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Abstract

Aims. Microbial samples are often serially diluted to estimate the number of microbes in a sample, whether as colony-forming units of bacteria or algae, plaque forming units of viruses, or cells under a microscope. There are at least three possible definitions for the limit of detection (LOD) for dilution series counts in microbiology. The statistical definition that we explore is that the LOD is the number of microbes in a sample that can be detected with high probability (commonly 0.95).

Methods and Results. Our approach extends results from the field of chemistry using the negative binomial distribution that overcomes the simplistic assumption that counts are Poisson. The LOD is a function of statistical power (one minus the rate of false negatives), the amount of over-dispersion compared to Poisson counts, the lowest countable dilution, the volume plated, and the number of independent samples. We illustrate our methods using a data set from *Pseuodomonas aeruginosa* biofilms.

Conclusions. The techniques presented here can be applied to determine the LOD for any counting process in any field of science whenever only zero counts are observed.

Significance and Impact of Study. We define the LOD when counting microbes from dilution experiments. The practical and accessible calculation of the LOD will allow for a more confident accounting of how many microbes can be detected in a sample.

Keywords: microbial counts; Poisson; overdispersion; negative binomial

1. Introduction

Microbiologists use the limit of detection (LOD) to describe the minimum number of microbes that can be detected by their analytical approach. Limits of detection have been widely discussed for decades, especially in the field of analytical chemistry. The theory of the LOD began to take shape in the 1920's (see Fisher, Thornton, and Mackenzie 1922, for example), but not until the 1960's did it begin to take precedence in research. LODs have been introduced into many different aspects of chemistry, including water analysis and spectrochemical analysis. In analytical chemistry, for example, one instrument may be able to detect a chemical concentration as small as one part per billion but another instrument may not be able to measure any concentrations less than one part per million. Although LODs have been used for many years in chemistry, a precise, broadly accepted definition of the LOD was not adopted until the mid-1990s. Before that, Currie (1996) reported that a review of literature in the 1960's demonstrated that LOD definitions spanned almost three orders of magnitude when used to measure the same quantity.

A universal definition of the LOD for microbiological purposes has not yet been established (see e.g., Duarte et al. 2015, Evers et al. 2010). One popular operational definition in dilution series used by microbiologists is to define the LOD as 1 colony forming unit (CFU) for bacteria or algae, or 1 plaque forming unit (PFU) for viruses (e.g., see Evers et al. 2010, Magnani 2021, Sutton 2011). We show that this definition may be too simplistic because it is not associated with any measure of statistical uncertainty (e.g., a confidence level). Many times, LOD refers to the suggested range to consider in a single plate when plate counting, though the range varies by plating method (e.g., 30 to 300 for some plating methods; ASTM International D5465, Ben-David and Davidson 2014, Magnani 2021, Sutton 2011). These ranges have been

established in part because lower counts can exhibit extra-Poisson variability (see, e.g., Jongenburger et al. 2010). Adding to potential confusion, the International Organization for Standardization (ISO) uses different terms to refer to the limit of detection for different types of microbiological methods. ISO uses 'LOD' to refer to qualitative methods (that assess presence/absence of microbes), and 'LOQ' (limit of quantification) to refer to quantitative microbiological methods (ISO 2016, p.5). In chemistry, however, LOQ is defined as a quantity that is greater than the LOD by a factor that is between five and ten (Thompson & Ellison 2013). In our paper, we use the term "limit of detection" (LOD) to describe quantitative methods that count microbes as suggested by AOAC International (AOAC International 2006, Wehling et al 2011) and is consistent with terminology used in other fields of science (e.g., Currie 1968, Currie 1987, Currie 1996, Koenig 2021, Thompson & Ellison 2013). Because the LOD is an important characteristic of any microbiological method, an accepted LOD definition among microbiologists would facilitate consistent communication.

