

 SANI MARC

BIOASSURE™

DIN 02456435

COMPARATIVE STUDY
ON THE EFFICACY OF DISINFECTANTS
AGAINST BACTERIAL
CONTAMINATION CAUSED BY
BIOFILM



WOOD WYANT™

Subsidiary of Sani Marc Group

ORIGINAL ARTICLE

COMPARATIVE STUDY ON THE EFFICACY OF DISINFECTANTS AGAINST BACTERIAL CONTAMINATION CAUSED BY BIOFILM

Marchand P., Desrosiers D., Côté A., Touchette M., Mamouret N.

Centre de recherche Groupe Sani Marc, 42 rue de l'Artisan, Victoriaville, Quebec, Canada

Corresponding author: Patrick Marchand, Sani Marc Group, 42 rue de l'Artisan, Victoriaville, Quebec, Canada G6P 7E3

ABSTRACT

The disinfection of surfaces containing biofilms is complex, since bacteria within biofilms use mechanisms of protection that lead to an increased bacterial resistance against commonly used disinfectants. To eliminate biofilm contamination, microorganisms within the biofilm must be destroyed and the biofilm structure itself must be removed from the surface. The study presented here uses a standard method (ASTM standard test method E2871-12) for measuring efficacy of six disinfectants (Hydrogen peroxide, peracetic acid, chlorine dioxide, sodium hypochlorite, quaternary ammonium compound and enzyme) on removing biofilm and killing the bacteria within it. Results show that some disinfectants are good to kill bacteria (e.g., sodium hypochlorite) where other are better at removing the biofilm structure from the surface (e.g., enzyme). However, two disinfectants, peracetic acid and chlorine dioxide, are able to have a dual action on killing the bacteria and removing the biofilm from the surface. We also conclude that diffusion controls the relative efficacy of each disinfectant.

KEY WORDS: Biofilm, disinfection, method, sodium hypochlorite, peracetic acid, quaternary ammonium compound, hydrogen peroxide, enzyme

INTRODUCTION

Most discussions on infection control or environmental contamination tend to centre on planktonic bacteria (free bacteria in the liquid environment) [Ofek I, 1994 #1; Ofek I, 1994 #1; Buckingham-Meyer, 2007 #11][1]. However, it is known that bacteria will naturally stick to a surface and create a micro community called a biofilm [2]. This natural way of life for bacteria is in large part to blame for recurrent contamination on specific surfaces and a clear indicator of how we should disinfect surfaces [3]. Recent advances in microbiology shed new light on how bacterial communities are organized, raising serious concerns around disinfection protocols [4].

When bacteria encounter a surface, they can attach themselves to it. At this point, they change their metabolism and start to produce new metabolites, this decision is made in reaction to their surrounding environment [2]. When there are plenty of resources, bacteria will grow and multiply until resource become scarce. Then, bacteria will start to create a "cocoon" in which they can be protected and form a viable community [5]. This "cocoon", which is called an exopolysaccharide matrix (EPS), is the main difference between planktonic bacteria and a biofilm. Though this differentiation may seem meaningless, it has a huge impact on the bacteria and on the disinfection of the surface containing this biofilm. Four protection mechanisms are now generally accepted [3]. Firstly, the matrix creates a physical barrier that prevents dangerous agents from reaching bacteria due to limited diffusion. Secondly, some of the cells in a biofilm produce stress responses at the expense of other cells, which are sacrificed. Thirdly, the cells lower their metabolic activity, which may make them less susceptible to antimicrobials. Finally, persistent cells accumulate in biofilms because they revert less readily and are physically retained by the biofilm matrix. These four mechanisms of protection lead to an increased bacterial resistance against commonly used disinfectants.

