Using a serial dilution experiment to estimate the density of organisms
by Milton Wayne Loyer

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Statistics
Montana State University

Abstract:
The serial dilution assay is a standard microbiological method for determining the density of organisms in a solution. This paper presents alternatives to current standard serial dilution confidence interval, point estimate and design recommendations.

Original exact confidence intervals are given which are narrower than those available in standard tables. Point estimates are given which have smaller mean squared error than the standard most probable number (MPN) maximum likelihood estimator. An algorithm is given which, for the techniques discussed and within certain researcher-chosen constraints, identifies the optimal design and the most efficient estimator.

This paper also gives the solution to the general finite population serial dilution problem, discusses finite population analogs of the confidence interval and point estimate techniques discussed, and compares the finite and the infinite population models.

The computer programs which were used to obtain the confidence intervals, point estimates and tables presented in the text are given in the Appendix. These programs, including the one for identifying the optimal design, generalize to any number of dilutions, any number of samples per dilution and any dilution factor.

USING A SERIAL DILUTION EXPERIMENT

TO ESTIMATE THE DENSITY OF ORGANISMS

by

MILTON WAYNE LOYER

A thesis submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Statistics

Approved:

Head, Major Department

Chairman, Examining Committee

Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

March, 1981

ACKNOWLEDGMENT

I wish to thank my thesis advisor Dr. Martin A. Hamilton for his advice and assistance throughout my graduate work. Thanks are also due to the Montana State University Research-Creativity Development Committee for funding computer work connected to the thesis and to Messiah College for assisting in the preparation of the final manuscript.

I also wish to acknowledge my wife and my parents for their encouragement and support throughout my education.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The serial dilution assay is a standard microbiological method for determining the density of organisms in a solution. This paper presents alternatives to current standard serial dilution confidence interval, point estimate and design recommendations.

Original exact confidence intervals are given which are narrower than those available in standard tables. Point estimates are given which have smaller mean squared error than the standard most probable number (MPN) maximum likelihood estimator. An algorithm is given which, for the techniques discussed and within certain researcher-chosen constraints, identifies the optimal design and the most efficient estimator.

This paper also gives the solution to the general finite population serial dilution problem, discusses finite population analogs of the confidence interval and point estimate techniques discussed, and compares the finite and the infinite population models.

The computer programs which were used to obtain the confidence intervals, point estimates and tables presented in the text are given in the Appendix. These programs, including the one for identifying the optimal design, generalize to any number of dilutions, any number of samples per dilution and any dilution factor.

1. INTRODUCTION

Halvorson and Ziegler (1933a) state "the use of dilution methods ...dates back to the early days of science" and note that Pasteur, for example, was using serial dilution techniques about 1875. Typically, one seeks to estimate the number of organisms per unit volume of solution under the assumptions that (1) the organisms are randomly distributed throughout the solution and (2) each sample from the solution, when incubated in the culture medium, is certain to exhibit fertility whenever the sample contains one or more organisms. If the solution averages λ organisms per unit volume and z is the dilution (multiple of the unit volume selected for analysis), then, under the Poisson probability model, P(sterile sample) = $e^{-\lambda z}$. In practice, one guards against obtaining samples which are likely to be either all sterile or all fertile by using more than one dilution. Letting X_i equal the number of fertile samples in n_i trials at the i^{th} dilution, $P(X_i=r) = \binom{n_i}{r}(1-e^{-\lambda z_i})^r(e^{-\lambda z_i})^{n_i-r}$.

In the first definitive study of the problem of estimating λ using serial dilutions, McCrady (1915) described the estimate $\hat{\lambda}$, the value of λ that maximizes the probability of obtaining the specific arrangement of fertile and sterile samples observed. McCrady called $\hat{\lambda}$ the "most probable number" (MPN) and presented the procedure, which today is known as maximum likelihood (ML) estimation, as Bayes estimation with an improper uniform prior on λ.[a] To justify the procedure, he cites, among others, distinguished late nineteenth century mathematician Richard L. Edgeworth who stated, "The assumption that any probability constant about which we know nothing in particular is as likely to have one value as another, is grounded upon the rough but solid experience that such constants do, as a matter of fact, as often have one value as another."