While many publications describe the empirical estimation of the LOD in microbiology (e.g., Corry et al. 2007, Feldsine et al. 2002, Reiske 2019, Uhlig and Gowik 2018, Vencia et al. 2014, Yáñez et al. 2005), the literature on the theoretical underpinnings of a microbiological LOD is limited. Standards setting organizations AOAC International and ISO give a probabilistic definition for the LOD for microbiological counts based on the Poisson distribution (AOAC International 2006, ISO 2016, Wehling et al 2011).

The Poisson distribution has been used for determining the LOD for other counting processes, for example, when counting asbestos fibers (ASTM D6620, Koenig 2021). Unfortunately, the use of the Poisson distribution for modeling microbiological counts may be overly optimistic when the counts exhibit extra-Poisson variability (Bliss & Fisher 1953,

Jarvis 2016). For the Poisson model, the microbes are assumed to be randomly distributed throughout the volume in the original beaker (see, e.g., Bliss and Fisher 1953, Jarvis 2016). This is the ideal case, although in reality, microbes may not always be distributed in this way. Sometimes there will be loose clusters of microbes that will develop in a dilution. When clusters are present, it is possible that a pipetted sample contains only a cluster or perhaps no clusters at all. The clumping causes the average count of microbes to vary from sample to sample. When there is clumping in the initial density, the extra-variability incurred from sample to sample is extra-Poisson variability. Another source of extra-Poisson variability could be differing pipette volumes (Chase and Hoel 1975). When the original sample is diluted and a volume is pipetted onto an agar plate at each dilution, we are assuming that all of the volumes taken are the same. Technology has evolved so that microbiologists can be accurate when pipetting, but, of course, there will always be some error. An important contribution of this work is to present a definition for the LOD that can account for this over-dispersion.

Counting processes are a crucial quantitative step in microbiological methods because often the goal is to estimate the number of microbes suspended in a volume or attached to a surface. These counts can be generated by plating, filtering, cytometry or microscopy. In many scenarios there is a high number of microbes in the initial sample, so the initial microbial sample is diluted repeatedly until a small number of microbes is counted at some convenient dilution. This count is then scaled up to estimate the number of microbes in the original sample (e.g., see Equation 2 in Garre et al. 2019, or Equation (5) below). Challenges can arise if the dilution(s) that were counted yield only zero counts. Simple statistical approaches would estimate zero organisms in the original sample with no associated measure of uncertainty. The LOD in this

scenario estimates how many microbes there could be in the original sample so that all zeros are observed with small probability.

The purpose of this paper is to review definitions of the LOD from chemistry and then suggest a definition for use in microbiology when counting microbes from dilution experiments. To overcome Poisson assumptions, we provide a definition of the LOD using the negative binomial distribution and show how to scale the LOD for dilution series. Previous work focuses on LOD for a single sample, where the LOD decreases as the volume plated increases. Here we show that the LOD also decreases as the number of replicate samples increases. The same LOD approach that we present may be applied whether the data are CFUs of bacteria or algae on agar plates from a viability assay; PFUs of viruses in host cells; or cells observed under a microscope. In our examples we explicitly focus on the case of CFUs.

2. Materials and Methods

2.1 Limits of Detection in Chemistry

In the 1960's, Kaiser used a hypothesis test to compute a LOD in spectrochemical analysis (Kaiser 1965). Kaiser discussed examining the null hypothesis that the sample taken is a "blank" (i.e., contains no microbes) versus the alternative that it is not a blank. For multiple samples, the null hypothesis is that the mean of the samples is the same as the mean of the blanks. Kaiser advocated the testing rule that the null hypothesis is rejected if the sample is more than three standard errors away from the blank mean (Kaiser 1965). Put another way, Kaiser sought to control the false positive (Type I) error rate. Calculating the LOD in this way is similar to the 'limit of quantification' defined by ISO 16140-2 (p. 24). Initially, Kaiser ignored the false negative error (Type II error) (Currie 1987). Many individuals recognize Kaiser's work in the

detection field as ground-breaking and many still utilize this concept for the LOD (Currie 1968, Thompson & Ellison 2013).

