Quantitative Microbiology 1 (1999): 29–45

Estimating the Antimicrobial Log Reduction: Part 1. Quantitative Assays

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Received July 13, 1998; Accepted February 24, 1999

Abstract. In quantitative antimicrobial assays, the responses are counts of viable microbes in two treatment groups. One group is treated with a chemical germicide and the other group is control, treated with an inactive chemical. This is part 1 of a pair of papers that pertain to assays that estimate the log reduction (LR), in the density of viable microbes, attributable to the germicide treatment (part 2 is concerned with presence/absence responses). Such assays are used by producers, consumers, and regulatory agencies to assess the efficacy of liquid germicides. We define and compare the two different mathematical formulations for LR that are commonly used in practice when there are replicate density measurements. One LR parameter is based on the mean of the log-transformed densities; the other is based on the logarithm of the mean of densities. We build a statistical model relating microbial count data to the LR parameters, derive maximum likelihood and method of moments estimators for each LR parameter, and compare the estimators according to both their asymptotic characteristics and the results of a simulation study utilizing realistic sample sizes. Standard error formulas for the estimators are derived, and they are evaluated via simulation studies. The results of this investigation lead us to recommend the method of moments estimator, regardless of which definition of LR is chosen.

Key words: antimicrobial, bioassay, count data, disinfectant, log reduction, statistical models

1. Introduction

Laboratory assays are used to assess the ability of liquid chemicals to kill pathogenic microorganisms, such as bacteria, spores, fungi, and viruses, that contaminate hard, nonporous surfaces in hospitals, restaurants, the home, etc. Each standard assay is conducted according to a protocol that clearly specifies the microbial species and strain, inoculum sizes, culture conditions, testing materials, and the carriers. The *carriers* are small objects constructed of glass, stainless steel, or other nonporous material, designed for ease of handling in the laboratory. They are inoculated with microorganisms to create laboratory surrogates for contaminated surfaces. The standardized carrier assays are designed to provide an inexpensive and reproducible laboratory assessment of each disinfectant's activity under conditions similar to real world applications.

Governmental regulatory agencies review data from antimicrobial assays when deciding whether a chemical formulation is sufficiently effective to be registered as a germicide.

When submitted to a laboratory antimicrobial assay, the chemical must pass the established performance standard in order to be registered for consumer usage. For this reason, such assays are often called antimicrobial tests. Laboratory assays are used for purposes other than registration, e.g., by industry for in-house screening during product development and by consumers for evaluating competing products.

In the past few years, microbiologists have made a concerted effort to develop new standardized antimicrobial assays that are intended to be more reproducible than the conventional assays (Sattar and Springthorpe, 1994; Sattar, 1997). The new carrier assays are designed to yield a quantitative measure of efficacy, in contrast to the conventional assays that produce only qualitative pass/fail conclusions. The new assays utilize multiple test carriers (exposed to the disinfectant) and multiple control carriers (exposed only to a non-germicidal liquid). Although different test species require different laboratory methods, the protocol for an assay typically follows the seven generic steps listed below.

Step 1. Prepare a culture of the selected species and strain of microorganism, and dilute it so that the microbes are at the density specified, and in a well-mixed suspension. Organic culture media, and possibly added organic soil (e.g., bovine blood), are present in the suspension.

Step 2. Select a sterilized carrier. For purposes of this description think of the carrier as a glass disk or the bottom of a flat-bottomed test tube.

Step 3. Inoculate the carrier in a prescribed way; e.g., pipette a specified volume from the suspension onto the carrier.

Step 4. Dry the inoculated carrier as described in the protocol. The microorganisms are now embedded in a protective, dry, organic film attached to the carrier. After drying, the targeted inoculum size ranges between 10^5 and 10^8 viable organisms, depending on the species.

Step 5t. Treat the test carrier by immersing it in the liquid disinfectant for a specified amount of time.

Step 5c. Treat the control carrier by immersing it in an inert liquid for the same amount of time as specified for a test carrier.

Step 6. Remove the disinfectant or liquid, or neutralize the disinfectant by chemical means, or remove the carrier and place it in a different vessel. Add appropriate media to the vessel that contains the carrier. Then sonicate, vortex, or scrape to remove organisms from the carrier into a well-mixed liquid suspension.

Step 7. Estimate the number of viable microorganisms per carrier using a specified microbiological technique. Usually, samples of the suspension are diluted to a known concentration and each sample is then plated or filtered, and incubated. During incubation, each viable organism divides many times, forming a single colony. Under magnification, the colonies can be counted and reported as counts of colony forming units (cfu). The dilution levels for each sample are recorded along with the associated cfu counts so that one can calculate an estimate of the number of viable organisms removed on each carrier after step 6.

As with all well-designed experiments, the new antimicrobial assays include control, replication, and randomization. Note that steps 2 through 7 are conducted on each carrier in the study, and that step 5 has two versions, one pertaining to test carriers (5t) and one pertaining to control carriers (5c). Control carriers provide information about the density of viable organisms expected if the disinfectant were completely inactive. The counts of viable organisms for control carriers (step 7) provide an estimate of the number of organisms that survive experimental manipulations other than disinfection. The control densities are required to determine whether low test carrier counts are due to a strong disinfectant or to a weak disinfectant applied to carriers that hold few recoverable organisms. This fact is emphasized because most of the previously-used antimicrobial assays did not include control carriers.

Each of the new antimicrobial assays requires multiple carriers for the control and test treatments. A series of carriers are inoculated and dried according to steps 2–4, then some are (randomly) chosen to be test carriers and the remainder are control carriers. In practice, the allocation is usually not strictly random, but is systematic, such as using every fourth carrier in the series as a control carrier.

The assay is called *quantitative* because the response data are counts of viable organisms (quantitative data) for both control and test carriers. Tables 1 and 2 provide an example of the type of data arising from a quantitative assay with three control carriers and ten test carriers. In this example, the control carriers contain approximately 10^8 viable

Table 1. Example—control carrier data.

Carrier number	Dilution volume per plate	Plate counts	Total volume sampled	Total colony counts	Density	Log density
1	10^{-6}	(63, 60, 66)	3×10^{-6}	189	6.3×10^7	7.799
2	10^{-6}	(59, 65, 71)	3×10^{-6}	195	6.5×10^7	7.813
3	10^{-6}	(76, 79, 64)	3×10^{-6}	219	7.3×10^7	7.863

Table 2. Example—test carrier data.

