APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1989, p. 1934–1942
0099-2240/89/081934-09$02.00/0

Vol. 55, No. 8

Estimation of Microbial Densities from Dilution Count Experiments

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Received 22 December 1988/Accepted 8 May 1989

Although dilution counts have been widely used in quantitative microbiology, their interpretation has always been widely discussed both in microbiology and in applied statistics. Maximum-likelihood (most-probable-number) methods have generally been used to estimate densities from dilution experiments. It has not been widely recognized that these methods are intrinsically and statistically biased at the sample sizes used in microbiology. This paper presents an analysis of proposed method for correction of such biases, and the method was found to be robust for moderate deviations from Poisson behavior. For analyses at greater variance with the Poisson assumptions, the use of the Spearman-Karber method is analyzed and shown to yield an estimate of density of lesser bias than that produced by the most-probable-number method. Revised methods of constructing confidence limits proposed by Loyer and Hamilton (M. W. Loyer and M. A. Hamilton, Biometrics 40:907–916, 1984) are also discussed, and charts for the three- and four- decimal dilution series with five tubes per dilution are presented.

The use of dilution counts for the determination of microbial densities has a long history in applied microbiology. In such a technique, a number of tubes are inoculated with various dilutions of a suspension containing microorganisms. Under the assumption of random sampling of enumerable units, leading to a Poisson probability distribution for the expected number of organisms inoculated in a given volume, the density of the original suspension can be estimated by using the maximum-likelihood technique (3, 10, 22, 23).

It has been known for many years, however, that the maximum-likelihood technique leads to biased estimates for parameters in all but the most simple cases (11, 15, 18). Indeed, in early comparisons of the membrane filter and most-probable-number (MPN) coliform techniques, the tendency for a positive bias in the MPN method was noted (20). Only more recently, however, has a method become available for the first-order correction of the inherent positive bias of this technique (17). In a related area, the estimation of confidence limits for MPN determinations has been reexamined by Loyer and Hamilton (14), who present alternative and more statistically rigorous methods for addressing this problem than those presently used in environmental microbiology (22).

Any procedure for the analysis of dilution experiments must be reasonably robust to the possible presence of deviations from the Poisson assumption. For example, a number of studies have indicated that replicate counts in environmental samples might be distributed according to the negative binomial, rather than the Poisson, distribution (2, 6, 8, 9, 16). The negative binomial distribution is an example of a discrete distribution with variance in excess of the Poisson—termed an overdisperse distribution. Such variance may be intrinsic to the enumeration methodology itself and thus may be experimentally correctable; one example of this type of situation is a possible variation in the efficiency of recovery between tubes. Alternatively, the variation may be a property of the microbial population itself. For example, if organisms exist in clumps possessing a frequency distribution of organisms per clump, and if clumps are randomly sampled but a dislodgement occurs during handling, the distribution of viable units will probably be overdisperse. If the latter type of situation forms the basis for the overdispersion, some method of analysis of dilution count data robust to the overdispersion is required.

It is the objective of this paper to explore the utility of the bias correction method and the alternative methods for constructing confidence limits to the common dilution series used in MPN determinations. First, the more recent techniques are examined and are found to be relatively robust for small to moderate deviations from the underlying assumptions of Poisson distribution of replication error. Then, alternatives methodologies for use when the replication error is severely different from Poisson are developed. In work previously published, diagnostic methods for detecting deviations from the Poisson distribution are presented (9).

MATERIALS AND METHODS

Likelihood estimation. If dilutions 1 through r, each with n_r tubes (not necessarily equal), are inoculated with a volume V_r of sample from a material containing an average concentration of microorganisms μ, standard theory (3) shows that the probability that a random vector $\langle p_1, p_2, \ldots, p_r \rangle$ of positive tubes will be observed is given by the following equation:

$$L = \sum_{i=1}^{r} \frac{n_i!}{(n_i - p_i)!p_i!}(p_{0,i})^{n_i - p_i}(1 - p_{0,i})^{p_i} \quad (1)$$

with

$$p_{0,i} = e^{-\mu V_i} \quad (2)$$

Equation 2 results from an independent Poisson distribution at each dilution for the distribution of microorganisms in a single volume of inoculum, and equation 1 expresses the independence of each replication and each dilution. If μ is known, L is the probability that a given combination of positive tubes will be obtained. If μ is unknown, the maximum-likelihood estimate (MLE) of its value can be obtained by finding the value of μ which, for the observed tube scores, maximize the value of L (termed the likelihood).

Should the sample be drawn under circumstances where the Poisson distribution does not apply, equation 1 can still be used to compute the probability that a given set of tube scores were obtained given the true distribution (and param-

eters of the distribution). However, in this case, equation 2 will be replaced by an alternative relationship for the probability of having no organisms in an inoculum. For example, if the distribution is negative binomial, the relationship will become

$$p_{0,i} = (1 + \mu V_i/k)^{-k} \quad (3)$$

with k being the dispersion parameter of the negative binomial distribution. The negative binomial distribution has been used to describe microbial distributions in which the variance between replicates is in excess of the Poisson distribution (2, 6, 16). As k increases to infinity, the negative binomial distribution approaches the Poisson distribution.

If a Poisson distribution is used to formulate the likelihood equation for an MLE of a sample drawn from a population which is, in fact, negative binomial, a systematic error in the MLE will result. Wadley (21) first noted that, for a single dilution ($r = 1$), the erroneous use of a Poisson assumption negatively biases the estimate of microbial densities of samples from negative binomially distributed populations. Bias is defined as the expected value of μ minus its true value. However, the robustness of the MLE (as well as to bias-corrected MLEs) to deviations from the Poisson assumption when $r > 1$ has not, apparently, been explored.

Bias correction methodology. Standard statistical theory predicts that the MLE estimator of the mean of a Poisson population should, for a sufficiently large number of samples (large n_r), approach the true mean at a rate proportional to $1/n_r$. However, in usual practice, n_r is not large, and bias in the estimator is to be expected. Salama et al. (17) have presented a method for correcting the bias to order $1/n_r^2$. However, these authors did not present the application of their method to the typical five-tube decimal dilution series used in environmental microbiology, nor did they examine the robustness of their correction to deviations from the Poisson assumption.