In 1996, Currie published a definition of the LOD that was accepted as the standard by the International Union of Pure and Applied Chemistry (IUPAC) and ISO stemming from work in analytical and radiochemistry (Currie 1996). Currie's (1996) definition of the LOD, or the minimum detectable value L_D, is the solution of Equation (1) for L_D, where the random variable $\hat{L}$ is the estimator of the quantity of interest, L is the true quantity of interest, L_C is the critical value, or " the minimum significant *estimated value* of the quantity of interest", and β is the probability of producing false negatives (i.e., indicating that quantity is not detectable when the level is really at $L = L_D$):

$$\Pr\left[\hat{L} \leq L_C \middle| L = L_D\right] = \beta. \qquad (1)$$

The numerical value of L_C is established by expert opinion of the associated maximum Type II error rate. In chemistry, the conventional value for β = 0.05 (Currie 1996).

2.2 The Need for Limits of Detection in Microbiology

A common goal in microbiology is to estimate the density of microbes in a volume in a beaker. The microbes in the beaker may have been harvested from an environmental sample, or from a benchtop reactor. The microbes may have been in a planktonic state or homogenized from a mature biofilm. An aliquot (sub-volume) is taken from the beaker with a calibrated pipette (often orders of magnitude less than the volume in the original sample) and placed into sterile diluent because, generally, the initial density is too large to be counted (see, e.g., Maturin & Peeler 1998). From this diluted sample, a portion is taken again with a calibrated pipette and the number of viable microbes in this liquid sample is typically found by plate counting techniques. Plate counting can be done with the pour plate, the spread plate, and the drop plate methods. For

the calculations, the main difference among these three methods is the volume plated; the pour plate volume is approximately 1mL, the spread plate volume is typically 100µL or 1mL, and the drop plate volume is typically 10µL. When spread plating, the volume in the pipette is placed onto an agar plate, and spread evenly with a sterile spreader. When the plate is incubated, the viable microbes divide and form colonies that are non-overlapping and can be counted. The number of CFUs of microbes are then counted on the agar plate and then scaled up by the dilution factor to estimate the number of microbes in the original suspension.

The LOD issue arises if, after dilution, there are no CFU on the agar plate. This does not necessarily mean that the original suspension has zero microbes. It is possible that there are microbes in the suspension, but the original suspension has been diluted to the point where there are no microbes in the sample plated. For example, suppose it is known that there are 100 CFUs in a beaker of a 10 mL suspension. A sample of 1 mL taken from the original suspension is placed into a beaker containing 9 mL of sterile diluent. From this 10 mL, a 1 mL sample is spread onto an agar plate so that CFUs can be counted. Suppose that there are no CFUs on the agar plate. When the count of zero CFUs is scaled up, the estimated number of microbes in the original sample would be zero although there are 100 microbes in the original sample. In this example, the LOD is the number of microbes in the original sample that assures non-zero CFU on the agar plate with high probability $(1 - \beta)$ or likewise assures zero CFU on the agar plate with low probability (β).

Such a probabilistic definition for a LOD for microbiology should be more universally established to help explain these problematic counts of zero and to give microbiologists a method to compare their laboratories more precisely. Like Duarte et al. (2015) and Thompson & Ellison (2013), we do not advocate that counts less than the LOD be excluded or censored. Niemela

(1983) may have said it best: "it is foolish to disregard colony counts below [the LOD] if they happen to be the only ones available." Indeed, the practice of excluding data, in this case microbial counts, merely because the counts are below the LOD has led some to advocate that the LOD should not be used at all (Thompson & Ellison 2013). The LOD is a useful concept because it gives the microbiologist a measure of the minimum number of microbes in a sample that can be detected with high probability. Generally, a count of zero will occur in subsequent dilutions if a count of zero was found for the first dilution plated. Therefore, we will focus on counts only at the first dilution plated when determining a LOD.