Car. no.	Diln 1	Plate counts diln 1	Diln 2	Plate counts diln 2	Total vol. samp.	Total colony counts	Dens.	Log dens.
1	10^{-1}	(60, 91, 92)	10^{-2}	(6, 8, 10)	0.33	267	809.09	2.908
2	10^{-1}	(69, 104, 94)	10^{-2}	(7, 10, 7)	0.33	291	881.18	2.945
3	10^{-1}	(32, 37, 30)	10^{-2}	(0, 0, 0)	0.33	99	300.0	2.477
4	10^{-1}	(72, 88, 89)	10^{-2}	(7, 8, 6)	0.33	270	818.18	2.913
5	10^{-1}	(65, 150, 79)	10^{-2}	(5, 12, 7)	0.33	318	963.64	2.984
6	10^{-1}	(29, 26, 44)	10^{-2}	(1, 0, 2)	0.33	102	309.09	2.490
7	10^{-1}	(75, 91, 107)	10^{-2}	(5, 9, 10)	0.33	297	900.0	2.954
8	10^{-1}	(35, 47, 32)	10^{-2}	(4, 4, 4)	0.33	126	381.82	2.582
9	10^{-1}	(66, 77, 58)	10^{-2}	(6, 7, 8)	0.33	222	672.73	2.828
10	10^{-1}	(145, 138, 161)	10^{-2}	(14, 14, 17)	0.33	489	1481.82	3.171

organisms and the test carriers contain approximately 1000 viable organisms—the difference in numbers of viable organisms between control and test carriers is presumably due to the germicide. These data will be used later to illustrate calculations.

The counts from a quantitative assay can be used to estimate an efficacy parameter such as the log reduction (LR), where LR = log (number of organisms per carrier before exposure to disinfection) − log (number of organisms per carrier after exposure to disinfection). Note that log denotes the base 10 logarithm, and ln denotes the natural logarithm. The preceding definition of LR is impossible to apply directly, because one cannot count the organisms on a carrier both before and after disinfection. In practice, the LR must be based on a comparison of control carrier counts to test carrier counts. Let the control carrier cfu count and test carrier cfu count be denoted by c and t, respectively. Then the conventional calculation of LR, for the single control carrier and single test carrier case, is $\mathrm{LR} = \log(c) - \log(t)$ (Robison *et al*., 1988). This quantity is also known as the microbicidal effect (Bloomfield *et al*., 1994) or the sterility assurance level (Oxborrow and Berube, 1991).

The new antimicrobial assays require multiple control and test carriers, in which case no established definition of LR exists. In fact, two different formulas for LR coexist in microbiology for multiple carrier data, but with no apparent recognition that they are different. Various intuitive estimators have been used to estimate the LR, but no one has previously constructed statistical models for a quantitative antimicrobial assay, nor described the statistical properties of the estimators.

The goals of this paper are: to propose statistical models for the quantitative carrier assay, to present the two alternative definitions of LR, to discuss the advantages and disadvantages of each definition, to derive for each definition the method of moments (MOM) estimator and the maximum likelihood estimator (MLE), to discuss associated standard error formulas, to present the large sample (many carriers) efficiencies of the estimators, to describe the results of a computer simulation study conducted to elicit the properties of the estimators when based on just a few carriers, and to evaluate via simulation the formulas for the standard errors of the estimates. Although the motivation for this work is provided by antimicrobial assays, the models, parameters, and estimation methods presented here may well be applicable to other experiments in microbiology where treatment and control groups are to be compared based on viable cell counts in a dilution series.

In a companion paper, we discuss estimators of LR for quantal assays in which the number of viable organisms per carrier are estimated from presence/absence responses in a dilution series (DeVries and Hamilton, 1999). Some antimicrobial assays are partially quantitative and partially presence/absence. For example, in the Hard Surface Carrier Test, viable cell counts are observed for each control carrier, and each test carrier is classified as presence or absence, where presence indicates one or more bacteria on the test carrier survived the disinfection step (Hamilton and DeVries, 1996).

2. Statistical model

The observations recorded for a quantitative antimicrobial assay are the cfu counts, and associated dilutions, for the dilution series prepared at step 7. We present here a probability

model for the observable data. Some notation is required. Let A denote the number of control carriers and B denote the number of test carriers. Let D denote the number of dilution stages for control carriers, G denote the number of dilution stages for test carriers, and F denote the number of samples per dilution for control or test carriers. For the example data in Tables 1 and 2, $A = 3$ (no. control carriers), $B = 10$ (no. test carriers), $D = 1$ (use data at one dilution for control carriers), $G = 2$ (use data at two dilutions for test carriers), and $F = 3$ (samples per dilution).

At each sample at each dilution stage, a fraction of the total suspension volume is used. Let u_{ijk} denote that fraction of the carrier's suspension and C_{ijk} denote the associated cfu count for control carrier i at dilution stage j and sample k, $i = 1, \ldots, A$, $j = 1, \ldots, D$, and $k = 1, \ldots, F$. Let c_{ijk} denote the realized value of the random variable C_{ijk}. Similarly, let v_{smn} denote the fraction of the carrier's suspension and T_{smn} denote the associated cfu count for test carrier s at dilution stage m and sample n, $s = 1, \ldots, B$, $m = 1, \ldots, G$, and $n = 1, \ldots, F$. Let t_{smn} denote the realized value of the random variable T_{smn}.

Let $C_i = \sum_{j=1}^{D} \sum_{k=1}^{F} C_{ijk}$ be a random variable denoting the sum of microbial counts across dilutions and samples for control carrier i. Let c_i denote the realized value of C_i. Let the vector $\vec{c} = (c_1, \ldots, c_A)$ denote the realized c_i values. Let $u_i = \sum_{j=1}^{D} \sum_{k=1}^{F} u_{ijk}$ denote the fraction of total suspension volume in the dilution series for control carrier i. Let the vector $\vec{u} = (u_1, \ldots, u_A)$ denote the u_i values. Let X_i denote the usual estimator of the number of viable organisms on control carrier i; specifically, $X_i = C_i/u_i$, $i = 1, 2, \ldots, A$.