For k dilutions, the likelihood function is given by

(1.1) $$L(x_1,x_2,\ldots,x_k;\lambda) = \Pi\binom{n_i}{x_i}(1-e^{-\lambda z_i})^{x_i}(e^{-\lambda z_i})^{n_i-x_i}$$

and the maximum likelihood estimate for λ, still most commonly referred to as the MPN, is the λ which solves $\Sigma(x_i z_i e^{-\lambda z_i})/(1-e^{-\lambda z_i}) = \Sigma(n_i-x_i)z_i$, which simplifies (deMan 1977) to

(1.2) $$\Sigma n_i z_i = \Sigma x_i z_i/(1-e^{-\lambda z_i}).$$

For $k>1$, the solution to (1.2) must be obtained by iterative methods. Several programs (e.g., Parnow 1972) to obtain the MPN for any k, any z_i and any n_i are readily available.

While the methods of all sections of this paper generalize to any k, any z_i and any n_i (except in Chapter 5 where it is required that $\Sigma n_i z_i \leq 1$), the numerical examples given are for the commonly encountered case of k=3 decimal dilutions $z_i=(.1)^i$ with $n_i=3$ for $i=1,2,3$. The 64 possible (X_1,X_2,X_3) sample results will be referred to by the codes 000,001,...,332,333. For these k, z_i and n_i, the first three columns of Table 1 summarize the results presented and recommended by standard reference works.

TABLE 1

95% Confidence Intervals: n=3, z_1=.1 z_2=.01 z_3=.001

result	MPN[a]	Woodward[b]	deMan[c]	Combining[d] Independent Results	Minimum[e] Expected Width
000	0.0	0-9[f]		0-12	0-13
001	3.0	0-9	<1-17	2-15	
002	6.0				
003	9.0				
010	3.0	.085-13	<1-17	<1-16	2-10
011	6.1			7-19	
012	9.2				
013	12				
020	6.2		2-22	4-17	
021	9.3			16-20	
022	12				
023	16				
030	9.4			17-17	
031	13				
032	16				
033	19				
100	3.6	.085-20	<1-21	<1-24	<1-25
101	7.2	.87-21	2-27	3-28	
102	11			26-28	
102	15				
110	7.3	.88-23	2-28	1-30	3-20
111	11	3-36	4-34	7-35	
112	15			35-35	
113	19				
120	11	2.7-36	4-35	5-32	
121	15		6-41	17-37	
122	20				
123	24				
130	16		6-42	18-32	
131	20				
132	24				
133	29				
200	9.1	1.0-36	2-38	1-42	<1-37
201	14	2.7-37	5-48	5-50	11-14
202	20			27-50	
203	26				
210	15	2.8-44	5-50	3-55	5-42
211	20	7-89	7-60	9-64	
212	27			36-65	
213	34				
220	21	3.5-47	8-62	7-61	10-32
221	28	10-150	11-74	18-71	
222	35			51-72	
223	42				
230	29		11-77	19-63	
231	36			40-74	
232	44				
233	53				
300	23	3.5-120	<10-130	3-137	4-120
301	39	6.9-130	10-180	10-175	14-69
302	64	15-380	20-230	42-183	
303	95				
310	43	7.1-210	10-210	5-257	7-200
311	75	14-230	20-280	15-320	21-180
312	120	30-380	40-350	51-340	
313	160			195-345	
320	93	15-380	30-380	11-456	12-360
321	150	30-440	50-500	26-594	38-400
322	210	35-470	80-640	69-659	120-260
323	290		110-790	208-687	
330	240	36-1300	<100-1400	27-1612	26-990
331	460	71-2400	100-2400	54->1800	70-2000
332	1100	150-4800	300-4800	115->1800	140-4070
333	∞	460-∞[f]		298-∞	370-∞

[a] American Public Health Association (1970, page 101)
[b] American Public Health Association (1971, page 676); see section 2.1
[c] deMan (1977); see section 2.2
[d] see section 2.3
[e] see section 2.4
[f] one-sided 95% confidence interval

This paper examines presently recommended serial dilution interval estimation (Chapter 2), point estimation (Chapter 3) and design (Chapter 4) techniques. In each chapter, alternatives are developed and compared to the currently standard methods. In Chapter 5, the exact solution is given to the finite population serial dilution problem.