The correction derived by Salama et al. (17) is essentially a Taylor series expansion. If μ^* is the maximum likelihood estimator of microbiol density, a bias-corrected density estimator (μ^b) is given by the following equation:

$$\mu^b = \mu^* - (1/2)\sum_{i=1}^{r}\left(\frac{\partial^2\mu^*}{\partial x_i^2}\right) n_i e^{-\mu^* V_i}(1 - e^{-\mu^* V_i})$$

with

$$\frac{\partial^2\mu^*}{\partial x_i^2} = \frac{V_i^2}{2(1 - e^{-\mu^* V_i})^2 D^3}\left\{\sum_{j=1}^{r}\frac{V_j^3 z_j \sinh(\mu^* V_j)}{[\cosh(\mu^* V_j) - 1]^2}\right\} - \frac{V_i^3}{(1 - e^{-\mu^* V_i})[\cosh(\mu^* V_i) - 1]D^2}$$

$$z_j = n_j(1 - e^{-\mu^* V_j})$$

$$D = \sum_{j=1}^{r}\frac{V_j^2 z_j}{2[\cosh(\mu^* V_j) - 1]}$$

Distribution-free estimation of densities. The problem of estimating the central point (50% effective dose or 50% lethal concentration) of a bioassay has been a longstanding one in biology and statistics. The MPN technique can be regarded as a bioassay with the ED_{50} being related to the microbial density. In particular, one can assume an underlying tolerance distribution (logit or probit) and use the method of maximum likelihood to estimate the median of this distribution (4). Alternatively, by using a variety of distribution-free methods, one can form estimates of the median of the empirical bioassay curve (1, 7, 12, 13). Distribution-free methods, although frequently less precise than other methods (in the statistical sense of producing greater estimating variance when the underlying assumptions are true), are very often insufficiently robust (being highly sensitive to deviations from the underlying assumptions).

One of the most widely used methods for producing a distribution-free estimate of the 50% effective concentration is the Spearman-Karber method (7, 12). In this work, the Spearman-Karber method is applied to locate the volume which is capable of infecting 50% of the dilution tubes. The reciprocal of this volume is defined as the Spearman-Karber (SK) estimate of microbial density. The procedure for construction of this estimate, given an MPN experiment in which sets of T tubes are exposed to a series of volumes (V_1, V_2, ..., V_n, with $V_1 > V_2$, etc.) producing positives (infected tubes) P_1, ..., P_n, is as follows. (i) A transformed dose parameter is computed to make the infectivity curve more symmetrical. In bioassay work, log-transforms are common (see, e.g., reference 12). In this work, the log-transform is used; thus, one computes $d_i = \ln(V_i)$. Coincidentally, this also renders the dose intervals equally spaced on an arithmetic scale (for common dilution sequences) with a separation ($d_i - d_{i+1}$) of Δ. (ii) The series is appended with two fictitious observations designed so that the series continues from complete infection to complete sterility. This procedure has no effect if the sequence is already bracketed by 100 and 0% infection. Mathematically,

$$d_0 = d_1 + \Delta,\ P_0 = T$$
$$d_{n+1} = d_n - \Delta,\ P_{n+1} = 0$$

(iii) Frequencies of positives are computed at each dose; i.e., $f_i = P_i/T$. (iv) The frequencies of positives are adjusted to produce a monotonically decreasing sequence of f's. For any i, if $f_i > f_{i-1}$, new frequencies (f^*) are defined by

$$f^*_i = f^*_{i-1} = (1/2)(f_i + f_{i-1});$$

after replacing the f's by the f^*'s, the process continues until the entire sequence is monotonic (12). (v) The SK estimate is then obtained by the following computation, which amounts to an integration of the dose-response polygon:

$$Q = \sum_{j=0}^{n}(f_j - f_{j+1})(d_j + d_{j+1})/2$$

where $SK = e^{-Q}$.

Comparison of point estimators. In this study, the performance of the MLE, the bias-corrected MLE estimator, and the SK estimator were studied by using a four-dilution, five-tube experiment with volumes of 10, 1, 0.1, and 0.01 ml. The range of μ values from 0.01 to 100/ml was subdivided into 40 intervals equally spaced on a log scale (i.e., $\log_{10}$ intervals of 0.1). For each assumed true μ value, the frequency of all possible tube scores (i.e., combinations of positive and negative tubes) was computed from equation 1. From the tube score, the value of each of the estimators (standard, bias-corrected, and SK) was then computed. The expected value of each estimator as a function of μ was then obtained by summing the products of the value of the estimator for a given tube score and the frequency with which that tube score is obtained at the value of μ. Similarly,

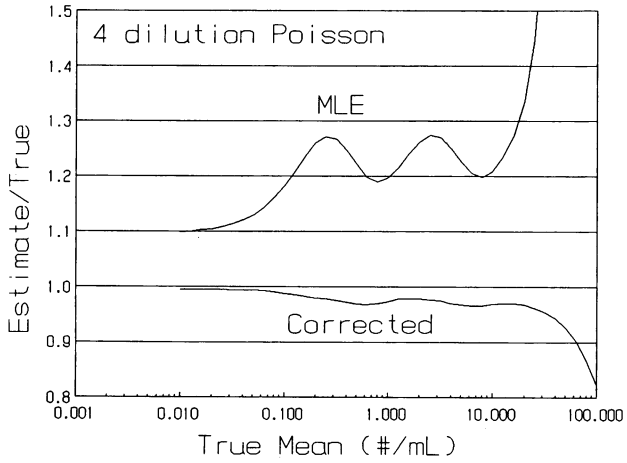

FIG. 1. Comparison of expected bias versus true mean for Poisson-distributed bacteria by using the MLE and corrected MLE methods.

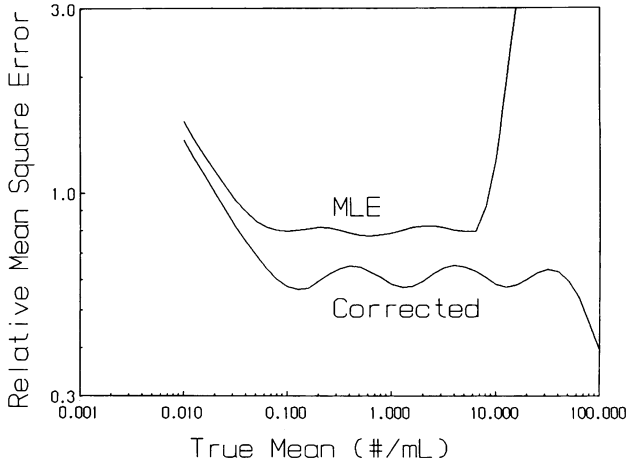

FIG. 2. Comparison of relative MSE (as a fraction of the true mean) for Poisson-distributed bacteria by using the MLE and corrected MLE methods.

the mean square error (MSE) of each estimator as a function of the true mean was also computed. The MSE is the average of the sum of the squares of the differences between the true value of microbial concentration and its estimate. For the score ⟨5, 5, 5, 5⟩, the MLE and the bias-corrected MLE were both assumed to be 10,000/ml. The MLE estimate was computed by a Newton-Raphson iteration method.