Let $T_s = \sum_{m=1}^{G} \sum_{n=1}^{F} T_{smn}$ be a random variable denoting the sum of microbial counts across dilutions and samples for test carrier s. Let the vector $\vec{t} = (t_1, \ldots, t_B)$ denote the realized T_s values. Let $v_s = \sum_{m=1}^{G} \sum_{n=1}^{F} v_{smn}$ denote the fraction of the total suspension volume in the dilution series for test carrier s. Let the vector $\vec{v} = (v_1, \ldots, v_B)$ denote the v_s values. Let Y_s denote the usual estimator of the number of viable organisms on test carrier s; specifically, $Y_s = T_s/v_s$, $s = 1, 2, \ldots, B$.

We will derive a plausible statistical model for the observable counts in steps that employ intermediate, unobservable random variables. Define the *recoverable count* on a carrier as the number of viable organisms removed into suspension (step 7) if the carrier were not exposed to a disinfectant. The recoverable count is not directly observable because control carriers contain too many microbes to count and test carriers have been exposed to a disinfectant.

Assumption 1. The (unobservable) recoverable counts for the A control carriers and the B test carriers, denoted by $\theta_i^{(C)}$, $i = 1, \ldots, A$ and $\theta_s^{(T)}$, $s = 1, \ldots, B$, respectively, are independent and identically distributed (iid) as gamma (mean $= \alpha\beta$, variance $= \alpha\beta^2$) random variables.

The recoverable count on a carrier is an integer-valued random variable that approximately follows a negative binomial distribution. The negative binomial distribution can be derived as a mixture of Poisson distributions (McCullagh and Nelder, 1989). The distribution of the inoculum counts is conceivably a mixture of Poisson distributions because the pipette volumes will vary, and, when a pipette volume is removed from a well-mixed suspension to inoculate a carrier (step 3), the number of organisms is a random variable following a Poisson distribution (Stearman, 1955). Differences among carriers in both the length and intensity of the drying (step 4) will also induce extra variability among

the numbers of organisms on the carriers. Similarly, inherent variability introduced at each subsequent step in the assay protocol will cause the recovery counts to have more variability than compatible with the Poisson distribution. Because the recoverable counts are typically large (between 10^5 and 10^8), it is appropriate to act as though the recoverable count is a continuous random variable, and the (continuous) gamma distribution closely approximates the (discrete) negative binomial distribution in these cases (Best and Gipps, 1974; Osaki and Li, 1988).

Assumption 2. The survivals of individual microbes on a randomly chosen test carrier are independent Bernoulli trials with common survival probability P which is a random variable that follows a distribution with mean ϕ and variance ζ^2. Also P and $\theta_s^{(T)}$, $s = 1, \ldots, B$, are statistically independent.

The quantity P is called the survival fraction and ϕ is the typical survival fraction. There are at least two reasons why the survival fraction might vary among test carriers. First, variation in the amounts of disinfectant added to the carriers and variation in the exposure period (step 5) could cause variable survival fractions. Second, the shape of the dried inoculum film could affect disinfection rates, with thicker films yielding lower disinfection rates (Stewart, 1996). In our experience, the variability among test carrier densities is usually larger than among control carriers, an observation consistent with Assumption 2. It is conceivable, however, that under ideal laboratory conditions, $P \equiv \phi$. We will use the term *variable survival fraction* to indicate the case where P has a positive variance, and the term *constant survival fraction* to indicate that $P \equiv \phi$.

Assumption 3. $(C_{ijk}|\theta_i^{(C)}, u_{ijk})$, $i = 1, \ldots, A$, $j = 1, \ldots, D$, $k = 1, \ldots, F$, are conditionally distributed independently as Poisson $(\theta_i^{(C)} \cdot u_{ijk})$.

Assumptions 1 and 3 imply that $(C_i|\theta_i^{(C)}, u_i)$, $i = 1, \ldots, A$, are conditionally distributed independently as Poisson $(\theta_i^{(C)} \cdot u_i)$, and the marginal distribution of C_i is negative binomial $(r = \alpha,\ p_i = 1/(\beta u_i + 1),\ q_i = \beta u_i/(\beta u_i + 1))$, $i = 1, \ldots, A$ (DeVries, 1997). Thus,

$$P(C_i = c_i|\alpha, \beta, u_i) = \frac{\Gamma(c_i + \alpha)}{\Gamma(\alpha)c_i!}\left(\frac{\beta u_i}{\beta u_i + 1}\right)^{c_i}\left(\frac{1}{\beta u_i + 1}\right)^{\alpha} \qquad c_i = 0, 1, 2, \ldots$$

The C_i are independent, and if the sample volumes, u_i, are constant, the C_i are identically distributed. We have chosen to parameterize the negative binomial distribution to have mean rq_i/p_i and variance rq_i/p_i^2. Thus, the mean and variance of the random variable C_i are $\alpha\beta u_i$ and $\alpha\beta u_i + (\alpha\beta u_i)^2/\alpha$, respectively. These results engender a model for $X_1, \ldots, X_A$, the estimated numbers of viable organisms on control carriers.

Assumption 4. $(T_{smn}|\theta_s^{(T)}, v_{smn}, P)$, $s = 1, \ldots, B$, $m = 1, \ldots, G$, $n = 1, \ldots, F$, are conditionally distributed independently as Poisson $(\theta_s^{(T)} \cdot v_{smn} \cdot P)$. Consequently, $(T_s|\theta_s^{(T)}, v_s, P)$, $s = 1, \ldots, B$, are conditionally distributed independently as Poisson $(\theta_s^{(T)} \cdot v_s \cdot P)$.

For assumptions 1, 2, and 4 DeVries (1997) showed that T_s, $s = 1, \ldots, B$, are marginally distributed independently with mean $= \alpha\beta\phi v_s$ and variance $\alpha\beta\phi v_s[1 + \alpha\beta\phi v_s[(\phi^2 + \zeta^2(\alpha + 1))/\alpha\phi^2)]]$. For convenience, define the parameter d, $d = [\phi^2 + \zeta^2(\alpha + 1)]/\phi^2$, where $d \geq 1$. If $d = 1$, then $\zeta^2 = 0$, which indicates a constant survival fraction. When $d = 1$, DeVries (1997) showed that T_s is marginally distributed independently as negative binomial $(r = \alpha, p_s = 1/[\beta\phi v_s] + 1, q_s = \beta\phi v_s/[\beta\phi v_s + 1])$, $s = 1, \ldots, B$. If $d > 1$, then $\zeta^2 > 0$, which indicates variable survival fractions. Because the distribution of P is unknown when $d > 1$, the marginal distribution of T_s cannot be derived; hence Assumption 5.