An approach to defining the LOD that focuses on controlling the probability of false positives (Type I errors) based on testing blanks (as proposed by Kaiser 1965) is not applicable to viability assays that count microbes. While some microbiological procedures would generate non-zero data from blanks (samples with no microbes), e.g., ATP or e-DNA assays, plate count assays would only generate zero CFU from blank samples (unless there was some contamination). Thus, a LOD based on Type I error control is not well defined and not an informative tool for microbiologists. Therefore, we use the LOD definition proposed by Currie (1996) that is nearly universal in chemistry that controls the probability of false negatives (Type II errors).

Our strategy is to adapt the Currie's definition to microbiological plate count assays (AOAC 2006, ISO 2016). That definition (Equation (1)), when adapted to counting CFU, leads to Equation (2), where X is a random variable denoting the number of CFUs of microbes counted at the first plated dilution, L is the number of microbes in the original beaker, β is the largest probability of incorrectly obtaining zero CFUs (specified by the microbiologist), and L_{original} is

the LOD for the CFUs in the original sample of microbes, which is the minimum of all values of L that satisfy

$$P\left[X = 0 \middle| L = L_{\text{original}}\right] \leq \beta. \qquad (2)$$

Note that L_{original} depends on β. For a probability of $\beta = 0.05$, L_{original} is the density of microbes in the original volume for which there is no more than a 5% chance of seeing zero CFU at the first plated dilution. This notation for the LOD utilizes a subscript to indicate the units. As we will see below, to calculate L_{original}, first the LOD per plated volume, L_{plate}, is calculated.

2.3 Detection Limit Formulation

LODs (L_{original}) can be found using Equation (2). Suppose X, the number of CFUs, is a Poisson random variable with rate parameter Λ. Occasionally, X can exhibit extra Poisson variability (Bliss and Fisher,1953; Jarvis 2016). To model this over-dispersion, we let Λ be a random variable. Suppose that Λ is distributed as a gamma random variable with parameters μ and coefficient of variation (CV). The CV is the standard deviation of Λ (σ) divided by the mean of Λ (μ): $CV = \frac{\sigma}{\mu}$. To write the probability density function of Λ in shape-scale form, let $d = \frac{1}{CV^2}$. Using the parameters d (shape) and $\tau = \frac{\mu}{d}$ (scale; Bain and Engelhardt 1987), the probability density function of Λ is

$$f_{\Lambda}(\lambda) = \frac{\lambda^{d-1} e^{-\lambda/\tau}}{\Gamma(d)\tau^d}$$

for $\lambda > 0$, $d, \tau > 0$. The conditional distribution of X given Λ follows a Poisson distribution with probability mass function

$$f_{X|\Lambda}(\mathrm{x} \mid \lambda) = \frac{e^{-\lambda}\lambda^x}{x!}$$

for $x = 0, 1, 2, ...$ The random variable *X* can then be shown to be from a negative binomial distribution with mean $\mu = d\tau$ and variance $d\tau + d\tau^2 = \mu + \frac{\mu^2}{d}$ (McCullagh and Nelder 1989):

$$f_X(x) = \int_0^\infty f_{X|\Lambda}(x|\lambda) f_\Lambda(\lambda) d\lambda$$

$$= \frac{\Gamma(x+d)}{\Gamma(d)x!}\left(\frac{\tau}{\tau+1}\right)^x \left(\frac{1}{\tau+1}\right)^d . \quad (3)$$

The term $\frac{\mu^2}{d}$ is the extra Poisson variability. Reparameterizing the function in Equation (3) using the shape and scale parameterization d (shape) and $\mu = d\tau$ (scale), the distribution of *X* is

$$f_X(x) = \frac{\Gamma(x+d)}{\Gamma(d)x!}\left(\frac{\mu}{\mu+d}\right)^x \left(\frac{d}{\mu+d}\right)^d \quad (4)$$

where $d = \frac{1}{CV^2}$.