2. CONFIDENCE INTERVALS

Sections 2.1-2.4 present two commonly used and two new methods for constructing exact 100(1-α)% confidence intervals. Several approximate confidence interval techniques are discussed briefly in section 2.5. The 95% confidence intervals obtained by the methods in sections 2.1-2.4 are given in the final four columns of Table 1. As apparent from the discussion below, the methods of sections 2.1-2.3 can be used to construct one-sided confidence intervals, and for these methods the endpoints given in Table 1 may be used as the endpoints for appropriate 97.5% one-sided confidence intervals.

2.1 Woodward's Method

Perhaps the most commonly used 95% confidence intervals are those given by the American Public Health Association (1971, page 676). Prepared by Woodward (1957) and appearing in the "Woodward" column of Table 1, these intervals are the accepted norm by which other procedures are often judged (e.g., Martins and Selby 1980). Woodward ranked each of the 64 possible $X_1X_2X_3$ outcomes according to the magnitude of the MPN and then constructed 95% confidence intervals (i.e., approximate intervals, since the outcome space is discrete) by testing $H_o:\lambda=\lambda_o$ for selected λ_o values in $[0,\infty)$. For a given $X_1X_2X_3$ outcome, Woodward rejected $H_o:\lambda=\lambda_o$ if and only if that $X_1X_2X_3$ outcome produced an MPN in the lower 2.5% or the upper 2.5% of the probability distribution of MPN's generated under H_o. The set of all

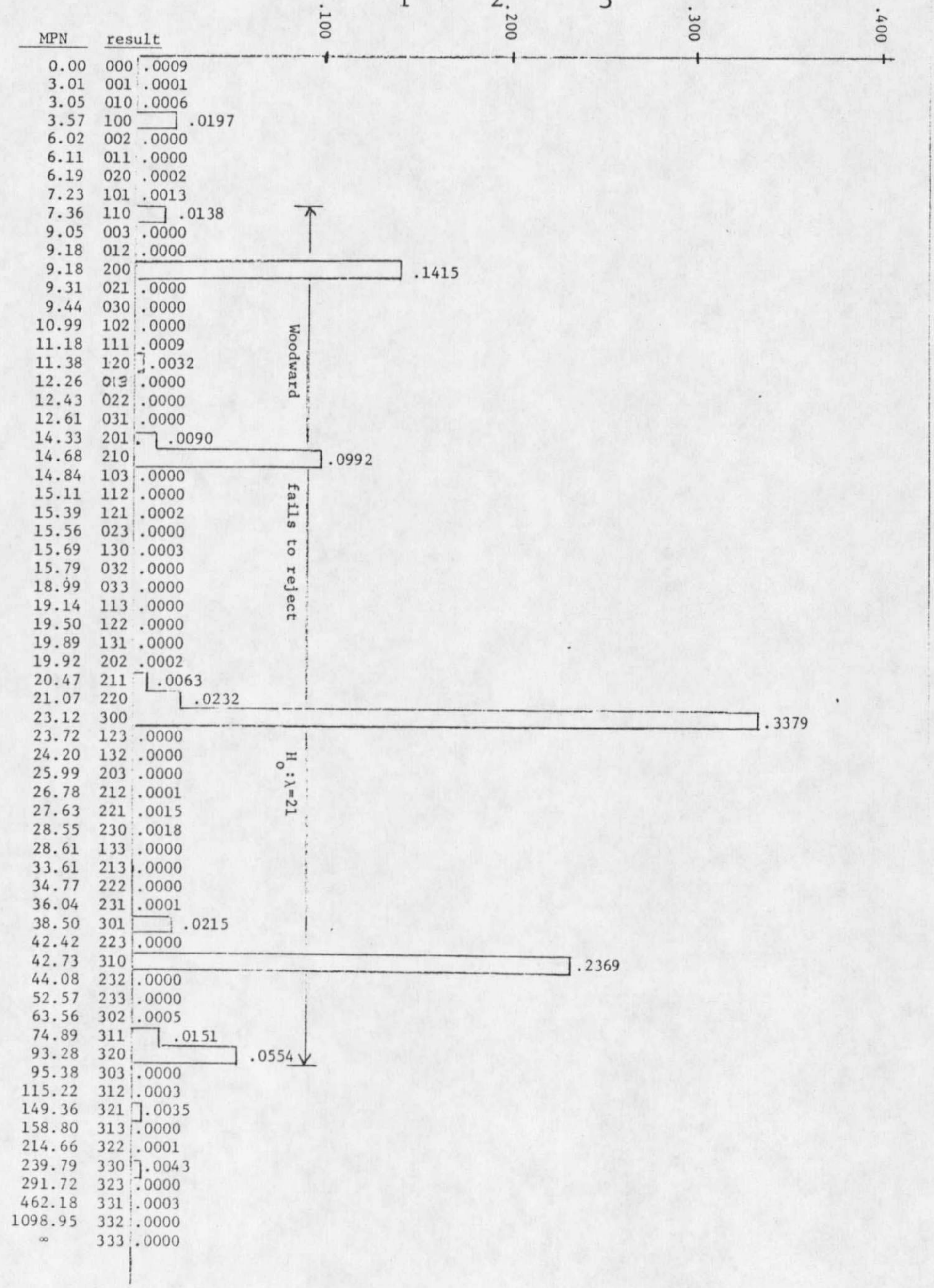
λ_o's not rejected for any $X_1X_2X_3$ outcome comprise the two-sided 95% confidence interval associated with that outcome.