Assumption 5. The distribution of T_s is closely approximated by a negative binomial $(r = \alpha/d, p_s = 1/[\beta\phi v_s d + 1], q_s = \beta\phi v_s d/[\beta\phi v_s d + 1])$ distribution.

The parameters of this negative binomial distribution were chosen to match the marginal mean and variance of test carriers. Note that the T_s will be identically distributed if the dilution levels, v_s, are constant. These results engender a model for Y_s, the observable test carrier densities.

Assumption 6. The carrier counts C_i, $i = 1, \ldots, A$, and T_s, $s = 1, \ldots, B$ are all statistically independent.

Under assumptions 5 and 6, the joint probability mass function (pmf) of the observable data is closely approximated by the product of A negative binomials times the product of B negative binomials. Let $\vec{\tau}$ denote the vector parameter $(\phi, \zeta, \alpha, \beta)'$. Let $l(\vec{\tau}|\vec{c})$ and $l(\vec{\tau}|\vec{t})$ denote the log likelihoods for the control and test carrier data, respectively. Then

$$l(\vec{\tau}|\vec{c}) = \sum_{i=1}^{A}\left[\ln(\Gamma(c_i + \alpha)) - \ln(\Gamma(\alpha)) - \ln(c_i!) + \alpha\ln\left(\frac{1}{\beta u_i + 1}\right) + c_i\ln\left(\frac{\beta u_i}{\beta u_i + 1}\right)\right],$$

and

$$l(\vec{\tau}|\vec{t}) = \sum_{s=1}^{B}\left[\ln\left(\Gamma\left(t_s + \frac{\alpha}{d}\right)\right) - \ln\left(\Gamma\left(\frac{\alpha}{d}\right)\right) - \ln(t_s!) + \frac{\alpha}{d}\ln\left(\frac{1}{\beta\phi v_s d + 1}\right) + t_s\ln\left(\frac{\beta\phi v_s d}{\beta\phi v_s d + 1}\right)\right].$$

The log likelihood function for the model, denoted by $l(\vec{\tau}|\vec{c}, \vec{t})$, is given in (1).

$$l(\vec{\tau}|\vec{c}, \vec{t}) = l(\vec{\tau}|\vec{c}) + l(\vec{\tau}|\vec{t}) \tag{1}$$

3. Two definitions of log reduction

To calculate the log reduction, first convert the cfu counts and dilutions for each carrier into an estimate of the density, which is the number of surviving microbes for the carrier.

Next compare on the log scale, the difference between the average density for control carriers and the average density for test carriers. This statement is not precise and can be interpreted in at least two ways, both of which have been used in antimicrobial research. One measure is the difference between the control and test means of log densities; the other measure is the difference between the log transformed means of densities. Although the mean of logs is not equal to the log of the mean, the distinction is not mentioned in the antimicrobial literature. Some notation is required to give these issues a mathematical foundation. Let

$$X_i^* = \begin{cases} \log(X_i), & \text{if } X_i > 0 \\ \log\left(\dfrac{1}{u_i}\right), & \text{if } X_i = 0 \end{cases}$$

denote the log transformed density for control carrier i, $i = 1, \ldots, A$. Note that the log density is undefined if $C_i = 0$, in which case we artificially set $C_i = 1$. Let $\bar{X} = 1/A \sum_{i=1}^{A} X_i$ denote the mean of control carrier densities, and let $\overline{X^*} = 1/A \sum_{i=1}^{A} X_i^*$ denote the mean of the log control carrier densities. Let

$$Y_s^* = \begin{cases} \log(Y_s), & \text{if } Y_s > 0 \\ \log\left(\dfrac{1}{v_s}\right), & \text{if } Y_s = 0 \end{cases}$$

denote the log transformed density for test carrier s, $s = 1, \ldots, B$. Let $\bar{Y} = 1/B \sum_{s=1}^{B} Y_s$ denote the mean of test carrier densities, and let $\overline{Y^*} = 1/B \sum_{s=1}^{B} Y_s^*$ denote the mean of the log test carrier densities.

Let the *mean of logs* definition of log reduction be denoted by Υ_1. Specifically, letting $E(\cdot)$ be the expectation operator,

$$\Upsilon_1 = \frac{1}{A} \cdot \sum_{i=1}^{A} E(X_i^*) - \frac{1}{B} \cdot \sum_{s=1}^{B} E(Y_s^*).$$

Let the *log of the mean* definition of log reduction be denoted by Υ_2. Specifically,

$$\Upsilon_2 = log\left[\frac{1}{A} \cdot \sum_{i=1}^{A} E(X_i) \div \frac{1}{B} \cdot \sum_{s=1}^{B} E(Y_s)\right].$$

An equivalent, concise definition is $\Upsilon_2 = -\log(\phi)$.

A reason for preferring Υ_1 is that it is the log of the geometric mean density for test carriers subtracted from the log of the geometric mean density for control carriers. Because the distribution of densities among carriers often exhibit positive skewness, the preferred statistic for summarizing such microbiological count data is the geometric mean (Eaton *et al.*, 1995).

Our model assumes negative binomial distributions for control and test carrier cfu counts, and the negative binomial distribution has positive skew. The log of a (zero-

adjusted) negative binomial random variable has a more symmetric distribution. Also, the log of a gamma has a more normal, and hence symmetric, distribution than the original gamma (Johnson, Kotz and Kemp, 1992). We believe that normal theory statistical inference can be applied to estimators of Υ_1 because the log transformation moves the data toward normality. Antimicrobial assay data support these statements. For example, our analyses of many assays showed that the skewness coefficients for densities were usually positive, but the skewness coefficients for log transformed densities were typically near zero (DeVries, 1997).

A reason for preferring Υ_2 is that it has a nice biological interpretation as the log transform of the mean survival probability. When marketing a disinfectant, manufacturers often use as a measure of efficacy the fraction of microbes killed, and that fraction equals $(1 - 10^{-\Upsilon_2})$. Because the fraction killed cannot be calculated easily from Υ_1, some microbiologists may prefer Υ_2.

4. Estimators of log reduction

We will now present a method of moments (MOM) estimator and a maximum likelihood estimator (MLE) for each definition of log reduction.