To obtain approval for a disinfection product's stated efficacy, one must show efficacy results based on different methods. However, these methods test product efficacy on newly grown bacteria that can be in a broth (e.g., EN1276, AOAC 960.09) or dried on a carrier (e.g., AOAC 955.14). These methods do not take into account the fact that, on environmental surfaces, bacteria tend to be hidden in a biofilm. Other microorganisms (i.e., viruses) can also hide in a biofilm, leading to increased protection [6, 7]. Various strategies have been suggested for attacking biofilms, but from the author's point of view, to eliminate biofilm contamination, the microorganism within the biofilm must be destroyed and the biofilm structure itself must be removed from the surface. If a disinfectant only kills the bacteria but leaves the biofilm structure in place, it may create a nest to which other bacteria can attach themselves in order to create a new biofilm more quickly. On the other hand, if a disinfectant removes the biofilm without killing the bacteria, it will release the planktonic bacteria, which will contaminate the surrounding environment.

Multiple studies have been conducted on biofilms over the years [3, 4, 8-14]. However, because of variability of methods and the resulting high disparity of outcomes, conducting a meaningful comparison across the studies is challenging [4]. The objective of this paper is to present the efficacy of common disinfectants in killing and removing bacterial biofilm on surfaces. The results

A FIRST IN CANADA

HEALTH CANADA
REGISTERED
PRODUCT AGAINST
BIOFILMS



BIOASSURE
DIN 02456435

KILLS BACTERIA IN BIOFILMS IN DRAINS IN 5 MINUTES
KILLS AND REDUCES THE SPREAD OF SUPERBUGS
VERSATILE AND SAFE FOR ALL HARD SURFACES

TAKE CONTROL!

Learn more

<http://www.sanimarc.com/bioassure/>

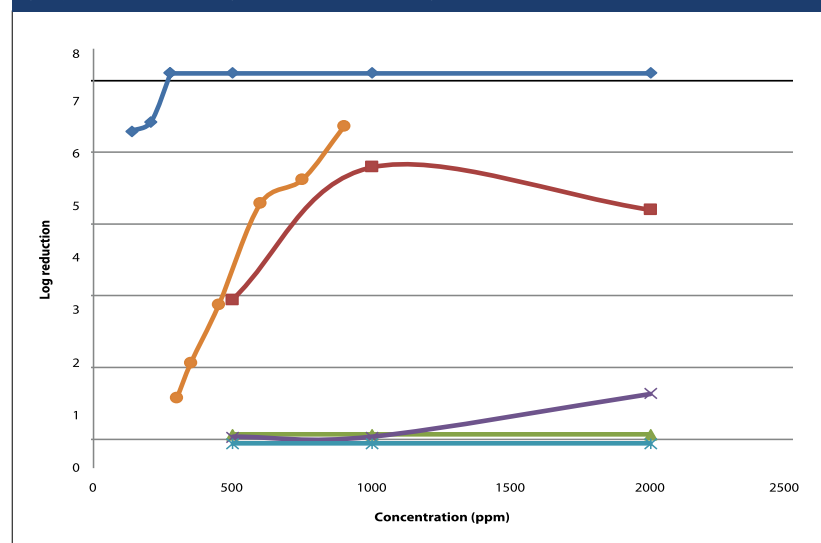
compare the effect of hydrogen peroxide, sodium hypochlorite, chlorine dioxide, quaternary ammonium compound (quat), enzymes and peracetic acid (PAA) on a pseudomonas biofilm. This will shed light on the efficacy of each method and demonstrate how the molecular characteristics of various disinfectants can explain these outcomes.

MATERIALS AND METHODS

Materials

The hydrogen peroxide solution was obtained by diluting 50% hydrogen peroxide from Arkema inc. (Colombes, France) to the required test concentration. The sodium hypochlorite solution was obtained by diluting 12% sodium hypochlorite from UBA Inc. to the required test concentration. The chlorine dioxide solution was prepared with the product Activator™ and the product Ecosant™ from Sani Marc Inc. (Victoriaville, Quebec, Canada). The quat solution was obtained by diluting an 80% first generation quat from Stepan Co. (Northfield, Illinois, États-Unis) to the required test concentration. A concentrated enzymatic solution was made using a mix of three types of enzymes: a protease, a lipase and an amylase from Novozymes Inc. (Gledsaxe, Denmark): Everlase 16 L, Termamyl 300 L and Stainzyme 12 L, respectively. The concentration of each enzyme was 0.8% for Everlase 16 L, 0.6% for Termamyl 300 L and 0.6% for Stainzyme 12 L. The concentrated enzymatic solution contained 0.5% fatty alcohol ethoxylates and pH was adjusted to 9.5 with sodium hydroxide. The enzymatic solution was made with this concentrated enzymatic solution. The PAA solution was obtained by diluting Oxygerm (5% PAA product from Sani Marc) to the required test concentration.

