[24] Development of a Standardized Antibiofilm Test

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Introduction

Existing standard methods for evaluating biocides, disinfectants, or antibiotics are inadequate for analyzing the performance of antibiofilm products. Methods designed for testing antibiofilm treatments are needed because biofilm bacteria respond differently to antimicrobials compared to suspended bacteria.[1-5]

These differences include the following:

1. Biofilm bacteria may express different genes than do suspended bacteria. Some of these genes may express antimicrobial resistance.[6,7]
2. Antimicrobials may not fully penetrate a biofilm.[8,2]
3. Biofilms exhibit heterogeneity such as gradients in physiology where slower growing organisms may be more resistant to antimicrobials.[8-11]
4. Biofilms in the field may accumulate contaminants from the surrounding medium. These contaminants can present a demand on the antimicrobial.[10]

For the preceding reasons, current methods (based on suspended cultures) for evaluating antimicrobials may exaggerate performance of the treatment. In addition to determining the ability to kill bacteria, which is typically the end point of standard antimicrobial tests, biofilm tests may have other end points that are unique to biofilm, such as the following:

1. The application of an antibiofilm product may be designed to remove the biofilm rather than kill the bacteria.

[1] J. W. Costerton, K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie, *Annu. Rev. Microbiol.* **41,** 435 (1987).
[2] D. R. de Beer, R. Srinivasan, and P. S. Stewart, *Appl. Environ. Microbiol.* **60,** 4339 (1994).
[3] D. J. Hassett, J. G. Elkins, J.-F. Ma, and T. R. McDermott, *Methods Enzymol.* **310,** 599.
[4] C. T. Huang, F. P. Yu, G. A. McFeters, and P. S. Stewart, *Appl. Environ. Microbiol.* **61,** 2252 (1995).
[5] P. S. Stewart and J. B. Raquepas, *Chem. Eng. Sci.* **50,** 3099 (1995).
[6] J. G. Elkins, D. J. Hassett, P. S. Stewart, H. P. Schweizer, and T. R. McDermott, *Appl. Environ. Microbiol.* **65,** 4594 (1999).
[7] D. J. Hassett, J. G. Elkins, J.-F. Ma, and T. R. McDermott, *Methods Enzymol.* **310,** 599.
[8] J. W. Costerton, A. K. Camper, P. S. Stewart, N. Zelver, and M. E. Dirckx, *Analyst* **6,** 18 (1999).
[9] C.-T. Huang, P. S. Stewart, and G. A. McFeters, *in* "Digital Image Analysis of Microbes" (M. H. F. Wilkinson and F. Schut, eds.), p. 411. Wiley, New York, 1998.
[10] R. Srinivasan, P. S. Stewart, T. Griebe, and C.-I. Chen, *Biotechnol. Bioeng.* **46,** 553 (1995).
[11] P. S. Stewart, L. Grab, and J. A. Diemer, *J. Appl. Microbiol.* **85,** 495 (1998).

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2. The antibiofilm product may be designed to prevent regrowth or resuscitation of the bacteria. The heterogeneity of a natural biofilm provides a rich and diverse ecological community of bacteria. This heterogeneity allows microniches where bacteria can reside and resist the antimicrobial application. Following application of the antimicrobial, the resistant biofilm bacteria may exhibit rapid regrowth.[10]
3. An antibiofilm product may be designed to prevent the biofilm from initially colonizing a surface rather than killing an established biofilm.

In summarizing the above issues, standard antibiofilm methods must use relevant biofilm as the test sample. Depending on the manufacturers' claims for antibiofilm products, methods are also needed to evaluate (1) kill of the biofilm bacteria, (2) removal of biofilm slime layer, and (3) rate of biofilm regrowth following antibiofilm application.

In this paper, we use the rotating disk reactor protocol with sodium hypochlorite treatment as a model for developing a standard *tier-one* antibiofilm test. A tier-one test is a quick, inexpensive method that is relevant to generic biofilm conditions. Since the tier-one test does not represent a specific field application, it may be most applicable to general screening of antibiofilm products. The tier-one test may also be used as the starting point in developing a *tier-two* test. The tier-two test is a more stringent test designed to represent a particular field system, such as a cooling tower, a swimming pool, or a medical implant device. For tier-two tests, defining parameters of the field system such as water chemistry, water temperature, water filtration, and types of inoculum must be represented in the test apparatus.