4.1 Estimators of Υ_1

Let $\hat{\Upsilon}_{1,\mathrm{MOM}}$ denote the MOM estimator of Υ_1, as defined in (2).

$$\hat{\Upsilon}_{1,\mathrm{MOM}} = \overline{X^*} - \overline{Y^*} \tag{2}$$

Let $\hat{\Upsilon}_{1,\mathrm{MLE}}$ denote the MLE of Υ_1. The computation of $\hat{\Upsilon}_{1,\mathrm{MLE}}$ involves finding the values of $\hat{\phi}, \hat{\zeta}, \hat{\alpha}, \hat{\beta}$ which maximize (1). The MLE equations cannot be solved in a closed form and an iterative solution is required. The details are described in DeVries (1997). Once the value of $\hat{\tau}$ has been found, $\hat{\Upsilon}_{1,\mathrm{MLE}}$ is given by (3), where $\hat{d} = [\hat{\phi}^2 + \hat{\zeta}^2(\hat{\alpha} + 1)]/\hat{\phi}^2$.

$$\begin{aligned}\hat{\Upsilon}_{1,\mathrm{MLE}} = &\left[-\frac{1}{A}\sum_{i=1}^{A} \log(u_i) + \frac{1}{A}\sum_{i=1}^{A}\sum_{c=1}^{\infty} \log(c)\, \frac{\Gamma(c+\hat{\alpha})}{\Gamma(\hat{\alpha})c!} \left(\frac{\hat{\beta}u_i}{\hat{\beta}u_i+1}\right)^{c} \left(\frac{1}{\hat{\beta}u_i+1}\right)^{\hat{\alpha}} \right] \\ &- \left[-\frac{1}{B}\sum_{s=1}^{B} \log(v_s) + \frac{1}{B}\sum_{s=1}^{B}\sum_{t=1}^{\infty} \log(t)\, \frac{\Gamma\left(t+\frac{\hat{\alpha}}{\hat{d}}\right)}{\Gamma\left(\frac{\hat{\alpha}}{\hat{d}}\right)t!} \left(\frac{\hat{\beta}\hat{\phi}\hat{d}v_s}{\hat{\beta}\hat{\phi}\hat{d}v_s+1}\right)^{t} \right. \\ &\left. \times \left(\frac{1}{\hat{\beta}\hat{\phi}\hat{d}v_s+1}\right)^{\hat{\alpha}/\hat{d}} \right].\end{aligned} \tag{3}$$

We have not found a closed form version of (3), however, reasonable approximations are given in DeVries (1997).

4.2 Estimators of Υ_2

The expectation of Y_s, is $\alpha\beta\phi$, and the expectation of X_i, is $\alpha\beta$, and the ratio of expectations is ϕ. Thus the negative logarithm of the ratio of expectations is $-\log(\phi)$, which is Υ_2. Let $\hat{\Upsilon}_{2,\mathrm{MOM}}$ denote the MOM estimator of Υ_2, given by (4).

$$\hat{\Upsilon}_{2,\mathrm{MOM}} = \log(\bar{X}) - \log(\bar{Y}) \tag{4}$$

Let $\hat{\Upsilon}_{2,\mathrm{MLE}}$ denote the maximum likelihood estimate of Υ_2. Find the values of $\hat{\phi}, \hat{\zeta}, \hat{\alpha}, \hat{\beta}$ that maximize (1) and substitute $\hat{\phi}$ into (5) to get $\hat{\Upsilon}_{2,\mathrm{MLE}}$.

$$\hat{\Upsilon}_{2,\mathrm{MLE}} = -\log(\hat{\phi}) \tag{5}$$

5. Standard error estimators

Let $S^2_{X^*}$ and $S^2_{Y^*}$ denote the sample variances for the log densities of control and test carriers, respectively. The sample variances are calculated in the standard way,

$$S^2_{X^*} = \frac{1}{A-1}\sum_{i=1}^{A}(X^*_i - \overline{X^*})^2; \qquad S^2_{Y^*} = \frac{1}{B-1}\sum_{s=1}^{B}(Y^*_s - \overline{Y^*})^2.$$

The standard error for $\hat{\Upsilon}_{1,\mathrm{MOM}}$ is given in (6).

$$\mathrm{SE}(\hat{\Upsilon}_{1,\mathrm{MOM}}) = \sqrt{\frac{S^2_{X^*}}{A} + \frac{S^2_{Y^*}}{B}} \tag{6}$$

Let CV_X and CV_Y denote the sample coefficients of variation for control and test carrier densities, respectively; i.e.

$$\mathrm{CV}_X = \frac{\sqrt{S^2_x}}{\bar{X}}, \qquad \mathrm{CV}_Y = \frac{\sqrt{S^2_Y}}{\bar{Y}}.$$

Application of the delta method leads to (7) for the $\mathrm{SE}(\hat{\Upsilon}_{2,\mathrm{MOM}})$.

$$\mathrm{SE}(\hat{\Upsilon}_{2,\mathrm{MOM}}) \doteq \log(e) \cdot \sqrt{\frac{\mathrm{CV}^2_X}{A} + \frac{\mathrm{CV}^2_Y}{B}}, \tag{7}$$

where $\log(e) \doteq 0.4343$.

DeVries (1997) suggests formulas for $\mathrm{SE}(\hat{\Upsilon}_{1,\mathrm{MLE}})$, and $\mathrm{SE}(\hat{\Upsilon}_{2,\mathrm{MLE}})$ based on the asymptotic variances of the MLEs. The details are quite complicated and, due to limitations of space, will not be presented here.

6. Example

This example illustrates the calculation of log reduction estimates, with associated standard errors. These are the intermediate statistics used to calculate the log reductions and standard errors: $\bar{x}^* = 7.825$, $\bar{y}^* = 2.825$, $\bar{x} = 6.7 \times 10^7$, $\bar{y} = 751.818$, $S^2_{x^*} = 0.001136513$, $S^2_{y^*} = 0.05366018$, $\mathrm{CV}_X = 0.07897765$, and $\mathrm{CV}_Y = 0.4790878$.