FIGURE 1: Concentration curve for killing bacteria within the biofilm (chlorine dioxide ◊, sodium hypochlorite ■, hydrogen peroxide ▲, quaternary ammonium compound ×, enzyme *, peracetic acid ●)



All disinfectants tested were aseptically diluted at appropriate concentrations with sterile water or hard water (hardness expressed as CaCO₃ concentration) just before performing the assays. Disinfectant samples were diluted and kept at room temperature.

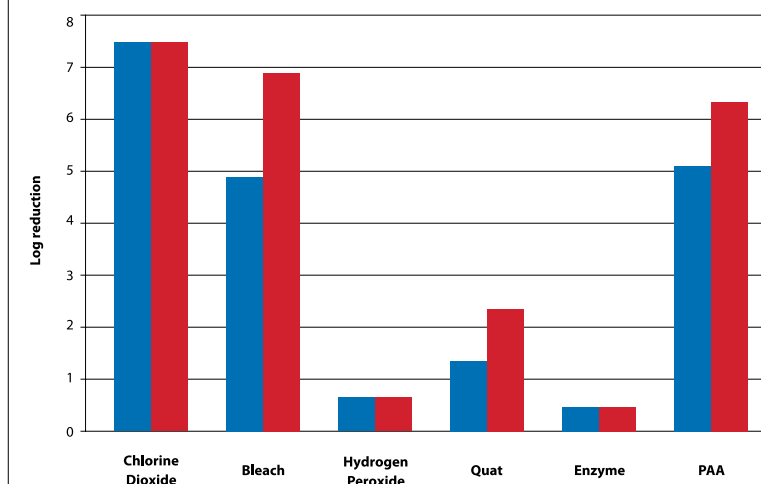
Reactor preparation

The reactor (CBR 90 Biofilm Reactor, BioSurface Technologies Corporation, Bozeman, USA) preparation and sterilization were done according to ASTM standard test method E2562-12. Borosilicate glass disc coupons were used for all tests and served as a carrier for the biofilm formation. A total of 24 coupons were used for each assay. The coupons were retained by height rods, each one holding three coupons.

Culture preparation

Pseudomonas aeruginosa ATCC 15442 was grown on a TGEa (Tryptone glucose extract agar) slant from a frozen stock culture. The inoculum was incubated for 24 ± 1 h at 37 ± 1°C under aerobic condition. A minimum of three daily transfers on TGE slants were done prior to the preparation of the final test suspension. For the final test culture step, bacteria were transferred to TGE broth and the viable cell density was determined using absorbance at 600 nm. The standardized culture was maintained at 4°C for a maximum of 30 minutes until the reactor inoculation step.

FIGURE 2: Effect of time on the efficacy of common disinfectants: chlorine dioxide (250 ppm, 5 min and 15 min); bleach (2000 ppm, 5 and 60 min); hydrogen peroxide (2000 ppm, 5 and 60 min); quat (2000 ppm, 5 and 60 min); enzyme (2000 ppm, 5 and 60 min); PAA+surfactants (600 ppm, 5 and 10 min)



Pseudomonas aeruginosa biofilm production

P. aeruginosa biofilm was produced on borosilicate glass disc coupons according to ASTM standard test method E2562-12 with some deviations. 350 mL of TGE broth were added to the sterile reactor. The reactor was then inoculated at 106 CFU/mL with the standardized *P. aeruginosa* culture. The inoculated reactor was incubated in batch mode at room temperature for 24 ± 1 h with a rotation speed of 125 ± 5 rpm. At the end of the batch mode, a continuous flow of 17 ± 2 L of TGE broth (flow rate around 702 mL/h) was started for another 24 h. The reactor was still maintained at room temperature under stirring at the speed of 125 ± 5 rpm. The drain spout of the reactor was connected to an empty carboy to allow the medium to drain and to maintain a constant volume (350 mL) in the reactor during the continuous phase.