Let

$$L_{\text{plate}} = k \times L_{\text{original}}$$

take the place of μ in (4). The quantity L_{plate} is the LOD for CFU in the volume plated, *u*. The factor *k* is the dilution factor, $k \leq 1$, to find L_{original}, the LOD for CFU in the original volume *V*,

$$k = \frac{u}{V \times 10^f}, \quad (5)$$

where f specifies the first (lowest) 10-fold dilution that was plated (f is one of 0,1,2, …). If multiple plates are used at dilution *f* (as is commonly the case), then *u* is the total volume plated across all plates at dilution *f*, and *X* is the total number of CFUs counted in all plates at dilution *f*. Then, following equation (2), L_{plate} is the smallest value satisfying the following equations:

$$P[X = 0] = \left[\frac{d}{L_{\text{plate}}+d}\right]^d \leq \beta \quad (6)$$

$$\Rightarrow \frac{d}{L_{\text{plate}}+d} \leq \sqrt[d]{\beta}$$

$$\Rightarrow L_{\text{plate}} = k \times L_{\text{original}} \geq \left(\frac{d}{d\sqrt{\beta}}\right) - d \qquad (7)$$

where $d = \frac{1}{CV^2}$.

If Λ does not vary (i.e., if there is no over-dispersion), then $CV = 0$, $\lim_{d\to\infty} \frac{\mu^2}{d} = 0$ and X is a Poisson random variable with mean L_{plate}. Thus, the LOD in the Poisson case is derived from

$$P[X = 0] = \lim_{d\to\infty} \left(\frac{d}{L_{\text{plate}} + d}\right)^d = \lim_{d\to\infty} \left(1 + \frac{L_{\text{plate}}}{d}\right)^{-d} = e^{-L_{\text{plate}}}$$

which shows that

$$L_{\text{plate}} = k \times L_{\text{original}} \geq -\ln(\beta). \qquad (8)$$

The equations above are used to calculate the LOD when there is only a single replicate beaker/sample. To consider the LOD for a microbiological method that includes *n* independent beakers/samples, each subjected to a dilution series (in many cases, *n* = 3), then equation (6) is replaced by

$$P[X = 0 \text{ in all } n \text{ replicate beakers}] = \left[\frac{d}{L_{\text{plate}} + d}\right]^{nd} \leq \beta$$

where $d = \frac{1}{CV^2}$. Equation (7) for finding the LOD when CFU counts follow a negative binomial distribution, when the coefficient of variation is non-zero (e.g., there is over-dispersion) is then replaced with

$$L_{\text{plate}} = k \times L_{\text{original}} \geq \left(\frac{d}{nd\sqrt{\beta}}\right) - d \qquad (9)$$

and equation (8) for finding the LOD when CFU counts follow a Poisson distribution is replaced with

$$L_{\text{plate}} = k \times L_{\text{original}} \geq -\frac{\ln(\beta)}{n}. \qquad (10)$$

2.4 Estimating the Coefficient of Variation

When modelling extra-Poisson variability of CFUs using the negative binomial model as we do, it is necessary to estimate the CV for the particular microbiological system. The experimentalist may have to perform several experiments to collect CFU data to estimate the CV. We propose an approach for estimating the CV. Given *J* experiments with the same experimental settings and the same number of samples *n* in each experiment, the Poisson rate is estimated by $\hat{\lambda}_j$ for each experiment by an arithmetic mean of the *n* counts if the same dilution was used for all samples, or otherwise by the weighted average described by Hamilton & Parker (2010). The mean and standard deviation of these rates are then estimated by $\hat{\mu} = {\sum_{j=1}^{J} \hat{\lambda}_j}/{J}$ and $\hat{\sigma} = \sqrt{{\sum_{j=1}^{J} \left(\hat{\lambda}_j - \hat{\mu}\right)^2}/{J-1}}$, respectively, and can be used to estimate *CV* with $\widehat{CV} = \hat{\sigma}/\hat{\mu}$. An expansion of this approach for a more accurate CV estimate, when *J* is large, is to use a bootstrapping procedure to find the mean and standard deviation of the sampling distribution of Λ (Efron and Tibshirani 1993).