The results are:

$$\hat{\Upsilon}_{1,\mathrm{MOM}} = 7.825 - 2.825 = 5.00;$$

$$\mathrm{SE}(\hat{\Upsilon}_{1,\mathrm{MOM}}) = \sqrt{\frac{0.001136513}{3} + \frac{0.05366018}{10}} = 0.0758.$$

$$\hat{\Upsilon}_{2,\mathrm{MOM}} = -\log\left(\frac{751.818}{6.7 \times 10^7}\right) = 4.95;$$

$$\mathrm{SE}(\hat{\Upsilon}_{2,\mathrm{MOM}}) = 0.4342945 \cdot \sqrt{\frac{(0.07897765)^2}{3} + \frac{(0.4790878)^2}{10}} = 0.0687.$$

$$\hat{\Upsilon}_{1,\mathrm{MLE}} = 5.00; \qquad \mathrm{SE}(\hat{\Upsilon}_{1,\mathrm{MLE}}) = 0.0714.$$

$$\hat{\Upsilon}_{2,\mathrm{MLE}} = 4.95; \qquad \mathrm{SE}(\hat{\Upsilon}_{2,\mathrm{MLE}}) = 0.0676.$$

7. Asymptotic relative efficiencies of the estimators

In this section, we define asymptotic efficiency, discuss formulas, and show the results of asymptotic efficiency calculations for a few specific cases. Let R be a constant denoting the ratio of numbers of test carriers to control carriers, $R = B/A$. Suppose we are using h_1 and h_2 as estimators of $\kappa(\vec{\tau})$ which is a specified function of the model parameters; e.g. $\kappa(\vec{\tau}) = \Upsilon_1$ or $\kappa(\vec{\tau}) = \Upsilon_2$. The asymptotic relative efficiency (ARE) of h_1 to h_2 is

$$\mathrm{ARE}(h_1 : h_2) = \frac{\mathrm{var}(h_2)}{\mathrm{var}(h_1)},$$

where $\mathrm{var}(h_1)$ and $\mathrm{var}(h_2)$ are the asymptotic normal variances of h_1 and h_2, respectively, as A and B go to ∞ (Huber, 1981). The ARE is a function of R, the sample volumes used in the dilution series, and $\vec{\tau}$.

DeVries (1997) derives formulas for calculating the AREs for estimators based on quantitative antimicrobial assay data. Here we apply those formulas to the 12 special cases

listed in Table 3. The parameter values for these cases cover a wide range of conditions. Two values of ϕ were chosen, 10^{-7} or $10^{-3.5}$, representing an active and moderately active disinfectant, respectively. We choose values for the mean (μ) and coefficient of variation (CV) for the recoverable counts, as well as the index of variability among survival fractions, d, after analyzing many quantitative antimicrobial assay data sets. Most of the observed control carrier densities for those assays were close to the target counts of 10^8 organisms per carrier. For this reason we set the mean recoverable count at 10^8 for each case. We allowed the CV for the true unobservable recoverable counts to vary over three values, 0.10, 0.30, and 0.75. The CV of 0.3 is typical of real data, and few real assays produce a CV as high as 0.75. Note that the parameters of Assumption 1 are uniquely determined by the mean and CV, $\alpha = \mathrm{CV}^{-2}$ and $\beta = \mu \cdot \mathrm{CV}^2$. The odd numbered cases correspond to the model with constant survival fraction ($d = 1.0$), and the even numbered cases correspond to the model with variable survival fractions. Table 3 also shows the volumes sampled, u and v, the CV values for the control carrier densities (C_i, $i = 1, \ldots, A$) and test carrier densities (T_s, $s = 1, \ldots, B$), as well as the true values of Υ_1 and Υ_2.

Column 2 of Table 4 shows $\mathrm{ARE}(\hat{\Upsilon}_{1,\mathrm{MOM}} : \hat{\Upsilon}_{1,\mathrm{MLE}})$ when $R = \frac{10}{3}$ for each of the twelve cases described in Table 3. Because $\mathrm{ARE}(\hat{\Upsilon}_{2,\mathrm{MOM}} : \hat{\Upsilon}_{2,\mathrm{MLE}}) = 1.0$ for all cases, this ARE is omitted from Table 4. A relative efficiency of 1.0 for $\mathrm{ARE}(\hat{\Upsilon}_{2,\mathrm{MOM}} : \hat{\Upsilon}_{2,\mathrm{MLE}})$ occurred for the odd numbered cases in Table 4 because the MOM and MLE estimators are identical for the model with constant survival fraction (DeVries, 1997). However, we did not anticipate that $\mathrm{ARE}(\hat{\Upsilon}_{2,\mathrm{MOM}} : \hat{\Upsilon}_{2,\mathrm{MLE}})$ would equal one for the model with variable survival fractions. We have been unable to construct a general proof, but it seems reasonable to infer that MOM is asymptotically fully efficient for Υ_2, at least for the spectrum of practical situations covered by Table 3.

The results in column 2 of Table 4 show that MOM is more than 90% efficient except for cases 5 and 11. Cases 5, 6, 11, and 12 represent greater variability of the recoverable counts than we expect to occur in practice. Also, cases 5 and 11 represent the less realistic

Table 3. Parameter settings for 12 representative cases.

Case	ϕ	d	u	v	CV(θ)	CV(C_i)	CV(T_s)	Υ_1	Υ_2
1	$10^{-3.5}$	1.0	10^{-6}	10^{-2}	0.10	0.141	0.115	3.498	3.5
2	$10^{-3.5}$	99.68	10^{-6}	10^{-2}	0.10	0.141	1.0	3.747	3.5
3	$10^{-3.5}$	1.0	10^{-6}	10^{-2}	0.30	0.316	0.305	3.498	3.5
4	$10^{-3.5}$	11.08	10^{-6}	10^{-2}	0.30	0.316	1.0	3.729	3.5
5	$10^{-3.5}$	1.0	10^{-6}	10^{-2}	0.75	0.757	0.752	3.497	3.5
6	$10^{-3.5}$	1.77	10^{-6}	10^{-2}	0.75	0.757	1.0	3.614	3.5
7	10^{-7}	1.0	10^{-6}	1.0	0.10	0.141	0.332	7.022	7.0
8	10^{-7}	215	10^{-6}	1.0	0.10	0.141	1.5	7.356	7.0
9	10^{-7}	1.0	10^{-6}	1.0	0.30	0.316	0.436	7.024	7.0
10	10^{-7}	23.89	10^{-6}	1.0	0.30	0.316	1.5	7.338	7.0
11	10^{-7}	1.0	10^{-6}	1.0	0.75	0.757	0.814	7.017	7.0
12	10^{-7}	3.82	10^{-6}	1.0	0.75	0.757	1.5	7.222	7.0

Table 4. Asymptotic relative efficiency values and observed ratios of MSEs for the cases of Table 3.