Disinfectant efficacy evaluation

Disinfectant efficacy was tested according to ASTM standard test method E2871-12. Rods were aseptically removed from the reactor and rinsed in 30 mL of sterile phosphate buffer (pH 7.2) in order to remove planktonic cells. The coupons, on which the biofilm was grown, were aseptically removed from rods using a flame-sterilized Allen wrench. Each coupon was placed in a sterile 50 mL conical tube. Then, 4 mL of disinfectant were added to each tube. For the initial population control, the disinfectant was replaced by 4 mL of sterile phosphate buffer (pH 7.2). Tubes were left at room temperature during the contact time targeted. At the end of the contact time, 36 mL of neutralizer (Lethen broth; 25.7 g/L, Tween 80; 100 g/L, L-alpha-lecithin; 11 g/L, sodium thiosulfate; 2 or 20 g/L depending on the disinfectant's concentration) were added to each tube. Tubes were then mixed with a vortex and placed at 4°C until the next step. For each disinfectant treatment, five coupons were used. The initial population control was made for each assay and four coupons were used.

In order to quantify the viable population on each coupon, the biofilm was removed with a sequence of vortex and sonication (30 seconds vortex, 30 seconds sonication at 40 ± 2.5 KHz, 30 seconds vortex, 30 seconds sonication at 40 ± 2.5 KHz and 30 seconds vortex). Tubes were then serially diluted with sterile phosphate buffer (pH 7.2). Quantification of the viable biofilm population was done using the spread plating method on TGEa. Plates were incubated at 37 ± 1°C for 24 h. The colony number was counted on each plate, and results were reported in log reduction (mean log of initial population control – mean log of population after disinfectant treatment).

Microscopy

Rods were aseptically removed from the reactor and gently rinsed in 30 mL of sterile phosphate buffer (pH 7.2) in order to remove planktonic cells. Coupons were then stained with safranin solution, 0.6% w/v, for 1 minute. Coupons were visualized at a 200X magnification with an optical microscope (Nikon, Eclipse E2000, Mississauga, Canada).

| Product | Killing Efficacy | Biofilm Removal Efficacy |
|-------------------|------------------|--------------------------|
| Hydrogen peroxide | Bad | Good |
| Bleach | Medium | Bad |
| Chlorine dioxide | Good | Good |
| Quat | Bad | Bad |
| Enzymes | Bad | Good |
| Peracetic acid | Good | Good |

RESULTS

All the data collected are presented in Figure 1 for disinfectant efficacy evaluation and in Figure 2 for microscopic observations. Concentration curves have been limited to 2000 ppm to keep standard conditions. The authors estimate that a good disinfectant should be able to reach a 5 log reduction at these concentrations without harming the surfaces. As we see in Figure 1, oxidizing products seem to be more effective than non-oxidizing products (enzyme and quat) at penetrating the biofilm and killing the bacteria. The only exception to this is hydrogen peroxide.

Hydrogen peroxide shows no efficacy in killing bacteria in a biofilm up to 2000 ppm, as shown in Figure 1. Even if we increase the time to 60 minutes, there is no increase in killing ratio. However, microscopy results show that hydrogen peroxide is effective at removing the biofilm matrix from the surface. This indicates that hydrogen peroxide can penetrate the biofilm to remove the matrix from the surface, but is unable to kill bacteria within it. This is not surprising since tests conducted on planktonic data in our lab show that unformulated hydrogen peroxide is not a good bactericide [15]. Adding surfactant is needed to achieve the correct killing ratio.