The performance of the estimators in the presence of overdispersion was also assessed by using the above procedure, in which the negative binomial distribution with a fixed k value was used to determine the tube score frequency via equations 1 and 3. However, the MLE was determined by using an assumed Poisson distribution and equations 1 and 2.

Confidence limits to the maximum likelihood estimator. The Sterne method for computing confidence limits (interval estimates) for the MPN estimator developed by Loyer and Hamilton (14) computes the set of tube scores $\langle P_1, p_2, \ldots, p_r \rangle$ which lie in a confidence limit by successively, for all values of the true density (μ), computing the most frequently observed scores ranked by their frequency. The α confidence limit (e.g., 0.05) for a tube score combination $\langle p_1, p_2, \ldots, p_r \rangle$ is defined as the range of μ values for which that score lies within the $(1 - \alpha)$ most frequently observed combinations. In this study, the confidence limits were determined by examining the range 0.01 to 1,000/ml over 500 equally spaced logarithmic intervals ($\log_{10}$ intervals of 0.01) for μ.

The Woodward method (22) by which confidence limits of most frequent use in sanitary microbiology have been obtained assumes that the MLE for the observed tube score is the true mean. The confidence limit for the true mean consists of the range of the MLE values for the central $(1 - \alpha)$ proportion of tube scores ranked by their MLE. One of the major criticisms of this method (14) is that it frequently includes highly improbable tube scores in the confidence region while rejecting more probable scores.

RESULTS

Bias reduction method. For a four-dilution experiment, over the entire range examined, the first-order bias reduction method produced point estimates of MPN of lower relative bias (defined as [mean estimator − true value]/true value) (Fig. 1) and MSE (Fig. 2) than the conventional MLE. These findings are similar to those of Salama et al. (17), who presented results for other experimental designs.

Prior workers have used a bias correction factor of 85% for the five-tube MPN test (19). The derivation of this factor is obscure, but probably involved an assumption about the underlying true MPN value itself. Examination of Fig. 1 shows that this correction factor is roughly the average of the bias over the midrange of microbial densities. It is also clear from this figure that over a similar range the bias in the corrected MLE is substantially less than 5%.

If the average relative bias and MSE are computed over the midrange of values for μ for negative binomial distributions of various degrees of dispersion (k values), it is found that the bias-corrected estimator is still superior to the MLE, provided that the negative binomial k value is greater than 6.0 (Fig. 3 and 4). Since tests are available to detect deviations from Poisson behavior which are sensitive to negative binomial k values at this level with sample sizes of about 1,000 (9), the correction for bias appears to be useful in conjunction with tests of homogeneity to confirm consistency with underlying Poisson statistics. This result is in

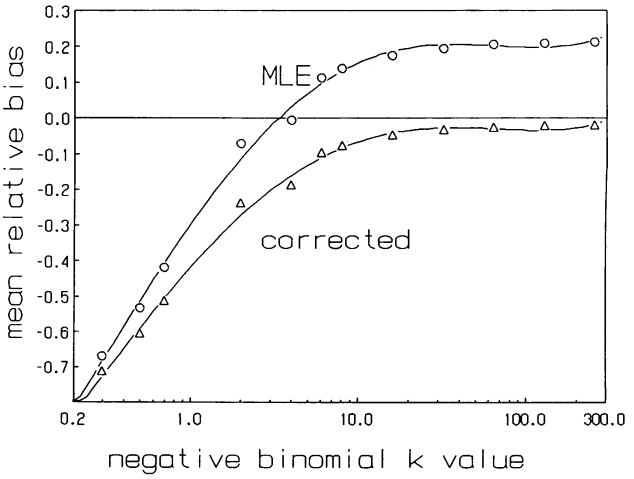

FIG. 3. Effect of negative binomial k value on the estimation bias by the MLE and corrected MLE methods.

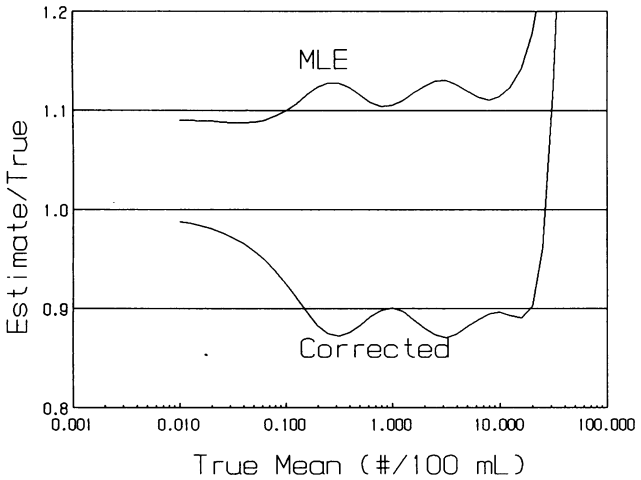

FIG. 4. Estimation bias versus true mean for a negative binomial distribution with $k = 6$.

qualitative accord with the results of Wadley (21), who noted that for single-dilution experiments, a negative binomially distributed population would be underestimated if the Poisson assumption were used to compute an MPN value.

Similar conclusions about the effect of deviation on the relative performance of the MLE versus the corrected methods were obtained for a five-tube, three-decimal-dilution experiment (results not shown). Furthermore, the reduction in bias and MSE was about as good in the three-decimal-dilution protocol as in the four-decimal-dilution protocol.

Based on this analysis, alternative MPN tables for the five-tube, three-decimal-dilution (10, 1, and 0.1 ml) and four-decimal-dilution (10, 1, 0.1, and 0.01 ml) protocols were constructed. Tables 1 and 2, respectively, present the MLE values along with bias-corrected MLE values. Also tabulated are the 5 and 1% confidence limits estimated by using the approach of Loyer and Hamilton (14). For the three-dilution protocol, the Woodward (22) confidence limits are also noted. As noted by Loyer and Hamilton (14), some tube combinations produce null confidence limits and should be regarded as improbable. In other words, no more than 5% of any set of samples should contain combinations with empty confidence limits at the 5% level and similarly for the 1% level. This is analogous to the consistency test suggested by Woodward (22).