Col. 1 Case	Col. 2 $\text{ARE}(\hat{\Upsilon}_{1,\text{MOM}} : \hat{\Upsilon}_{1,\text{MLE}})$	Col. 3 $\frac{\widehat{\text{MSE}}(\hat{\Upsilon}_{1,\text{MLE}})}{\widehat{\text{MSE}}(\hat{\Upsilon}_{1,\text{MOM}})}$	Col. 4 $\frac{\widehat{\text{MSE}}(\hat{\Upsilon}_{2,\text{MLE}})}{\widehat{\text{MSE}}(\hat{\Upsilon}_{2,\text{MOM}})}$
1	0.990	0.993	0.999
2	0.987	1.029	1.000
3	0.950	0.972	0.999
4	0.999	1.038	1.000
5	0.735	0.903	1.012
6	0.991	0.946	1.002
7	0.938	1.028	1.000
8	0.931	1.003	1.000
9	0.915	0.968	1.001
10	0.940	0.970	0.993
11	0.766	0.883	1.003
12	0.963	0.955	0.998

situation where the test carriers have constant survival fractions. Cases 1–4 and 7–10 represent customary levels of variability in the recoverable counts. For these cases, the MOM estimator of Υ_1 has a reasonably high efficiency relative to MLE.

8. Simulation study: evaluating estimators and standard errors

We conducted computer simulation experiments to assess the properties of the various estimators of Υ_1 and Υ_2, and the associated standard errors, for the 12 cases in Table 3. Each of the 12 experiments consisted of 1000 simulated quantitative assay data sets. The simulated data were generated according to the distributions described in section 2 with the aid of the random number generator functions from the statistical software package, *S-plus* (Splus User's Guide, 1997). All simulated assays used $A = 3$ control carriers and $B = 10$ test carriers.

Summaries of the results of the simulation study are shown in Tables 4–6. Table 5 shows the empirical bias and mean squared error (MSE) values, along with a comparison of the mean SE to the observed standard deviation (SD) of the estimator, for $\hat{\Upsilon}_{1,\text{MOM}}$ and $\hat{\Upsilon}_{1,\text{MLE}}$, and Table 6 shows the same information for $\hat{\Upsilon}_{2,\text{MOM}}$ and $\hat{\Upsilon}_{2,\text{MLE}}$. Columns 3 and 4 of Table 4 show ratios of the empirical MSEs.

8.1 Comparing $\hat{\Upsilon}_{1,\text{MOM}}$ *and* $\hat{\Upsilon}_{1,\text{MLE}}$

The estimator $\hat{\Upsilon}_{1,\text{MOM}}$ is unbiased for Υ_1, a fact supported by the simulation results. The small sample bias of $\hat{\Upsilon}_{1,\text{MLE}}$ is negligible for all practical purposes.

Column 3 of Table 4 shows the empirical small sample relative efficiencies as measured by the ratios of $\widehat{\text{MSE}}$s, $\hat{\Upsilon}_{1,\text{MLE}}$ to $\hat{\Upsilon}_{1,\text{MOM}}$. The ratios ranged between 0.88 and 1.04, and generally were well approximated by the the ARE values of Table 4, although the small

Table 5. Simulation results for $\hat{\Upsilon}_{1,MOM}$ and $\hat{\Upsilon}_{1,MLE}$ for the cases in Table 3.

	Results for $\hat{\Upsilon}_{1,MOM}$				Results for $\hat{\Upsilon}_{1,MLE}$			
1. Case	2. Bias $\hat{\Upsilon}_1$	3. Mean SE($\hat{\Upsilon}_1$)	4. SD($\hat{\Upsilon}_1$)	5. $\widehat{MSE}$ $\hat{\Upsilon}_1$	6. Bias $\hat{\Upsilon}_1$	7. Mean SE($\hat{\Upsilon}_1$)	8. SD($\hat{\Upsilon}_1$)	9. $\widehat{MSE}$ $\hat{\Upsilon}_1$
1	0.000	0.03689	0.03850	0.00148	0.001	0.03400	0.03836	0.00147
2	−0.002	0.1716	0.1749	0.0306	−0.002	0.1684	0.1774	0.0315
3	0.000	0.08660	0.09108	0.00829	0.006	0.07032	0.08964	0.00806
4	0.001	0.1885	0.1987	0.0394	0.003	0.1821	0.2023	0.0409
5	0.010	0.2284	0.2462	0.0606	0.035	0.1779	0.2315	0.0547
6	−0.002	0.2677	0.2664	0.0709	0.012	0.2320	0.2589	0.0671
7	0.001	0.05915	0.0.05930	0.00351	0.006	0.05978	0.05984	0.00361
8	0.000	0.1835	0.1768	0.0312	0.009	0.1666	0.1768	0.0313
9	−0.006	0.1012	0.1117	0.0125	0.001	0.08667	0.1102	0.0121
10	0.002	0.1959	0.2000	0.0400	0.007	0.1754	0.1971	0.0388
11	0.005	0.2375	0.2585	0.0668	0.036	0.1882	0.2403	0.0590
12	−0.004	0.2749	0.2838	0.0805	0.004	0.2400	0.2775	0.0769

Table 6. Simulation results for $\hat{\Upsilon}_{2,MOM}$ and $\hat{\Upsilon}_{2,MLE}$ for the cases in Table 3.

	Results for $\hat{\Upsilon}_{2,MOM}$				Results for $\hat{\Upsilon}_{2,MLE}$			
1. Case	2. Bias $\hat{\Upsilon}_2$	3. Mean SE($\hat{\Upsilon}_2$)	4. SD($\hat{\Upsilon}_2$)	5. $\widehat{MSE}$ $\hat{\Upsilon}_2$	6. Bias $\hat{\Upsilon}_2$	7. Mean SE($\hat{\Upsilon}_2$)	8. SD($\hat{\Upsilon}_2$)	9. $\widehat{MSE}$ $\hat{\Upsilon}_2$
1	−0.001	0.03665	0.03822	0.00146	−0.001	0.03381	0.03819	0.00146
2	0.020	0.1316	0.1436	0.0210	0.020	0.1323	0.1436	0.0210
3	−0.005	0.08351	0.08816	0.00780	−0.003	0.06939	0.08820	0.00780
4	0.013	0.1497	0.1609	0.0261	0.014	0.1463	0.1608	0.0260
5	−0.023	0.1911	0.2253	0.0512	−0.015	0.1646	0.2273	0.0518
6	−0.017	0.2117	0.2329	0.0545	−0.015	0.1974	0.2333	0.0546
7	0.002	0.05596	0.05632	0.00317	0.002	0.05626	0.05633	0.00317
8	0.052	0.1830	0.2187	0.0505	0.052	0.2012	0.2187	0.0505
9	−0.009	0.09374	0.1056	0.0112	−0.009	0.08134	0.1057	0.0112
10	0.046	0.1987	0.2369	0.0582	0.046	0.2064	0.2360	0.0578
11	−0.023	0.1977	0.2293	0.0531	−0.022	0.1752	0.2298	0.0533
12	0.004	0.2501	0.2837	0.0804	0.006	0.2564	0.2834	0.0803