The antibiofilm test demonstration uses the rotating disk reactor (RDR) system described previously by the authors.[12] The RDR, shown in Fig. 1, consists of a 1 liter glass beaker fitted with a drain spout. The bottom of the reactor contains a magnetically driven rotor with six 1.27 cm^2 biofilm test-surface coupons. The rotor is constructed from a star-head magnetic stir bar to which a Teflon and Viton rubber disk is attached that holds the coupons. The coupons rotate continuously to provide fluid shear and mixing. Biofilm growth nutrients are continuously pumped into the reactor.

Continuous Stirred Tank Reactor Chemostat Approach to Growing Biofilm

The RDR is operated as a continuous stirred tank reactor (CSTR) chemostat. As a chemostat, the RDR is a perfectly mixed environment with continuous feed

[12] N. Zelver, M. Hamilton, B. Pitts, D. Goeres, D. Walker, P. Sturman, and J. Heersink, *Meth. Enzymol.* **310,** 608.

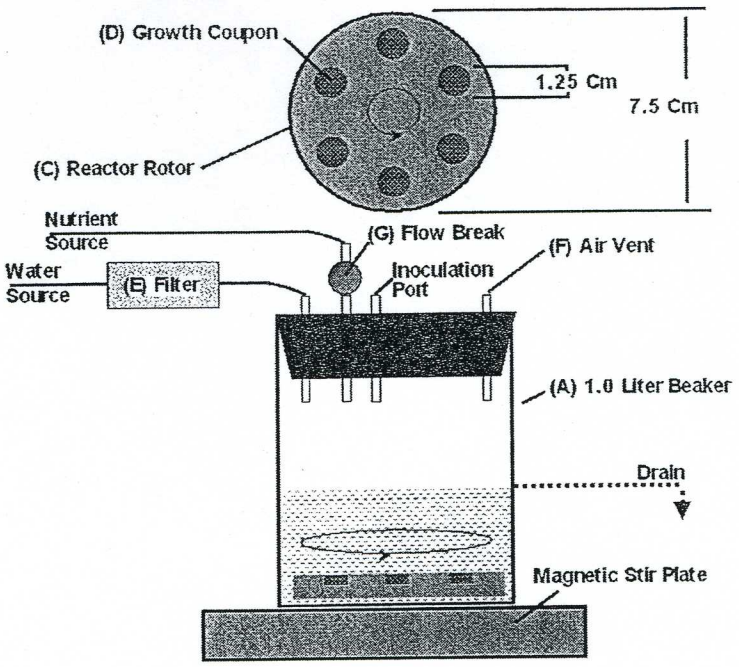

FIG. 1. Rotating disk reactor (RDR) for laboratory biofilm assays.

rate matching the effluent rate. The rate at which the nutrients are added to a chemostat is referred to as the dilution rate.

Controlling the dilution rate controls the rate of bacterial growth. Above a critical dilution rate, when nutrients are flowing through the reactor faster than the bacteria can divide, suspended bacteria are flushed out of the reactor. Operating a chemostat beyond the critical dilution rate selects for biofilm growth since suspended organisms are not available to compete for the nutrients.[13] The RDR is a biofilm growth reactor because it is operated at a high dilution rate. The RDR is operated until a steady-state biofilm bacterial cell density is reached (biofilm growth equals biofilm sloughing). The steady-state assumption provides a repeatable model biofilm.

Impact of Inoculation of the RDR on Repeatability

In the protocol presented previously by the authors, the RDR was inoculated at the beginning of an experiment by injecting a thawed culture of test organisms into the reactor and then operating the RDR in batch mode for 24 hr to establish a biofilm. Subsequently, we found this inoculation procedure to be a source of variability and we established a more repeatable inoculation protocol where the

[13] W. G. Characklis, *in* "Biofilms" (W. G. Characklis and K. C. Marshall, eds.), p. 37. John Wiley and Sons, New York, 1990.

thawed organisms are cultured to a known bacterial cell density prior to inoculation of the RDR. In this new protocol, a colony of *Pseudomonas aeruginosa (ERC-1)* is inoculated into a flask containing 100 ml of sterile tryptic soy broth (TSB) at 300 mg/liter. The flask is incubated in an orbital mixer at 35° for a maximum of 24 hr to attain a viable bacterial cell density of $\sim 10^8$ CFU/ml. The inoculation culture is serially diluted and plated to ensure that the cell bacterial cell density is correct for each RDR test. We believe the new inoculation procedure is a microbiological improvement because of the following reasons:

1. The initial method of injecting frozen inoculum was associated with additional nutrients being added from nutrients in the inoculum.
2. The new inoculation method allows the organisms to be acclimated to the type of environment found in the RDR so that the organisms are not shocked during inoculation.
3. The new inoculation method allows the technician to check for a pure culture when inoculating.