We demonstrate the approach to estimate the coefficient of variation, CV, using data from one of the labs in a study of *Pseudomonas aeruginosa* biofilms described in Goeres et al. (2019). In this study, there were six treatments (high and low levels of bleach, phenol, quat-alcohol) and two sets of untreated controls, each with three replicate samples (*n=3*), two plates per sample (100µL per plate, *u* = 200µL), in each of three experiments (*J*=3). Each biofilm sample was put into a *V*=40mL original volume into which biofilm bacteria were harvested and homogenized. Plate counts were summed to give a total CFU count per treatment, sample, experiment combination and then scaled by dividing using $k = \frac{0.2}{40 \times 10^f}$ (Equation (5)). We

estimated the CV for each treatment and then estimated the LOD for experiments when there are n=1 and n=3 samples using $\hat{d} = \frac{1}{\widehat{CV}^2}$ as in Equations (7) and (9).

3. Results

3.1 Calculating the LOD

LODs per plated volume (L_{plate}) were computed for varying values of the CV and Type II error rate (β) using equations (7) and (8), see Table 1. To find the LOD, one must choose the desired probability of detection ($1-\beta$ = power), the CV for the microbes being analyzed, and the dilution factor k. For example, suppose that one would like to have a probability of $\beta = 0.10$ of seeing no CFUs when there really are microbes present, and u=100µL is plated at the 0^{th} dilution (f=0) from an original sample with volume V=10mL. Suppose that, when running the experiment, the $CV = 0.2$. For $CV = 0.2$ and $\beta = 0.10$, the LOD in the 100uL plated volume is $L_{\text{plate}} = 2.41195$ (Table 1). To calculate L_{original}, the LOD in the original volume, the dilution factor to use in equation (7) is $k = \frac{0.1}{10 \times 10^0} = 0.01$. Dividing $L_{\text{plate}} = 2.41195$ by k=0.01 shows that $L_{\text{original}} = 241$ CFUs is the LOD in the original volume. That is, for this experiment, there must be at least 241 microbes in the initial sample to have a 90% chance of seeing microbes at the 0^{th} dilution.

Table 1. Values of LOD for CFUs per volume plated, $L_{\text{plate}} = k \times L_{\text{original}}$, for several combinations of the coefficient of variation (*CV*) for the Poisson rate parameter Λ (*CV* = (SD of Λ)/(mean of Λ)), and the probability of a false negative β (0.05 up to 0.65) (see Equations (5), (7) and (8)) with *n*=1 sample per experiment.

		β													
CV	$\boldsymbol{d = \frac{1}{CV^2}}$	**0.05**	**0.1**	**0.15**	**0.2**	**0.25**	**0.3**	**0.35**	**0.367879**	**0.4**	**0.45**	**0.5**	**0.55**	**0.6**	**0.65**
2.0	0.25	40,000	2,500	494	156	64	31	16.41	13.40	9.52	5.85	3.75	2.48	1.68	1.15
1.5	0.44	376	79	31	16.17	9.61	6.23	4.27	3.77	3.05	2.24	1.67	1.26	0.96	0.73
1.0	1	19.00	9.00	5.67	4.00	3.00	2.33	1.86	1.72	1.50	1.22	1.00	0.82	0.67	0.54
0.9	1.23	12.74	6.74	4.51	3.31	2.56	2.04	1.65	1.54	1.36	1.12	0.93	0.77	0.63	0.52
0.8	1.56	9.07	5.26	3.70	2.81	2.23	1.81	1.50	1.40	1.25	1.04	0.87	0.73	0.60	0.50
0.5	4	4.46	3.11	2.43	1.98	1.66	1.40	1.20	1.14	1.03	0.88	0.76	0.64	0.54	0.45
0.2	25	3.18	2.41	1.97	1.66	1.43	1.23	1.07	1.02	0.93	0.81	0.70	0.61	0.52	0.43
0.1	100	3.04	2.33	1.92	1.62	1.40	1.21	1.06	1.01	0.92	0.80	0.70	0.60	0.51	0.43
0.0	Poisson	3.00	2.30	1.90	1.61	1.39	1.20	1.05	1.00	0.92	0.80	0.69	0.60	0.51	0.43

One popular operational definition used by microbiologists is to define the LOD in the volume plated as $L_{\text{plate}} = 1$ CFU (see Evers et al 2010, Magnani 2021, Sutton 2011) which, as pointed out above, is different than the recommended ranges for determining the number of colonies to count on a plate (e.g., 30-300; Ben-David and Davidson 2014). Under the Poisson model, this corresponds to $\beta = 0.37$ (Table 1). This is a high error rate for observing zero CFU when there really are microbes in the sample compared to $\beta = 0.05$ typically used in chemistry. In other words, stating the LOD as $L_{\text{plate}} = 1$ CFU may be misleading.