Sodium hypochlorite shows good efficacy in killing bacteria in biofilms, achieving almost 6 log of reduction at 1000 ppm. However, this tends to diminish to 5 logs at 2000 ppm. It is reasonable to think that a protective layer is formed by oxidation of the matrix at higher concentrations, lowering the diffusion of hypochlorite ions through the biofilm. This can be corroborated with the total kill of the bacteria in the biofilm at 60 min. Contrary to peroxide, hypochlorite is unable to remove the polysaccharide structure on the surface. Consequently, treatment with bleach will produce excellent initial activity, but the biofilm can regrow very quickly on the surfaces.

Chlorine dioxide shows a very strong effect on the biofilm killing ratio and on removal. Total kill has been observed for concentrations as low as 138 ppm. This could be explained by the fact that chlorine dioxide is a gas and could diffuse more readily in the matrix, accessing the bacteria more easily. Microscopy also shows a good removal of the biofilm at low temperature. The major problem with chlorine dioxide stems from its instability. It has to be produced at the same time and place where the treatment is done. This might be conceivable for large open spaces, but not for specific applications like hospital rooms.

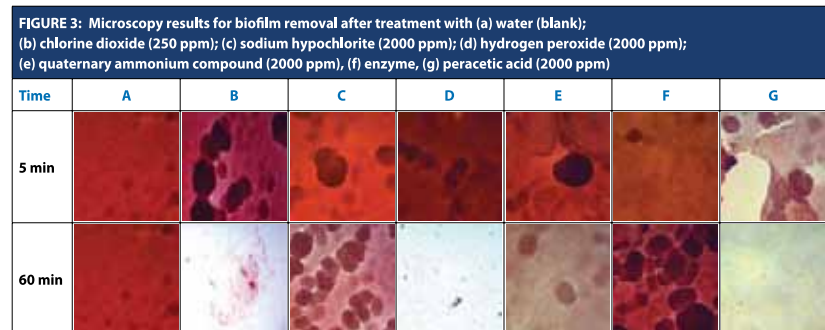
Quat has shown a negligible effect on both killing and removal. Even if a reduction has been observed at 2000 ppm, efficacy is very low. This can be explained by the limited diffusion of Quat throughout the matrix, explaining the increase of log reduction with a prolonged time. This limited diffusion is due to the size and charge of the Quat. Quat is also unable to remove biofilm from the surface. A possible explanation for this is that positively charged quats create a layer on the negatively charged biofilm, leading to a repulsive interaction with other quat molecules.

The enzyme mix used showed no effect on antibacterial activity against biofilm. This result is not surprising since it is common knowledge that these kinds of enzymes are not harmful to bacteria [16]. However, microscopy reveals that the biofilm is completely removed from the surface of the coupon. We can therefore presume that the enzyme action will solely be on digesting the EPS matrix, liberating the bacteria in the liquid. Combining enzymes with a common sanitizer could do a good job of eradicating biofilm from a surface, as long as the disinfecting agent does not attack the enzymes.

Peracetic acid is the last product tested in this study. Results show that peracetic acid is very good at diffusing within the biofilm and killing bacteria in it. We observed a total kill of bacteria at 900 ppm of peracetic acid. Moreover, we noted that adding surfactant to the PAA allows us to reduce the concentration of PAA to 600 ppm. This is explained by the lowering of the solution's interfacial tension, which allows for better diffusion of the product in the biofilm. Microscopy also shows that the surfactant is effective at removing more of the biofilm structure.

DISCUSSION

These results show that efficacy varies greatly depending on the technology used. However, there is a clear pattern. The more a molecule can diffuse into the biofilm, the higher the efficacy will be [17]. We have also noted that adding compounds, like a



surfactant, to the solution will help disinfecting products reach the bottom of the biofilm more efficiently, allowing them to attack the microorganisms before they acquire resistance [13, 18]. These results are highly significant when having to choose products that can efficiently clean a highly contaminated area where biofilm will be present.

To help validate the theory that limited diffusion impairs the efficacy of products, we have tested two different contact times as shown in Figure 2. For chlorine dioxide, we see that diffusion is too rapid in the biofilm and that there's no change in results over time, since a total kill was achieved in 5 min. For PAA, however, we see an increase in efficacy over time, with a total kill in 10 min. Quat and bleach at 2000 ppm also show a limited diffusion profile; efficacy increases between 5 and 60 min. Finally, enzymes and hydrogen peroxide show no change over time, revealing their low efficacy in killing bacteria within the biofilm.