sample MOM performed slightly better than the ARE values indicate. The biggest discrepancies between the $\widehat{MSE}$ occurred for cases 5, 6, 11, and 12, which are cases with the highest variability of recoverable counts. We believe that real assays will seldom produce such high variability. However, when it does occur, the results in Tables 4 and 5 suggest that $\hat{\Upsilon}_{1,MLE}$ has a smaller MSE than $\hat{\Upsilon}_{1,MOM}$. For the cases 1–4 and 7–10 that are

more typical of situations in practice, neither $\hat{\Upsilon}_{1,\mathrm{MOM}}$ nor $\hat{\Upsilon}_{1,\mathrm{MLE}}$ is superior to the other on the basis of mean squared error comparisons.

The standard error formula for $\hat{\Upsilon}_{1,\mathrm{MOM}}$ appears to be less biased than that of $\hat{\Upsilon}_{1,\mathrm{MLE}}$. For cases 5 and 11 $\mathrm{SE}(\hat{\Upsilon}_{1,\mathrm{MLE}})$ badly underestimated $\mathrm{SD}(\hat{\Upsilon}_{1,\mathrm{MLE}})$, but these are cases representing extremely variable data.

All things considered, we recommend $\hat{\Upsilon}_{1,\mathrm{MOM}}$ over $\hat{\Upsilon}_{1,\mathrm{MLE}}$ because the MOM method is easy to calculate, is unbiased, has a reliable standard error formula, and has an MSE not much different from the MSE for $\hat{\Upsilon}_{1,\mathrm{MLE}}$ for cases likely to be met in practice.

8.2 Comparison of $\hat{\Upsilon}_{2,\mathrm{MOM}}$ *and* $\hat{\Upsilon}_{2,\mathrm{MLE}}$

Table 6 shows that both $\hat{\Upsilon}_{2,\mathrm{MOM}}$ and $\hat{\Upsilon}_{2,\mathrm{MLE}}$ have negligible bias for the cases simulated. As seen in Tables 4 and 6, the MSEs for these estimators are nearly identical for each case. Thus from a MSE criterion neither $\hat{\Upsilon}_{2,\mathrm{MOM}}$ nor $\hat{\Upsilon}_{2,\mathrm{MLE}}$ is superior to the other.

Table 6 shows that, for each estimator and each of the twelve cases, the mean of the 1000 standard errors was less than the empirical standard deviation of the point estimates. The standard error formulas for both estimators appear to be biased downwards. In general, $\mathrm{SE}(\hat{\Upsilon}_{2,\mathrm{MOM}})$ was less biased than $\mathrm{SE}(\hat{\Upsilon}_{2,MLE})$ for the model with constant survival fractions, but slightly more biased for the model with variable survival fractions. However, $\hat{\Upsilon}_{2,\mathrm{MOM}}$ and $\mathrm{SE}(\hat{\Upsilon}_{2,\mathrm{MOM}})$ are far easier to compute than their MLE counterparts.

Overall, we recommend using $\hat{\Upsilon}_{2,\mathrm{MOM}}$ as the estimator of Υ_2. The MOM and MLE estimators have about the same statistical characteristics, and we believe microbiologists will feel much more comfortable using a procedure they can compute with the aid of a spreadsheet rather than with a complicated maximum likelihood computer program.

9. Discussion

We believe that most microbiologists will follow the *Standard Methods for the Examination of Water and Wastewater* (Eaton *et al*., 1995) and choose the geometric mean over the arithmetic mean as a measure of the typical density of recoverable counts among carriers; i.e., they will choose the parameter Υ_1 as the measure of log reduction. We note, however, that one referee strongly prefers Υ_2. Also, DeVries(1997) showed that Υ_2 is the more easily estimated parameter in the sense that, for most of the cases considered, the estimators of Υ_2 had smaller relative mean squared errors than the estimators of Υ_1. This result is interesting but irrelevant to the main practical issue—which parameter is more meaningful to microbiologists.

Regardless of which parameter the microbiologist prefers, we recommend the MOM estimator and the associated SE. The MOM is very easy to calculate. The results of asymptotic relative efficiency calculations and a small simulation study indicate that the MOM is unbiased, and nearly fully efficient, for the models of section 2. The MOM is model independent in the sense that it is valid for any probability distributions that have the same means and variances as the models of section 2. The MOM is accompanied by a

simple, fairly reliable SE formula. It may well be possible, however, to find a better SE formula; such an investigation would be a worthy research project for the future.

The SE discussed here measures the uncertainty of the estimator within a single assay. There are other sources of uncertainty to consider. For example, environmental microbiologists routinely conduct collaborative studies to assess the variability of standard assay techniques. In a collaborative study, different laboratories conduct blind replicate assays of a disinfectant, where all assays are conducted according to the same protocol. We have analyzed the data from a few collaborative assays. The results were that the total variance of $\hat{\Upsilon}_{1,\mathrm{MOM}}$ could be partitioned into three components, the variance within assays, the variance among replicate assays within a laboratory, and the variance among laboratories. The variance within assays usually amounted to less than 10% of the total variance, whereas the variance among laboratories amounted to as much as 80% of the total variance. For this reason, it is important to include all relevant variance components when calculating the standard error of the estimate.

Although the motivation for this work was provided by our interest in antimicrobial carrier assays, we believe that the MOM estimator is of potential use in other microbiological experiments conducted to compare two treatments based on cfu counts in dilution series. For example, suspension tests and biofilm tests for antimicrobial activity might be properly summarized using MOM estimators of the log reduction.

Our conclusions are based on a few cases studied in detail, however we believe that many real experimental conditions will fall within the range of conditions studied in this paper. Nevertheless, our conclusions are circumscribed by the accuracy and scope of the cases on which they were based.

Acknowledgment

This research was supported in part by U.S. Environmental Protection Agency (EPA) Contract No. 68W50061 and National Science Foundation Cooperative Agreement No. EEC-8907039 with the Center for Biofilm Engineering at Montana State University. This paper does not necessarily represent the views of the EPA.

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