Confidence limits. The sensitivity of both methods of computing confidence limits to deviations from the Poisson assumption was assessed. As in the comparison of bias, a negative binomial k of 6 indicates an approximate point at which unacceptable performance is crossed. Figures 5 and 6, respectively, show the confidence limits (95%) plotted under an underlying Poisson assumption (lines) versus the exact confidence limits for the negative binomial (points). These figures are drawn from the three-dilution protocol. In Fig. 5, using the Woodward method (22), the ordinate represents the true mean of the underlying population, whereas in Fig. 6, the ordinate is the bias-corrected estimate for a given tube combination. Note that the Loyer and Hamilton method yields more choppy confidence bands, owing to the diverse likelihood of individual tube scores. However, the effect of the negative binomial distribution is not different on the shift between the lines (Poisson confidence intervals) and the points (negative binomial intervals) for either method.

TABLE 1. Corrected estimates and confidence limits for five-tube, three-decimal-dilution experiment

Score	No./ml		Loyer and Hamilton method				Standard methods (5% limits)	
			5% limits		1% limits			
	MLE	Bias	Low	High	Low	High	Low	High
000	0.00	0.00	0.00	0.09	0.00	0.12		
001	0.02	0.02			<0.01	0.05	0.01	0.1
010	0.02	0.02	<0.01	0.07	<0.01	0.11	0.01	0.1
020	0.04	0.03			0.03	0.05	0.01	0.13
100	0.02	0.02	<0.01	0.14	<0.01	0.18	0.01	0.11
101	0.04	0.04			0.02	0.10	0.01	0.15
110	0.04	0.04	<0.01	0.13	<0.01	0.18	0.01	0.15
111	0.06	0.05			0.07	0.08	0.02	0.18
120	0.06	0.05			0.03	0.13	0.02	0.18
200	0.04	0.04	<0.01	0.19	<0.01	0.25	0.01	0.17
201	0.07	0.06	0.06	0.10	0.02	0.18	0.02	0.2
210	0.07	0.06	0.02	0.20	<0.01	0.26	0.02	0.21
211	0.09	0.08			0.05	0.19	0.03	0.24
220	0.09	0.08	0.05	0.16	0.03	0.23	0.03	0.25
230	0.12	0.10			0.11	0.17	0.05	0.29
300	0.08	0.07	0.02	0.27	<0.01	0.34	0.03	0.24
301	0.11	0.09	0.07	0.17	0.04	0.27	0.04	0.29
310	0.11	0.09	0.03	0.30	0.02	0.37	0.04	0.29
311	0.14	0.12	0.13	0.14	0.06	0.29	0.06	0.35
320	0.14	0.12	0.06	0.27	0.04	0.36	0.06	0.35
321	0.17	0.14			0.11	0.26	0.07	0.4
330	0.17	0.14			0.08	0.31		
400	0.13	0.11	0.03	0.42	0.02	0.51	0.05	0.38
401	0.17	0.14	0.09	0.28	0.05	0.41	0.07	0.45
410	0.17	0.14	0.04	0.49	0.03	0.60	0.07	0.46
411	0.21	0.17	0.11	0.34	0.07	0.49	0.09	0.55
420	0.22	0.17	0.08	0.51	0.05	0.68	0.09	0.56
421	0.26	0.20	0.20	0.30	0.11	0.54	0.12	0.65
430	0.27	0.21	0.14	0.45	0.09	0.65	0.12	0.67
431	0.33	0.24			0.18	0.47	0.15	0.77
440	0.34	0.24			0.17	0.52	0.16	0.8
500	0.23	0.18	0.05	0.78	0.04	1.07	0.09	0.86
501	0.31	0.23	0.13	0.62	0.08	0.98	0.1	1.1
502	0.43	0.30			0.26	0.56	0.2	1.4
510	0.33	0.24	0.07	1.23	0.05	1.59	0.1	1.2
511	0.46	0.32	0.14	1.12	0.10	1.41	0.2	1.5
512	0.63	0.49			0.24	1.15	0.3	1.8
520	0.49	0.35	0.10	1.78	0.07	2.19	0.2	1.7
521	0.70	0.57	0.20	1.78	0.14	2.34	0.3	2.1
522	0.94	0.81	0.52	1.15	0.27	2.00	0.4	2.5
530	0.79	0.67	0.16	2.40	0.12	3.09	0.3	2.5
531	1.09	0.94	0.28	2.69	0.18	3.55	0.4	3
532	1.41	1.19	0.71	2.19	0.39	3.24	0.6	3.6
533	1.75	1.44			1.00	2.63	0.8	4.1
540	1.30	1.11	0.28	3.55	0.18	4.47	0.5	3.9
541	1.72	1.42	0.43	4.27	0.29	5.25	0.7	4.8
542	2.21	1.74	0.89	4.68	0.52	6.17	1	5.8
543	2.78	2.05	1.82	3.24	1.10	5.76	1.2	6.9
544	3.45	2.37			3.16	3.89	1.6	8.2
550	2.40	1.84	0.50	6.92	0.35	9.13	1	9.4
551	3.48	2.38	0.78	10.48	0.49	14.80	1	13
552	5.42	3.69	1.17	16.61	0.87	22.41	2	20
553	9.18	7.49	1.82	25.14	1.45	33.92	3	29
554	16.09	12.09	3.31	45.76	2.24	61.73	6	53
555			7.08	∞	3.98	∞	16	∞

Hence, it is concluded that at and below this level of deviation (i.e., for $k > 6$), both methods provide reasonably robust interval estimates of microbial density.