CONCLUSION

Table 1 shows a summary of all the results obtained for the efficacy of common disinfectants on biofilm. As we can see, peracetic acid and chlorine dioxide seem to be best at killing bacteria within a biofilm, followed by bleach. These three compounds are composed of small molecules that can easily diffuse in the polysaccharide matrix. They are also recognized as having great efficacy on planktonic bacteria. On the other hand, molecules like quat are bigger and positively charged. These two characteristics will lead to difficulty in diffusing through the matrix.

REFERENCES

- Ofeik I, D.R., *Bacterial adhesion to cells and tissues*, ed. S. US. 1994, New York: Springer US.
- Donlan, R.M., *Biofilms: microbial life on surfaces*. Emerg Infect Dis, 2002. 8(9): p. 881-90.
- Stewart, P.S. and J.W. Costerton, *Antibiotic resistance of bacteria in biofilms*. Lancet, 2001. 358(9276): p. 135-8.
- Buckingham-Meyer, K., D.M. Goeres, and M.A. Hamilton, *Comparative evaluation of biofilm disinfectant efficacy tests*. Journal of Microbiological Methods, 2007. 70(2): p. 236-244.
- Donlan, R.M., *Biofilm formation: a clinically relevant microbiological process*. Clin Infect Dis, 2001. 33(8): p. 1387-92.
- Quignon, F., et al., *Poliovirus-1 inactivation and interaction with biofilm: a pilot-scale study*. applied and environmental microbiology, 1997. 63(3): p. 978-982.
- Skraber, S., et al., *Pathogenic viruses in drinking-water biofilms: a public health risk?* Biofilms, 2005. 2(2): p. 105-117.
- Chorianopoulos, N.G., et al., *Use of titanium dioxide (TiO₂) photocatalysts as alternative means for Listeria monocytogenes biofilm disinfection in food processing*. Food Microbiology, 2011. 28(1): p. 164-170.
- Cogan, N.G., *Two-Fluid Model of Biofilm Disinfection*. Bulletin of Mathematical Biology, 2008. 70(3): p. 800-819.
- F P Yu, G.A.M., *Physiological responses of bacteria in biofilms to disinfection*. applied and environmental microbiology, 1994. 60(7): p. 2462-2466.
- LeChevallier, M.W., C.D. Lowry, and R.G. Lee, *Disinfecting Biofilms in a Model Distribution System*. Journal (American Water Works Association), 1990. 82(7): p. 87-99.
- Stewart, P.S., et al., *Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates*. Journal of Applied Microbiology, 2001. 91(3): p. 525-532.
- Henoun Loukili, N., et al., *Effect of different stabilized preparations of peracetic acid on biofilm*. Journal of Hospital Infection, 2006. 63(1): p. 70-72.
- Belessi, C.-E.A., et al., *Efficiency of different sanitation methods on Listeria monocytogenes biofilms formed under various environmental conditions*. International Journal of Food Microbiology, 2011. 145: p. S46-S52.
- Toté, K., et al., *Evaluation of hydrogen peroxide-based disinfectants in a new resazurin microplate method for rapid efficacy testing of biocides*. Journal of Applied Microbiology, 2009. 107(2): p. 606-615.
- Johansen, C., P. Falholt, and L. Gram, *Enzymatic removal and disinfection of bacterial biofilms*. Applied and Environmental Microbiology, 1997. 63(9): p. 3724-8.
- Stewart, P.S., *Diffusion in Biofilms*. Journal of bacteriology, 2003. 185(5): p. 1485-1491.
- Cottenye, N., et al., *Interactions between non-phospholipid liposomes containing cetylpyridinium chloride and biofilms of Streptococcus mutans: modulation of the adhesion and of the biodistribution*. Biofouling, 2013. 29(7): p. 817-827.



TAKE CONTROL!

Ask for a demo in your facility



<http://www.sanimarc.com/bioassure/>