Biofilm Sampling and Enumeration in the RDR

Biofilm samples from the RDR are obtained by aseptically removing the test coupon from the rotor and removing the biofilm with a sterile wooden applicator stick. The stick is stirred vigorously into a test tube containing 9 ml of sterile buffered water. The entire coupon surface is scraped approximately three times for 1–2 min. The coupon is rinsed with 1 ml of sterile buffered water into the original 9 ml, bringing the final volume of the tube to 10 ml. Prior to enumeration, the cells are disaggregated by homogenization at a speed of 20,500 rpm for 30 sec to eliminate biofilm clumps.

Alternatively, a Teflon scraper can be used to remove the biofilm. Figure 2 shows comparisons of RDR biofilm coupons following the 24 hr CSTR growth phase. Figure 2a shows a coupon prior to sampling, Fig. 2b shows a coupon following sampling using a wooden scraper, and Fig. 2c shows a coupon following sampling using a Teflon scraper. With the wooden scraper, we measured biofilm (untreated) on 13 coupons sampled from the RDR and obtained a standard deviation of 0.47. With the Teflon scraper, we measured biofilm (untreated) on seven coupons sampled from the RDR and obtained a standard deviation of 0.57.

Enumeration of the biofilm bacteria is achieved using standard microbiological practices. Samples are diluted and plated using a modification of the spread plate method called the drop plate method.[12,14] If a mixed culture was used, viable cell enumeration would occur via the spread plate method.[15]

[14] A. A. Miles and S. S. Misra, *J. Hygiene* **38,** 732 (1938).

[15] A. L. Koch, *in* "Methods for General and Molecular Bacteriology" (P. Gerhardt, ed.), p. 255. ASM Press, Washington, D.C., 1994.