Figure 1 shows the probability mass functions (Equation (4)) for the microbial count data that provided the LODs of $L_{\text{plate}} = 3$, 4.46 and 19.00 from Table 1 when the CV = 0, 0.5 and 1, respectively, when there is only n=1 sample and β=0.05. When $CV = 0$, then the count data follow a Poisson distribution, and the Poisson rate is L_{plate}= 3 (i.e., LOD = 3 per plated volume). When the CFU data are distributed according to a negative binomial distribution with CV = 0.5 and 1, the extra Poisson variability in the counts is evident by more severe right skew (i.e., thicker tails) and higher means of $L_{\text{plate}} = 4.46$ and 19.00 (i.e., LODs of 4.46 and 19.00 per plated volume). In each of these cases, the probability of observing a zero count is β=0.05.

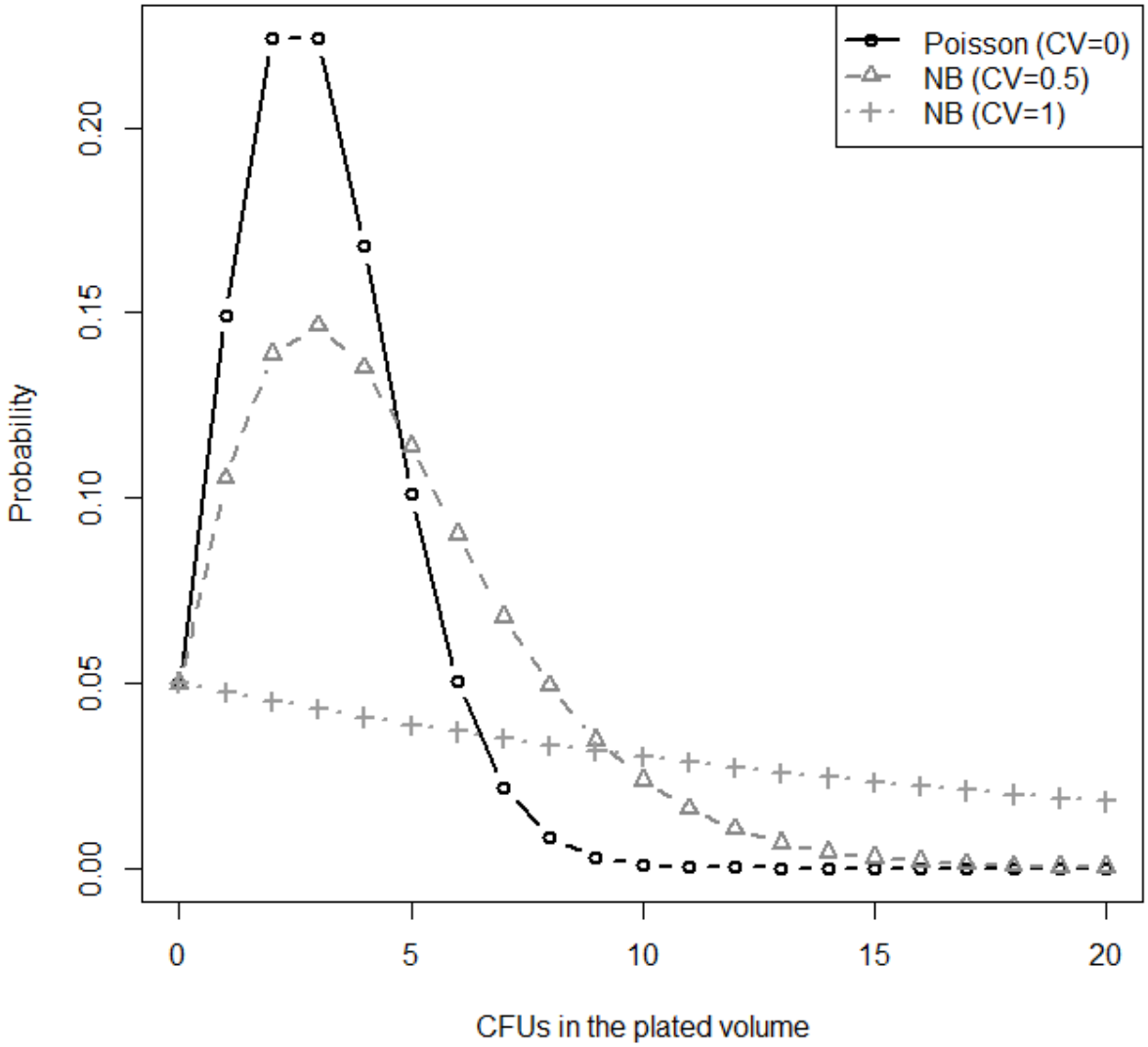

Figure 1. Distributions of the count data (CFUs) under Poisson (CV =0 when LOD = 3 per plated volume) and negative binomial distributions (CV =0.5, 1 when LOD = 4.46, 19 per plated volume respectively) that were used to compute some of the LODs in Table 1 when there is n=1 sample and the Type II error is β=0.05.

To make clear the effect on the LOD by increasing the plate volume, we consider the LOD per mL by dividing the LOD per plated volume (L_{plate}) by the dilution and volume plated (u): LOD per mL is $\frac{L_{\text{plate}}}{u \times 10^f}$. Experimentalists can decrease the LOD per mL by plating the f=0$^{\text{th}}$ dilution and increasing the volume plated, commonly done by using multiple plates at each dilution (see Equations (5), (7) and (8)), and by increasing the number of independent replicate samples n (Equations (9) and (10)). The latter point is relevant because microbiological methods usually include more than n=1 replicate sample. Changes in how the LOD per mL for