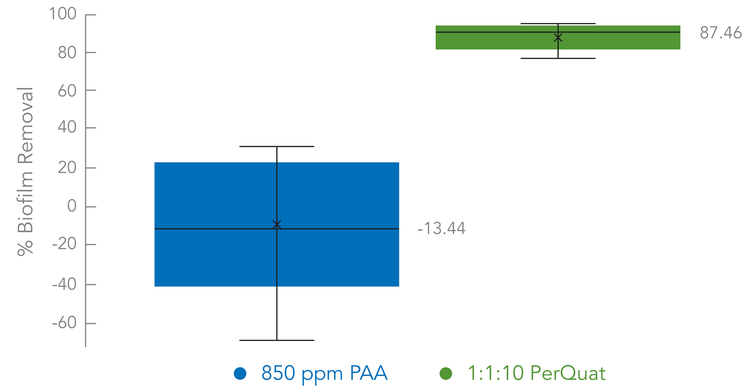


Pseudomonas aeruginosa biofilm at 40x magnification after 10 minute exposure to **(A)** hard water, **(B)** 850 ppm PAA in hard water and **(C)** 1:1:10 Ultra Disinfectant Cleaner Solution 1 and Ultra Activator Solution (PerQuat) in hard water. Addition of the PAA resulted in higher voxel measurements, on average, than the control with little-to-no evidence of removal. In contrast, **the biofilm treated with PerQuat had an altered structure with noticeable evidence of removal**.

Table 1 – Imaging measurements

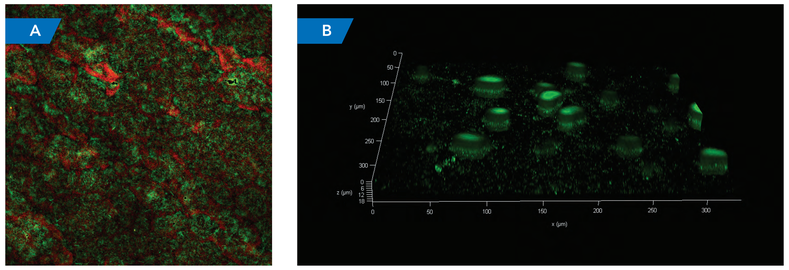
Image	Stack Depth (µm)			Large Microcolony Diameter (µm)			Total Voxels		
	Control	PAA	PerQuat	Control	PAA	PerQuat	Control	PAA	PerQuat
1	12	24	7	302	129	48	876,511	1,510,293	58,949
2	7	20	7	201	139	65	515,986	1,062,367	95,463
3	15	14	9	247	178	71	1,205,084	1,054,596	88,738
4	12	13	10	145	95	66	878,633	850,983	128,817
5	17	8	11	192	114	92	1,101,601	714,914	202,168
Mean	–	–	–	–	–	–	915,563	1,038,631	114,827

Figure 3 – *Pseudomonas* biofilm removal results



Using this quantification method, it was shown that the PerQuat-based disinfectant was able to remove over 80% of biofilm on average, while 850 ppm PAA showed no evidence of removal. Variation in biofilm formation or structure may account for negative removal values seen for 850 ppm PAA.

Figure 4 – Quantifying other biofilm models



Pseudomonas-Listeria multispecies biofilm was developed and analyzed. **(A)** Fluorescent Gram stain showing colonization of both *Listeria* (red) and *Pseudomonas* (green). **(B)** A 40x image z-stack of the co-biofilm.

DISCUSSION/CONCLUSIONS

Effective biofilm management is critical in food safety. Standardized methods now exist to assess biofilm kill, and EPA approval of these methods allows companies to make biofilm kill claims for their products. While biofilm kill is important, biofilm removal is also important in preventing the re-establishment of a biofilm. Currently, there are no standardized EPA approved methods for assessing biofilm removal and making biofilm removal claims. To make a biofilm removal claim it is likely the EPA would require a combination of data including viable cell counts, staining (e.g., crystal violet), and microscopy.

In this study, a method was developed to qualitatively and quantitively assess biofilm removal using a combination of fluorescent microscopy and imaging software. The method does not require the use of an expensive confocal microscope, but it does require the use of an epifluorescent microscope combined with imaging software.

Once developed, the method was used to evaluate the biofilm removal capability of PAA and PerQuat. At 850 ppm, PAA is effective in killing *P. aeruginosa* biofilm (data not shown) but in this study it had no effect on removal of the biofilm. In contrast, **PerQuat at the concentration tested is not only effective in killing the biofilm (data not shown) but also removing it**.

Preliminary results obtained with a *Pseudomonas-Listeria* dual species biofilm suggests that this method could be used for assessing the removal of more complex biofilms such as those found in food production environments.