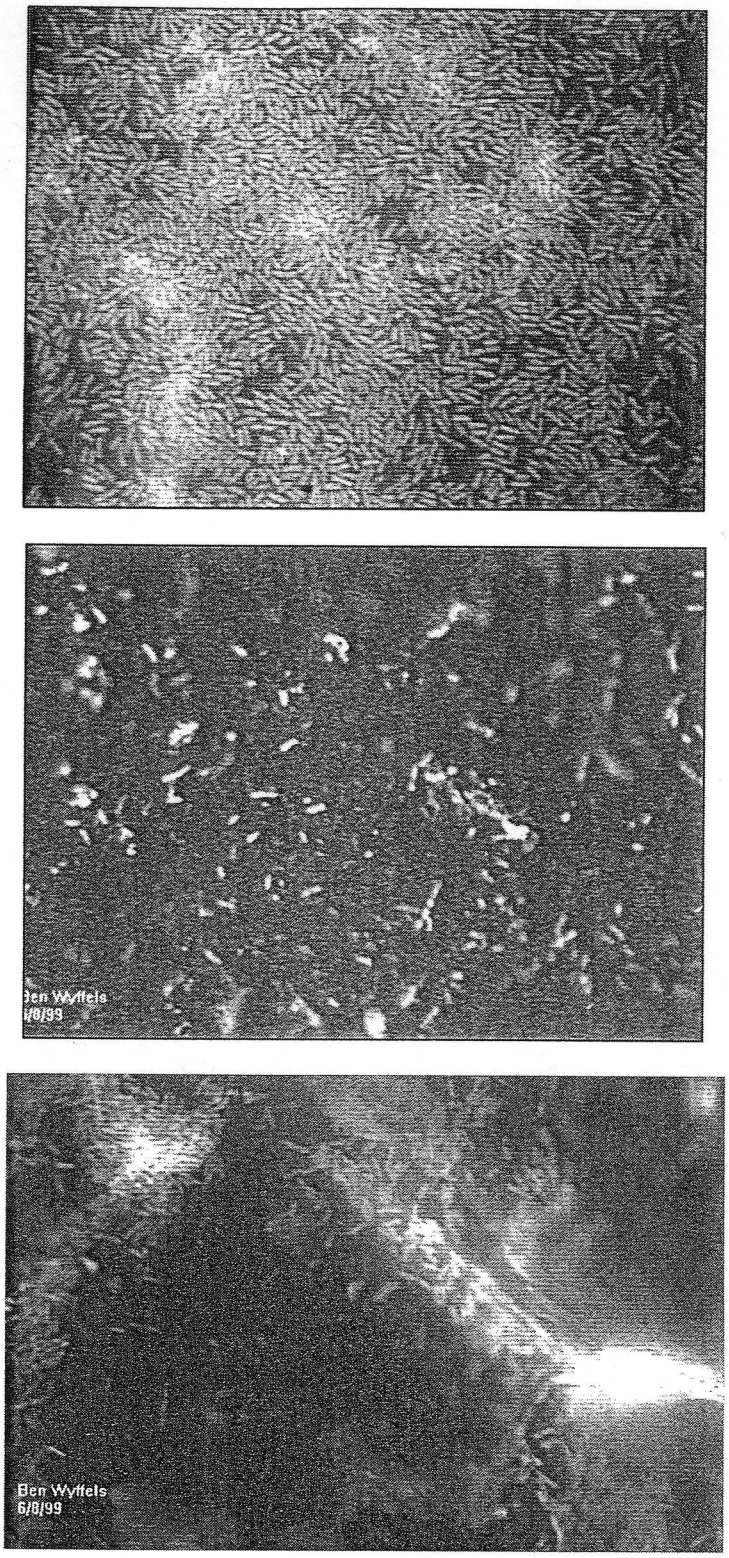

FIG. 2. Digital epifluorescent images of biofilm samples grown in the RDR and stained with DA
(1000× magnification).

Treatment Protocol using Sodium Hypochlorite as a Model Antibiofilm Treatment

To demonstrate the ability of the RDR to test the efficacy of disinfectants on biofilm, we conducted a series of tests to determine the log reduction in biofilm viable bacterial cell density resulting from a 10 min treatment of 5 mg/liter total chlorine added as NaOCl (bleach). Chlorine was chosen as the model antibiofilm treatment since the efficacy of the biocide has been previously studied on biofilm, chlorine water chemistry is understood, and a standard method exists for measuring bulk fluid concentrations.[2,8,11,16–18] Sodium thiosulfate will neutralize chlorine, and neutralization is required so that the biocide reaction does not continue following the applied dosing time.

Chlorine is a nondiscriminating oxidizing agent; therefore, the protocol includes a rinse step to help reduce the chlorine demand in the reactor prior to the treatment. The form in which chlorine is used in the treatment is important because various types of chlorines affect the water chemistry differently.

In this protocol, the chlorine is added as NaOCl, which will increase the alkalinity of the bulk water to the extent that the OCl^- reacts with the H_2O.[16] A change in the alkalinity is important because at pH < 7.5 HOCl dominates and at pH > 7.5 OCl^- dominates. HOCl is known to have the greater efficacy. In the protocol, the bulk water is buffered with K_2HPO_4 and KH_2PO_4 to a pH of 7.2.

The following treatment protocol was used to evaluate the performance of chlorine in killing biofilm organisms using the RDR system as described previously by the authors.[12] For the treatment protocol, two parallel RDRs are set up such that one is used as a control with no treatment and the other receives the chlorine treatment.

Setup of RDR Reactor Vessels

1. Assemble two sterile RDR reactors in parallel. Attach a "Y" joint to the water filter on the effluent end so both reactors can be run off of the same filter and pump. Adjust the flow rate to 16 ml/min.
2. Set up a single carboy for nutrient supply to both RDRs.
3. Set up separately pumped nutrient lines to each RDR.
4. Set flow rate for nutrients at 1 ml/min.

Batch Nutrients Preparation

The 24-hr batch phase is used to establish the biofilm.

1. Mix 0.15 g TSB, 3.5 g K_2HPO_4, and 1.0 g KH_2PO_4 in 500 ml reverse osmosis (RO) water for each reactor. K_2HPO_4 and KH_2PO_4 added to buffer the

[16] V. L. Snoeyink and D. Jenkins, "Water Chemistry." Wiley, New York, 1980.

[17] A. Camper, M. Burr, B. Ellis, P. Butterfield, and C. Abernathy, *J. Appl. Microbiol. Symposium Suppl.* **85,** 1S (1999).

solution are at the following concentrations: 7 g/liter K_2HPO_4 and 2 g/liter KH_2PO_4.

2. Check pH and adjust to 7.2.
3. Autoclave on liquid setting for 25 minutes.

CSTR Nutrients

These nutrients are used during the 24-hr CSTR growth period following batch operation. Buffer and nutrient concentrations are determined using Eq. (1):

$$C_1 \times Q_1 = C_2 \times Q_2 \tag{1}$$

(Q, flow rate; C, concentration; 1, influent; 2, effluent)