The confidence limits obtained by using the Loyer and Hamilton approach (14) are, in general, narrower than those assigned by Woodward (22). Additionally, fewer codes were determined to be admissible to the 95% class than those

TABLE 2. Corrected estimates and confidence limits for five-tube four-decimal-dilution experiment

Score	No./ml		Loyer and Hamilton method			
			5% limits		1% limits	
	MLE	Bias	Low	High	Low	High
0000	0.00	0.00	<0.01	0.09	<0.01	0.13
0010	0.02	0.02			<0.01	0.06
0100	0.02	0.02	<0.01	0.07	<0.01	0.12
0200	0.04	0.03			0.03	0.07
1000	0.02	0.02	<0.01	0.14	<0.01	0.19
1010	0.04	0.04			0.02	0.12
1100	0.04	0.04	<0.01	0.13	<0.01	0.19
1110	0.06	0.05			0.06	0.10
1200	0.06	0.05			0.02	0.16
2000	0.04	0.04	<0.01	0.20	<0.01	0.26
2010	0.07	0.06	0.05	0.10	0.02	0.20
2100	0.07	0.06	0.02	0.21	<0.01	0.28
2110	0.09	0.08			0.04	0.20
2200	0.09	0.08	0.05	0.17	0.03	0.26
2300	0.12	0.10			0.08	0.20
3000	0.08	0.07	0.02	0.28	<0.01	0.36
3001	0.11	0.09			0.08	0.16
3010	0.11	0.09	0.07	0.18	0.03	0.29
3100	0.11	0.09	0.03	0.32	0.02	0.40
3110	0.14	0.12	0.10	0.18	0.06	0.32
3200	0.14	0.12	0.06	0.28	0.04	0.41
3210	0.17	0.14			0.10	0.30
3300	0.17	0.14			0.07	0.35
4000	0.13	0.11	0.03	0.44	0.02	0.52
4001	0.17	0.14			0.10	0.27
4010	0.17	0.14	0.09	0.32	0.05	0.45
4100	0.17	0.14	0.04	0.51	0.03	0.65
4101	0.21	0.17			0.13	0.34
4110	0.21	0.17	0.11	0.39	0.06	0.52
4200	0.22	0.17	0.08	0.54	0.05	0.69
4201	0.26	0.20			0.20	0.31
4210	0.26	0.20	0.18	0.39	0.10	0.56
4300	0.27	0.21	0.14	0.50	0.08	0.71
4310	0.33	0.24			0.16	0.50
4400	0.33	0.24			0.15	0.60
5000	0.23	0.18	0.05	0.89	0.04	1.17
5001	0.31	0.23			0.13	0.63
5010	0.31	0.23	0.13	0.69	0.07	1.07
5020	0.42	0.30			0.20	0.72
5100	0.33	0.24	0.07	1.38	0.05	1.82
5101	0.45	0.32	0.33	0.46	0.14	1.07
5110	0.45	0.32	0.14	1.32	0.09	1.78
5111	0.62	0.48			0.35	0.85
5120	0.62	0.49	0.51	0.58	0.20	1.35
5200	0.49	0.35	0.10	1.95	0.07	2.45
5201	0.69	0.56	0.40	0.98	0.20	1.78
5210	0.69	0.56	0.18	2.00	0.12	2.57
5211	0.92	0.80			0.37	1.86
5220	0.93	0.81	0.45	1.55	0.25	2.34
5230	1.19	1.03			0.87	1.70
5300	0.78	0.66	0.16	2.69	0.11	3.39
5301	1.06	0.92	0.59	1.66	0.28	2.69
5310	1.07	0.93	0.28	2.95	0.17	3.72
5311	1.37	1.16	1.35	1.41	0.54	2.88
5320	1.38	1.18	0.54	2.69	0.32	3.63
5321	1.70	1.41			1.10	2.63
5330	1.72	1.43			0.72	3.09
5400	1.28	1.09	0.27	4.17	0.17	5.13
5401	1.66	1.38	0.89	2.75	0.51	4.07
5410	1.69	1.41	0.38	4.90	0.26	6.03
5411	2.12	1.70	1.12	3.39	0.65	4.90
5420	2.16	1.73	0.76	5.13	0.49	6.76
5421	2.64	2.02	2.04	3.02	1.10	5.37
5430	2.71	2.06	1.41	4.47	0.89	6.46
5431	3.26	2.36			1.82	4.68
5440	3.35	2.41			1.74	5.25
5500	2.31	1.82	0.47	7.76	0.30	10.72
5501	3.14	2.30	1.32	6.17	0.74	9.77
5502	4.27	2.97			2.63	5.62
5510	3.29	2.38	0.69	12.30	0.42	15.85
5511	4.56	3.19	1.45	11.22	0.98	14.13
5512	6.31	4.94			2.40	11.48
5520	4.93	3.51	1.00	17.78	0.66	21.88
5521	7.00	5.70	2.00	17.78	1.38	23.44
5522	9.44	8.13	5.25	11.48	2.69	19.95
5530	7.92	6.69	1.58	23.99	1.17	30.90
5531	10.86	9.36	2.82	26.92	1.82	35.48
5532	14.06	11.88	7.08	21.88	3.89	32.36
5533	17.50	14.37			10.00	26.30
5540	12.99	11.07	2.75	35.48	1.82	44.67
5541	17.24	14.19	4.27	42.66	2.88	52.48
5542	22.12	17.36	8.91	46.77	5.25	61.66
5543	27.81	20.50	18.20	32.36	10.96	57.54
5544	34.54	23.70			31.62	38.90
5550	23.98	18.45	5.01	69.18	3.55	91.20
5551	34.77	23.81	7.76	107.15	4.90	147.91
5552	54.23	36.88	11.75	165.96	8.71	223.87
5553	91.78	74.94	18.20	257.04	14.45	338.84
5554	160.94	120.93	33.11	457.09	22.39	616.60
5555			70.79	∞	39.81	∞

reported by Woodward (22). In view of the more rigorous statistical validity of the Loyer and Hamilton method for computing confidence limits and the fact that this approach leads to narrower confidence bands, the use of Tables 1 and 2 for reporting results from MPN determinations is recommended.

Estimation with gross overdispersion. Figures 7 and 8, respectively, present the bias and MSE of the SK and MLE bias-corrected estimators as a function of true density for negative binomial sampling errors for $k = 0.7$. At high values of the true mean, the bias and MSE for the MLE dramatically increase owing to the increasing preponderance of the

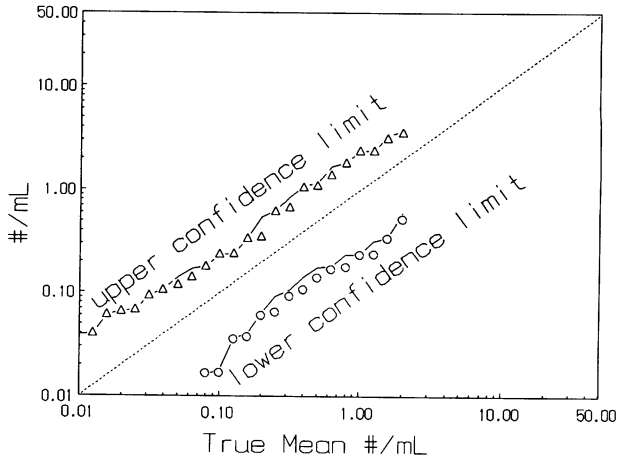

FIG. 5. Confidence limits from the Woodward (22) procedure for Poisson-distributed microorganisms (solid lines) and for negative binomially distributed ($k = 6$) microorganisms (circles and triangles).