Figure 1 illustrates the Woodward method. When testing $H_o:\lambda=21$ vs. $H_a:\lambda\neq21$, one obtains the distribution of MPN's shown. Rejecting the .025 most extreme results in each tail, Woodward rejects $\lambda=21$ for $X_1X_2X_3$=000,001,010,100,002,011,020,101 in the lower tail and for $X_1X_2X_3$=333,332,331,323,330,322,313,321,312,303 in the upper tail. The Woodward 95% confidence intervals of Table 1 for these results (when given) should not include the value 21. This is true for all cases except $X_1X_2X_3$=101, which represents an error in Woodward's calculations. Nor is this the only error in Woodward's work, as deMan (1975) notes. "In the table presented by Woodward (1957)," he states, "a few mistakes were also found, but they were minor. Undoubtedly, this table should have been given more attention than it apparently received."[b] The program used to generate the probabilities for Figure 1 is given in Appendix I. The MPN's in Figure 1 were obtained by Newton's method within the program of Appendix III.

Two additional comments regarding Woodward's confidence intervals need to be made. First, a caveat given by Woodward but often omitted by those reproducing his tables should be repeated. For the 000 (333) result, Woodward rejects $H_o:\lambda=\lambda_o$ if and only if the MPN is in the upper (lower) 5% of the sampling distribution and presents only upper (lower) 95% confidence intervals.

FIGURE 1
Distribution of Possible Sample Results (arranged by magnitude of MPN)

$\lambda=21$ $n=3$, $z_1=.1$ $z_2=.01$ $z_3=.001$



Secondly, while Woodward's method provides a 95% confidence interval for each of the $X_1X_2X_3$ possible outcomes, his 1957 table includes confidence intervals only for what he determines to be the 22 *most* likely $X_1X_2X_3$ outcomes. The remaining 42 $X_1X_2X_3$ outcomes he calls "improbable" and recommends that they not be used for making inferences. In other words, there are some $X_1X_2X_3$ outcomes (e.g., the result 003 -- no organisms present in the more concentrated .1 or .01 dilutions, but organisms present in all three samples at the weakest .001 dilution) for which Woodward's method gives a 95% confidence interval in which he apparently does not have 95% confidence. The last two methods of Table 1 eliminate this subjectivity by inherently failing to give confidence intervals (i.e., by giving empty confidence intervals) for improbable results.