Using a 4 liter carboy, the following calculations are made:

$$\begin{aligned} C_1 \times (1 \text{ ml/min}) &= (30 \text{ mg/liter TSB}) \times (17.0 \text{ ml/min}) \\ &= 510 \text{ mg/liter TSB} \\ &= (2040 \text{ mg/liter TSB}) \times (1 \text{ g}/1000 \text{ mg}) \\ &= 2 \text{ g TSB added to 4 liter RO water} \end{aligned}$$

$$\begin{aligned} K_2HPO_4 \text{ buffer: } C_1 \times (1 \text{ ml/min}) &= (7 \text{ g/liter } K_2HPO_4) \times (17 \text{ ml/min}) \\ &= (119 \text{ g/liter } K_2HPO_4) \times (4 \text{ liter water}) \\ &= 476 \text{ g } K_2HPO_4 \text{ added to 4 liters RO water} \end{aligned}$$

$$\begin{aligned} KH_2PO_4 \text{ buffer: } C_1 \times (1 \text{ ml/min}) &= (2 \text{ g } KH_2PO_4) \times (17 \text{ ml/min}) \\ &= (34 \text{ g/liter } KH_2PO_4) \times (4 \text{ liter water}) \\ &= 136 \text{ g } KH_2PO_4 \text{ added to 4 liters RO water} \end{aligned}$$

1. Add 3 liters RO water and a stir bar to a 4 liter carboy.
2. Add 1 liter RO water to a 2000 ml Erlenmeyer flask and mix the calculated amounts of TSB and buffering agents until dissolved and autoclave for 20 min on liquid setting. Because TSB caramelizes, the concentrated buffers and nutrients are autoclaved separately.
3. Autoclave the 4 liter carboy for 90 min on liquid setting.
4. Aseptically pour contents of the flask into the carboy.

Rinse Water

Rinse water is used to flush any demand on chlorine out of the RDR following the CSTR operation. The rinse water is buffered at the same concentrations as the nutrients (i.e., 7 g/liter K_2HPO_4 and 2 g/liter KH_2PO_4).

[18] G. A. McFeters, F. P. Yu, B. H. Pyle, and P. S. Stewart, *J. Indust. Microbiol.* **15,** 333 (1995).

Calculations.

$$7 \text{ g/liter} \times 10 \text{ liters RO water} = 70 \text{ g } K_2HPO_4$$

$$2 \text{ g/liter} \times 10 \text{ liter RO water} = 20 \text{ g } KH_2PO_4$$

1. Add 10 liters RO water to a large carboy.
2. Mix the buffering agents until dissolved.
3. Foil and tape tubing attached to the carboy.
4. Autoclave for 90 min on liquid setting.

Validating Chlorine Treatment Concentration

It is necessary to determine the total chlorine concentration of the NaOCl solution before treating the reactor because of the instability of NaOCl. Chlorine demand-free glass and water are used.

1. Using the DPD colorimetric method documented in *Standard Methods for the Examination of Water and Waste Water,* determine the actual concentration of total chlorine.[19]
2. Use the above determined concentration of total chlorine to calculate the volume of NaOCl needed to dose the reactor at 5 mg/liter total chlorine. For example, if the concentration of total chlorine in the NaOCl equals 69,920 mg/liter, use the relationship in Eq. (2),

$$C_1 \times V_1 = C_2 \times V_2 \tag{2}$$

to calculate the volume of NaOCl required to achieve a bulk fluid concentration of 5 mg/liter total chlorine in the reactor.

$$(69{,}920 \text{ mg/liter total chlorine}) \times V_1 = (5 \text{ mg/liter total chlorine}) \times (500 \text{ ml}) = 0.036 \text{ ml} = 36 \text{ } \mu\text{l NaOCl}.$$

Thus, the reactor is dosed with 5 mg/liter total chlorine by adding 36 μl NaOCl to the bulk fluid.

Preparing Sodium Thiosulfate Neutralization Solution

Note: 1 ml of a 2.1 g/liter sodium thiosulfate solution neutralizes 0.3 mg/liter solution of chlorine.

[19] Anonymous, *in* "AHPA, Standard Methods for the Examination of Water and Wastewater" (A. D. Eaton, L. S. Clesceri, and A. E. Greenberg, eds.), 19th ed., pp. 4–45. AHPA, ANWA, REF, Washington, D.C., 1995.

Calculations

0.3 mg/liter Cl/2.1 mg/liter sodium thiosulfate = 0.14 mg Cl/1.0 mg sodium thiosulfate

For this study, 500 ml of 5 mg/liter total chlorine must be neutralized.

(5 mg Cl/liter) × (1 mg sodium thiosulfate/0.14 mg Cl) = 35.71 mg/liter sodium thiosulfate solution is required to neutralize 5 mg/liter total chlorine.

Test the solution to ensure that it has been properly neutralized.