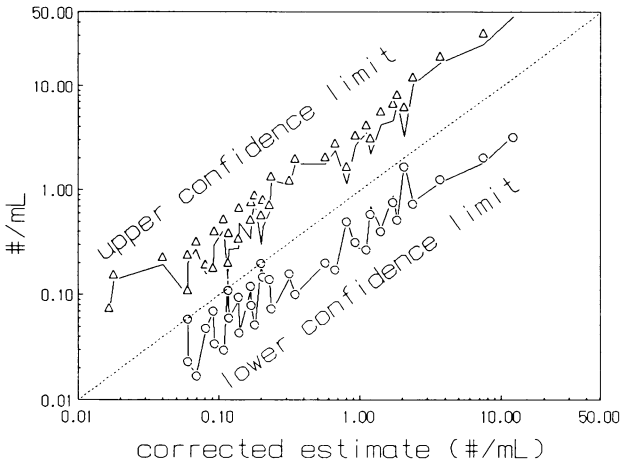

FIG. 6. Confidence limits from the Loyer and Hamilton (14) procedure for Poisson-distributed microorganisms (solid lines) and for negative binomially distributed (k = 6) microorganisms (circles and triangles).

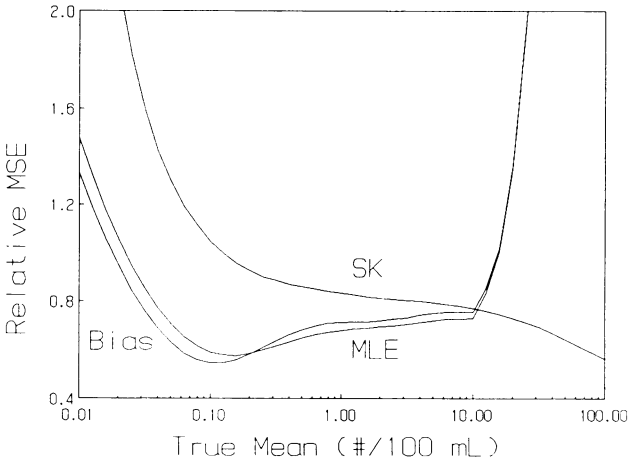

FIG. 8. Relative MSE for various estimators with an underlying negative binomial distribution (k = 0.7).

score ⟨5, 5, 5, 5⟩. However, the SK estimator is less sensitive to this score (owing to the bracketing of the sequence, effectively converting it to the sequence ⟨5, 5, 5, 5, 5, 0⟩). In the range of intermediate densities, it is clear that the SK estimator is substantially less biased than the MLE. The MSE is not substantially greater for the SK estimator than for the MLE in this broad intermediate range (Table 3).

From a more global perspective, the average values of the relative bias and the relative MSE over true densities of 0.02 to 20/ml are shown in Fig. 9 and 10. It is clear that the SK estimator leads to a lowered MSE (Fig. 10) over the entire range of k values studied (0.3 to 256). Furthermore, as the k value decreases and deviations from the Poisson assumption increase, the reduction in MSE becomes more pronounced. With respect to bias, below a k value of approximately 1, the absolute value of mean relative bias is less for the SK estimator than for the MLE (and, although not shown, also for the bias-corrected MLE).

Nonetheless, there is significant negative bias for all estimators studied at low values of the negative binomial k (and high degrees of excess dispersion relative to the Poisson distribution). Thus, further research is desirable to explore means of reducing this bias at high overdispersions.

The tendency of the SK estimator to produce results in excess of the MLE is probably a direct consequence of the assumption that the observed sequence is bracketed by complete infection (at the next highest volume) and complete sterility (at the next lowest volume), thus introducing a slight positive bias (4). The reasonable performance of the SK estimator for assessing negative binomial means stems from the consistency of the negative binomial distribution with the underlying assumptions in the range $0.1 < k < 1$. The tolerance distribution can be written as follows:

$$P = [1 + \exp(\Phi)/k]^{-k}$$

where $\Phi = \log \mu V$.

Table 4 presents the mean and median values of Φ for various values of k. The closeness of the mean and the median down to $k = 0.2$ (where they are within 0.3 log unit, or a factor of 2) indicates the existence of a reasonably symmetric distribution. Furthermore, a $k < 1$, the median value of Φ is positive; hence, by the above definition, the true mean (μ) would be greater than that estimated by the

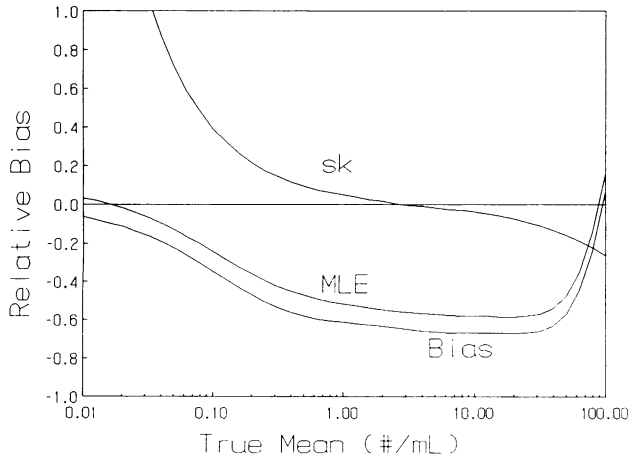

FIG. 7. Relative bias for various estimators with an underlying negative binomial distribution (k = 0.7).