A further inspection of Woodward's method reveals some serious practical flaws. Note from Figure 1 that ordering the $X_1X_2X_3$ results by the magnitude of the MPN does not yield a unimodal sampling distribution. According to most statistical inference texts (e.g., Cox and Hinkley 1974, page 66), this means that the MPN is not an acceptable test statistic since more extreme values of the MPN do not necessarily give stronger evidence of departure from H_o. Fisher (1956, page 98) objected to a procedure of Bartlett for similar reasons since his statistic "does not increase or decrease monotonically for changes in the weight of the evidence."

The difficulty caused by a multi-modal sampling distribution can be seen from Figure 1. Woodward's rejection region includes the result 100 for which $P(X_1X_2X_3=100) = .0197$ but fails to include the less likely result 110 for which $P(X_1X_2X_3=110) = .0138$. In fact, Woodward cannot place in his rejection region any result, no matter how unlikely, which gives an MPN larger than 3.57 unless the result 100 were already in the rejection region. Woodward's intervals, then, form a "staircase" based on the magnitude of the MPN so that the lower (upper) confidence limit associated with one sample result cannot be lower (higher) than the limit associated with another sample result yielding a lower (higher) MPN.[c] Consequently, the width of an interval is not determined by the precision associated with the sample result, and preliminary calculations verify that the actual level of Woodward's intervals is greater than 95%.

2.2 DeMan's Method

Another set of commonly used confidence intervals is given by deMan (1975) and appears in the "deMan" column of Table 1. Even though deMan uses the term "confidence interval," his procedure does not meet the necessary and sufficient conditions given by Neyman (1941), the originator of the concept of confidence intervals as presently employed. In the opinion of many authors (e.g., von Mises 1942), however, this is not necessarily to deMan's detriment. DeMan does, in fact, provide the limits of the middle 95% of the likelihood distribution for each $X_1X_2X_3$ result or, equivalently, the Bayesian interval for λ under an improper uniform prior. While the posterior function $f(\lambda;x_1,x_2,x_3)$ is defined continuously for $\lambda\varepsilon[0,\infty)$, deMan used discrete approximations in both directions from the MPN and truncated the posterior distribution whenever an additional tail histogram area contributed less than .000005 of the cumulative total.

DeMan's method, like Woodward's, provides a 95% confidence interval for each of the 64 possible $X_1X_2X_3$ results. Also like Woodward, deMan sates that "MPN tables should be restricted to results having a defined minimal probability" and gives no confidence intervals for "improbable" $X_1X_2X_3$ results. In their original articles, deMan and Woodward agree in all but two cases on what is improbable (deMan considers the result 312 improbable, but not the result 211). It is clear that each finds himself deciding which of his 95% intervals he chooses not to accept with nominal level 95 per cent.

If one desires to use a Bayesian procedure, of course, he is not limited to an improper uniform prior. In general, the more specific prior information the researcher has (or is willing to assume) about λ, the narrower he can make his "confidence interval." Even if the researcher begins with complete ignorance about λ, however, the uniform prior may not be the appropriate prior. Box and Tiao (1973) discuss Bayesian interval estimation in general and define a "noninformative prior," based on Fisher's information, that they recommend for the researcher with little or no prior information.

2.3 Methods of Combining Independent Results

Since each of the three dilutions gives results independent of those of the other dilutions, the total serial dilution experiment yields three independent point estimates and three independent confidence intervals for λ. First impressions might suggest constructing $\sqrt[3]{.95}$ confidence intervals C_1, C_2 and C_3 for each of the three dilutions and using the intersection $C_1 \cap C_2 \cap C_2$ as an experiment-wide 95% confidence interval. This is certainly statistically acceptable and has the advantage of permitting certain unlikely results to produce empty confidence intervals whenever $C_1 \cap C_2 \cap C_3 = \emptyset$. There are, however, at least two disadvantages major enough to discourage the use of this procedure.

First, the three independent confidence intervals have at most three distinct lower endpoints and three distinct upper endpoints. This means that the 64 possible $X_1X_2X_3$ results generate a maximum of $3^2=9$ distinct non-empty confidence intervals. Certainly, there exists the possibility of different $X_1X_2X_3$ results yielding identical confidence intervals. This would not be undesirable if the minimal sufficient statistic were some function of the X_i (e.g., $Y=\Sigma X_i$) that could assume only some number of values considerably less than 64.