Conducting the Antibiofilm Test

1. Operate the RDR reactors for 24 hr in batch, followed by 24 hr in CSTR operation (48 residence times).
2. After the 24 hr of CSTR operation, turn off the nutrients and dilution water and connect the rinse water to the dilution water pump. Run the rinse water through the reactors for six residence times (to speed up this process, the flow rate on the pump can be increased to 32 ml/min).
3. Turn the pumps off.
4. Extract 5 ml of the bulk water from each reactor and analyze pH.
5. Clamp off the effluent tubing on each reactor.
6. Add 5 mg/liter total chlorine to the RDR reactor designated for treatment.
7. Immediately collect 5 ml samples from each reactor and measure chlorine concentration.
8. Treat the reactors for 10 min.
9. After 10 min, collect samples from each reactor and measure the chlorine concentration.
10. Neutralize both reactors with 1 ml sodium thiosulfate solution for 2 min.
11. Ensure that the reactors are neutralized by collecting samples from each reactor and analyzing for chlorine.
12. Scrape three randomly chosen coupons from each reactor using the protocol described previously by the authors.[12]
13. Plate samples on R2A agar plates using the drop plate method.
14. Samples incubate for 17–20 hr in 35° incubator.
15. Enumerate the colony forming units (CFU) following incubation, and calculate the number of cfu/cm^2.
16. Calculate the log reduction associated with the treatment (see the next section for details on the calculation).

Repeatability of the Rotating Disk Reactor Tier-One Antibiofilm Test Protocol

Performance of the antibiofilm agent is expressed as a log reduction (LR) value comparing measurements on both antimicrobially treated and untreated biofilm in the same test. After repeated experiments, one can use the variability of the LR values as the basis for determining repeatability; specifically, one can calculate a repeatability standard deviation. An experimental method that produces a small repeatability standard deviation has good repeatability, and a big standard deviation indicates poor repeatability. One difficulty with this approach is that there is no generally accepted cutoff value for the repeatability standard deviation that separates repeatable from nonrepeatable methods.

In this paper, we will follow the terminology of the Association of Official Analytical Chemists—International.[20] The term "repeatability" standard deviation is used for the case where the replicates of the same experiment are all done within one laboratory. The term "reproducibility" standard deviation is used when the replicates are done in different laboratories. The next section shows how to calculate the LR, the standard error of the LR, and the repeatability standard deviation. For illustrative purposes, we apply these techniques to data from the RDR tier-one Antibiofilm Test Protocol using the sodium hypochlorite protocol described in the previous section of this paper.

Calculating the Log Reduction and the Standard Error: Formulas and Examples

Following the RDR antibiofilm test protocol, CFU counts are recorded for control (untreated) coupons and for test (treated with an antimicrobial chemical) coupons. The steps in calculating the log reduction (LR) are as follows:

1. Find the bacterial cell density (CFU/cm^2) for each coupon.
2. Find the mean of the $\log_{10}$ densities for control coupons and the mean of the $\log_{10}$ densities for the treated coupons.
3. Calculate the LR by subtracting the mean log densities for treated coupons from the mean log densities for control coupons. The standard error (SE) formula requires calculation of the control coupon and RDR biofilm coupon variances of log densities, as well as the means.

Calculating the Bacterial Cell Density for Each Coupon. For each dilution in a dilution series, CFU counts are recorded for each "location," where a location is a drop for the drop plate method. The counts need to be scaled up according to the volume represented by the location to provide an estimate of the total viable cells

[20] Anonymous, *in* "Official Methods of the Association of Official Analytical Chemists" (K. Helrich, ed.), p. 681. AOAC, Arlington, VA, 1990.

in the beaker, then converted to the number of colony forming units per square cm of the coupon, called the bacterial cell density. The appropriate formula is given by Eq. (3)[21–23]:

$$\text{Density} = \frac{\text{Avg count}}{\text{Volume Plated}} \times \frac{1}{\text{Dilution}} \times \text{Beaker Volume} \times \frac{1}{\text{Surface area}} \quad (3)$$

where

Avg count = average CFU across for a dilution (CFU),
Volume plated = volume plated for each raw data counting location (ml),
Dilution = the 10^{-k}(for 10-fold dilutions), where k is an integer (no units),
Beaker Volume = volume of liquid containing the biofilm removed from the coupon (ml),
Surface area = surface area of the coupon from which the biofilm was removed (cm^2)

Note:

$$\frac{\text{Volume Plated} \times \text{Dilution}}{\text{Beaker Volume}} = \text{fraction of the beaker volume for each raw data count}$$