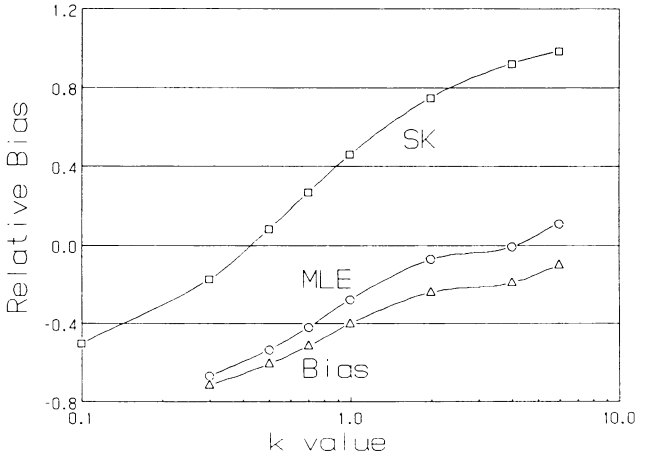

FIG. 9. Average relative bias (0.02 to 20/ml true density) versus negative binomial k value.

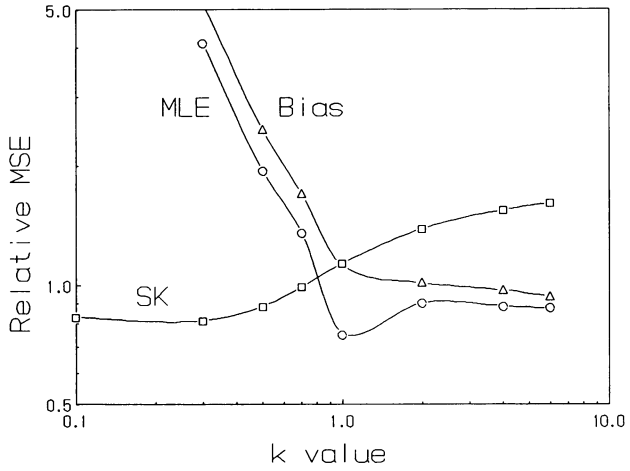

FIG. 10. Average relative MSE (0.02 to 20/ml true density) versus negative binomial k value.

reciprocal of the Spearman Karber volume for 50% infected tubes. In other words, the tendency to a negative bias as k decreases is a direct outcome of the tolerance distribution.

On the basis of this analysis, it is concluded that for negative binomial deviations leading to $k \leq 1.0$, the SK estimator is a better indicator of microbial densities than is the MLE. Although the SK estimator can readily be computed by hand, for convenience, Table 3 presents values for various tube combinations. For comparison, the MLE estimator is also tabulated.

DISCUSSION

The tables presented in this paper can be used as is, as substitutes for the usual MPN tables, in the analysis of data from dilution count experiments. For other tube combinations, the procedures discussed above can be used to construct alternative tables. The use of these tables is illustrated by an example. Assume that 10 replicate analyses, each using a four-decimal-dilution, five-tube protocol, have been performed on each of three different water samples. All three water samples have a mean density of 0.15/ml. However, one water sample has a Poisson distribution of microorganisms, whereas the other two have negative binomial distributions with $k = 1.0$ and 0.4. Table 5 indicates the tube scores recorded from each set of samples (at volumes of 10, 1, 0.1, and 0.01 ml). Each set of tube scores was a random sample, given the assumed distribution.

The first step is to use Table 2 (to four significant figures) to obtain, from each tube score, the MLE and the bias-corrected estimates, along with the 95% confidence limits from the Sterne procedure. The SK estimates for each tube score are obtained from Table 3. It is noted, first, that one of the tube scores for the negative binomial ($k = 0.4$) case, 0200, has no confidence limits. This indicates that this tube score is rare (not appearing in the 95% most frequent set of scores at any value of μ). With only 10 observations, little can be done; however, if such a rate of infrequent samples appeared consistently in a larger data set, a just reason for rejecting the consistency with the Poisson assumption would be provided.

When the three methods are used, the conclusions noted above are seen. The average of the bias-corrected MPN in the case of the true Poisson distribution is much closer to the true value than the MLE itself. However, in the case of the two negative binomial samples, the SK method gives an result closer to the true mean. Perhaps as importantly, in all cases (except for the one rare tube score combination), the true mean was contained within the 95% confidence limits obtained by using the Sterne intervals.

TABLE 3. SK method for estimating densities in four-dilution,[a] five-tube protocol

Score	Estimated no./ml		Score	Estimated no./ml	
	MLE	SK		MLE	SK
0000	0.0000	0.0316	5200	0.4890	0.7943
0010	0.0180	0.0501	5201	0.6851	1.2589
0100	0.0182	0.0501	5210	0.6920	1.2589
0200	0.0367	0.0794	5211	0.9221	1.9953
1000	0.0198	0.0501	5220	0.9322	1.9953
1010	0.0399	0.0794	5230	1.1896	3.1623
1100	0.0402	0.0794	5300	0.7820	1.2589
1110	0.0606	0.1259	5301	1.0570	1.9953
1200	0.0612	0.1259	5310	1.0709	1.9953
2000	0.0446	0.0794	5311	1.3651	3.1623
2010	0.0676	0.1259	5320	1.3842	3.1623
2100	0.0683	0.1259	5321	1.6963	5.0119
2110	0.0920	0.1995	5330	1.7216	5.0119
2200	0.0930	0.1995	5400	1.2756	1.9953
2300	0.1188	0.3162	5401	1.6577	3.1623
3000	0.0777	0.1259	5410	1.6888	3.1623
3001	0.1054	0.1995	5411	2.1161	5.0119
3010	0.1055	0.1995	5420	2.1609	5.0119
3100	0.1069	0.1995	5421	2.6442	7.9433
3110	0.1363	0.3162	5430	2.7084	7.9433
3200	0.1382	0.3162	5431	3.2597	12.5893
3210	0.1694	0.5012	5440	3.3512	12.5893
3300	0.1719	0.5012	5500	2.3116	3.1623
4000	0.1273	0.1995	5501	3.1391	5.0119
4001	0.1652	0.3162	5502	4.2665	7.9433
4010	0.1654	0.3162	5510	3.2906	5.0119
4100	0.1685	0.3162	5511	4.5619	7.9433
4101	0.2107	0.5012	5512	6.3085	12.5893
4110	0.2111	0.5012	5520	4.9322	7.9433
4200	0.2156	0.5012	5521	6.9964	12.5893
4201	0.2632	0.7943	5522	9.4351	19.9526
4210	0.2638	0.7943	5530	7.9243	12.5893
4300	0.2701	0.7943	5531	10.8645	19.9526
4310	0.3250	1.2589	5532	14.0557	31.6228
4400	0.3341	1.2589	5533	17.4979	50.1187
5000	0.2303	0.3162	5540	12.9934	19.9526
5001	0.3111	0.5012	5541	17.2382	31.6228
5010	0.3124	0.5012	5542	22.1159	50.1187
5020	0.4239	0.7943	5543	27.8097	79.4328
5100	0.3274	0.5012	5544	34.5437	125.8925
5101	0.4499	0.7943	5550	23.9790	31.6228
5110	0.4529	0.7943	5551	34.7668	50.1187
5111	0.6197	1.2589	5552	54.2256	79.4328
5120	0.6249	1.2589	5553	91.7842	125.8925
			5554	160.9442	199.5262

[a] The dilutions are 10, 1, 0.1, and 0.01 ml.