Example of a Bacterial Cell Density Calculation. An RDR coupon biofilm sample was scraped into a beaker volume of 10 ml; the surface area scraped from the coupon was 1.267 cm^2. The CFUs were counted in each of 10 drop locations using the drop plate method with an individual drop volume of 0.01 ml. At the 10^{-3} dilution, the CFU counts were 27, 32, 22, 22, 25, 21, 14, 24, 17, and 13. The average of these counts is 21.7. Then

$$\text{Density} = \frac{21.7}{0.01} \times \frac{1}{10^{-3}} \times 10 \times \frac{1}{1.267} = 1.71 \times 10^7 \frac{\text{CFU}}{\text{cm}^2}$$

and $\log_{10}$ density $= 7.234$.

Calculating LR and SE. Let N denote the number of control coupons and M denote the number of treated coupons for which densities have been calculate. The estimated LR for the treatment is to be based on these $N + M$ coupons. Take the $\log_{10}$ transformation of each of the $N + M$ densities. Denote the control coupon $\log_{10}$ densities by $C_1, C_2, \ldots, C_N$, and denote the treated coupon $\log_{10}$ densities

[21] B. Jarvis, "Statistical Aspects of the Microbiological Analysis of Foods." Elsevier, New York, 1989.
[22] F. J. Farmiloe, S. J. Cornford, J. B. M. Coppock, and M. Ingram, *J. Sci. Food Agric.* **5**, 292 (1954).
[23] S. Niemelä, *in* "Statistical Evaluation of Results from Quantitative Microbiological Examinations," 2nd ed. Nordic Committee on Food Analysis, Uppsala, Sweden, 1983.

by $T_1, T_2, \ldots, T_M$. Calculate $\bar{C}$, the mean of the log densities for control coupons, and $\bar{T}$, the mean of the log densities for RDR biofilm coupons, using Eq. (4):

$$\bar{C} = \frac{C_1 + C_2 + \cdots + C_N}{N}; \quad \bar{T} = \frac{T_1 + T_2 + \cdots + T_M}{M} \tag{4}$$

The variances of the log densities are needed to calculate the SE. Calculate S_C^2, the variance of the log densities for control coupons, and S_T^2, the variance of the log densities for treated coupons, using Eq. (5):

$$\begin{aligned} S_C^2 &= \frac{(C_1 - \bar{C})^2 + (C_2 - \bar{C})^2 + \cdots + (C_N - \bar{C})^2}{N - 1}; \\ S_T^2 &= \frac{(T_1 - \bar{T})^2 + (T_2 - \bar{T})^2 + \cdots + (T_M - \bar{T})^2}{M - 1} \end{aligned} \tag{5}$$

Calculating the LR and the Associated SE. Calculate the log reduction (LR) using Eq. (6)[24]:

$$\mathrm{LR} = \bar{C} - \bar{T} \tag{6}$$

The standard error of LR depends on the variances among the control and treated biofilm coupons. Calculate the SE using Eq. (7)[24]:

$$\mathrm{SE} = \sqrt{\frac{S_C^2}{N} + \frac{S_T^2}{M}} \tag{7}$$

This SE formula provides a measure of the inherent, within-test variability, due to the variability among coupons and the variability associated with forming dilutions, plating, and counting CFUs.

Example of LR and SE Calculations. Calculations will be demonstrated using observed $\log_{10}$ densities of 7.79934, 7.23045, and 7.86332 for N = 3 control coupons and 4.41497, 4.85733, and 3.98677 for M = 3 treated coupons. The means of the log densities are $\bar{C} = 7.63104$ and $\bar{T} = 4.41969$. The variances are $S_C^2 = 0.12138$ and $S_T^2 = 0.18749$. Here, as is usual in antibiofilm tests, the variance for treated coupon log densities is larger than the variance for control coupon log densities. The main results are

$$\mathrm{LR} = 7.63104 - 4.41970 = 3.21134 \quad \text{and}$$

$$\mathrm{SE} = \sqrt{\frac{0.12138}{3} + \frac{0.18749}{3}} = 0.32790$$

It is advisable to carry extra digits during intermediate calculations, then use 1 or 2 decimal places for the final answer; e.g., LR $\pm$ SE = 3.2 $\pm$ 0.3 = the interval (2.9, 3.5).

[24] T. A. DeVries and M. A. Hamilton, *Quant. Microbiol.* **1**, 29 (1999).