Conclusions. Correction for bias in the MLE for microbial densities in dilution experiments is a sufficiently robust procedure to small deviations from Poisson behavior to be adopted. The Loyer and Hamilton procedure (14) for estimating confidence limits leads to smaller intervals and is also robust to small deviations from the Poisson assumption. Alternative MPN tables for the three- and four-decimal-dilution, five-tube series, are presented; these methods were used to construct the tables.

As a practical manner, methods to correct for the well-known positive bias of the MPN technique can result in greater comparability between methods for measuring mi-

TABLE 4. Mean and median of negative binomial-based tolerance distribution as a function of k

k	Log_{10} Φ	
	Median	Mean
10	−0.14403	−0.22855
8	−0.14022	−0.22291
6	−0.13384	−0.21343
4	−0.12100	−0.19409
2	−0.08174	−0.13321
1	0	0.000047
0.8	0.042469	0.071473
0.7	0.073447	0.123994
0.5	0.176091	0.296226
0.4	0.270152	0.444273
0.3	0.435176	0.665360
0.2	0.792391	0.973700
0.1	2.009875	1.212624

croorganisms in water by using dilution and colony count methods. The tables presented in this article should be used in conjunction with statistical tests of conformity of the data set with the underlying assumptions of Poisson replication error.

In the face of more substantial deviations from Poisson behavior, which may be intrinsic to the distribution of microorganisms in an environment, a distribution-free technique to estimate microbial densities must be used.

It is clear that the SK estimator leads to lowered MSE (Fig. 10) over the entire range of k values studied (0.3 to 256). Furthermore, as k decreases and deviations from the Poisson assumption increase, the reduction in MSE becomes more pronounced. Below a k value of approximately 1, corresponding to 100% overdispersion, the absolute value of mean relative bias is less for the SK estimator than for the MLE estimator (and, although not shown, also for the bias-corrected MLE).

The following rules are suggested for analysis of dilution count data. (i) If the degree of overdispersion is less than that for a negative binomial k value of 6.0 ($k > 6$), the maximum likelihood estimator with bias correction should be used. (ii) If the degree of overdispersion is greater than that for a negative binomial k value of 1.0 ($k \leq 1.0$), the Spearman-Karber estimate is superior. (iii) In the intermediate case, the ordinary maximum likelihood estimator appears to be superior to either bias correction or the Spearman-Karber estimate. (iv) The conformity of the underlying microbial distribution with the Poisson distribution can be tested using the modified Stevens range statistic suggested by Haas and Heller (9).

It should be stressed that, depending upon the cause of the deviations from Poisson statistics, although the Spearman-Karber method can provide a better estimate of the actual mean, the mechanism leading to the overdispersion may have resulted in a general reduction in microbial counts. For example, if microbial counts are actually distributed according to Poisson statistics, but if the dilution tube medium is partially inhibitory (and if this inhibition is variable), a negative binomial distribution (or similar overdisperse distributions) may be mimicked. As stressed by Eisenhart and Wilson (5), a finding of deviations from the Poisson assumption always requires a determination of the mechanism for such deviation before the proper interpretation of microbial enumeration data can be made.

TABLE 5. Illustration of application of bias correction, SK, and interval estimates

Distribution[a] and tube score	MLE	Bias-corrected MLE	Sterne confidence limits (5%)		SK estimate
			Low	High	
Poisson					
4100	0.1680	0.1405	0.0427	0.5129	0.3162
5200	0.489	0.3492	0.0955	1.9498	0.7943
5100	0.3274	0.2379	0.0692	1.3804	0.5012
4100	0.1685	0.1405	0.0427	0.5129	0.3162
4100	0.1685	0.1405	0.0427	0.5129	0.3162
4000	0.1273	0.1095	0.0295	0.4365	0.1995
4000	0.1273	0.1095	0.0295	0.4365	0.1995
4100	0.1685	0.1405	0.0427	0.5129	0.3162
3000	0.0777	0.0688	0.0166	0.2754	0.1259
3000	0.0777	0.0688	0.0166	0.2754	0.1259
Mean	0.1900	0.15057			0.32111
NB (k = 1)					
3200	0.1382	0.1179	0.0603	0.2754	0.3162
4100	0.1685	0.1405	0.0427	0.5129	0.3162
1100	0.0402	0.0363	0.0129	0.1349	0.0794
2000	0.0446	0.0402	0.01	0.1995	0.0794
4010	0.1652	0.1381	0.0891	0.3236	0.3162
4000	0.1273	0.1095	0.0295	0.4365	0.1995
3100	0.1069	0.0931	0.0339	0.3162	0.3162
2000	0.0446	0.0402	0.01	0.1995	0.0794
3000	0.0777	0.0688	0.0166	0.2754	0.1259
2100	0.0683	0.0609	0.0229	0.2138	0.1259
Mean	0.0982	0.08455			0.19543
NB (k = 0.4)					
2100	0.0683	0.0609	0.0229	0.2138	0.1259
3000	0.0777	0.0688	0.0166	0.2754	0.1259
2000	0.0446	0.0402	0.01	0.1995	0.0794
4100	0.1685	0.1405	0.0427	0.5129	0.3162
0200	0.0367	0.0332	—[b]	—	0.0794
2000	0.0446	0.0402	0.01	0.1995	0.0794
2100	0.0683	0.0609	0.0229	0.2138	0.1259
3000	0.0777	0.0688	0.0166	0.2754	0.1259
3110	0.1363	0.1165	0.1047	0.1778	0.3162
3100	0.1069	0.0931	0.0339	0.3162	0.1995
Mean	0.083	0.07231			0.15737

[a] NB, Negative binomial.
[b] —, Not in region.

ACKNOWLEDGMENTS

This work was completed while I was on sabbatical at the University of Illinois at Urbana Champaign, under the support of the Advanced Environmental Control Technology Research Center and the Racheff Chair in Environmental Engineering.

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