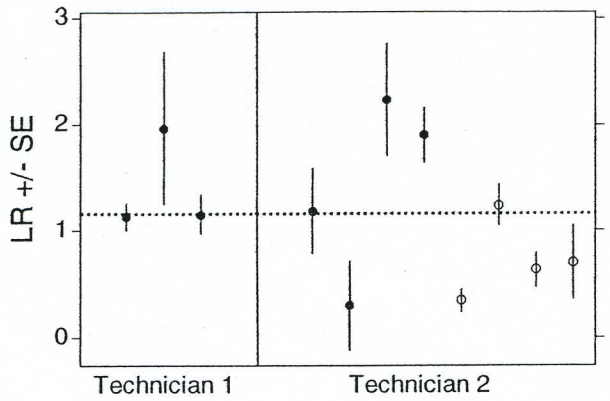

FIG. 3. Log reduction (LR) values for 5 mg/liter chlorine administered for 10 min to biofilms grown on coupons in the rotating disk reactor. Technician 1 conducted 3 experiments and Technician 2 conducted 8 experiments; the vertical line separates the results. Each LR is based on 3 control coupons and 3 treated coupons. The biofilms were grown in either a 500 ml fluid volume (closed circles) or a 200 ml fluid volume (open circles). Over all 11 experiments, the mean LR is 1.16 (horizontal dotted line) and the standard deviation is 0.66. The error bars show the within-experiment standard error (SE).

Repeatability Standard Deviations

The repeatability of an antimicrobial test will depend on the test protocol, the species of organism, and the disinfectant. A literature survey[25] of standard antimicrobial suspension and dried surface tests showed that the repeatability standard deviation of LR values varied between 0.2 and 1.2 with a median repeatability standard deviation of 0.5. It is important to check the repeatability standard deviation of antibiofilm testing protocols. For this reason the LR values for a 5 min application of 5 mg/liter sodium hypochlorite were measured on separate occasions using the RDR protocol (Fig. 3). Figure 3 shows the LR $\pm$ SE for 11 separate repeats of the test. The repeatability standard deviation of the LR values was 0.66. Because two technicians conducted the test, it was possible to conduct a random effects analysis of variance and estimate the three sources of variability that make up the repeatability standard deviation (i) within assay, (ii) among assays within a technician, and (iii) among technicians. On a percentage basis, the variation among repeated RDR tests can be attributed 30% to within-assay variability, 70% to variability among assays within a technician, and 0% to variability among technicians. The lack of variability between the technicians suggests that the RDR protocol can be learned and applied. The relatively large variability among assays within a technician suggests there are factors in the experimental technique that vary when the RDR is set up and run on different days, and that if the method is to be improved, it will be important to find and control those factors. Note, however, that the RDR

[25] N. Tilt and M. A. Hamilton, *J. Assoc. Official Anal. Chem.* **82,** 384 (1999).

protocol is as repeatable as the typical standard suspension or dried surface test, and for that reason, it appears to be sufficiently repeatable for routine use.

The literature survey[25] of standard antimicrobial suspension and dried surface tests also showed that the reproducibility standard deviation of LR values varied between 0.3 and 1.5 with a median reproducibility standard deviation of 0.9. The reproducibility standard deviation is made up of (i) the within-lab variation as measured by the repeatability standard deviation and (ii) lab-to-lab variation. Typically, there was a 50–50% split between these two sources. We have not yet collaborated with other laboratories in round robin testing of disinfectants with the RDR protocol, so we do not know either the lab-to-lab variance or the reproducibility standard deviation for the RDR.

Conclusions

Current tests for evaluating antimicrobials were developed for evaluating the efficacy of antimicrobials on suspended bacteria. Because biofilms have unique characteristics and can be more resistant than suspended bacteria, new and more rigorous test methods must be developed. Methods for evaluating antibiofilm tests must be conducted using relevant biofilm that represents the genetic, transport, and heterogeneity antimicrobial resistance properties of biofilm. Depending on the antibiofilm product claim being tested, the antibiofilm test may need to determine kill, removal, or prevention of regrowth of a biofilm. All of these considerations result in the practitioner having to make more decisions when developing or applying tests for evaluating antibiofilm tests. Additional considerations include whether the test should use a CSTR chemostat approach and how the biofilm organisms are sampled and enumerated.

Because an antibiofilm test is more complicated than traditional antimicrobial tests on suspended bacteria, we expect it will be difficult to develop antibiofilm tests with adequate quality control such as repeatability. The authors have demonstrated the use of a simple rotating disk reactor (RDR) chemostat in evaluating hypochlorite as a model biocide for killing biofilm bacteria. Preliminary work presented by the authors shows that it is possible to achieve an acceptable repeatability of log reduction values for the RDR biofilm test system using NaOCl.

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