different volumes plated (u), different numbers of independent samples (n), and differing values for the CV are depicted in Figure 2. The results displayed in Figure 2 illustrate the LOD per mL when the counts for each sample follow a Poisson distribution (Figure 2(a); i.e., $CV = 0$ and Equation (10)); when the counts for each sample exhibit moderate extra-Poisson variability (Figure 2(b); i.e., $CV = 0.5$ and Equation (9)); and when the counts for each sample exhibit a high level of extra Poisson variability (Figure 2(c); i.e., $CV = 1$ and Equation (9)). For example, when a single beaker/sample (n = 1) is assessed in an experiment resulting in 0 CFUs in a single 100µL plate volume, the LOD per plated volume is L_{plate}= 3 CFU/(100uL) (Table 1) when the counts are Poisson ($CV = 0$), depicted by the black curve in Figure 2(a) as the LOD = 30 CFU/mL. The LOD decreases to 15 CFU/mL when there are zero CFUs in the 100uL plate volume in each of n = 2 independent samples, and decreases further to 10 CFU/mL when there are zero CFUs in the 100µL plate volume in each of n = 3 independent samples (black curve in Figure 2(a)). Note that the 100µL plate volume can be attained by either spread-plating 100uL or by drop plating ten 10µL drops. Other common plating volumes are also considered in Figure 2. The 200µL plate volume can be attained by spread-plating 100µL in each of 2 plates at the f=0$^{\text{th}}$ dilution for each independent beaker/sample. The 2mL plate volume can be attained by spread-plating 1mL in

each of 2 plates at the f=0th dilution for each independent beaker/sample. The LOD is substantially reduced when using microbiological methods that utilize n=3 independent replicate samples with a 1mL volume plated at the lowest dilution (f = 0). Higher values of CV (i.e., higher over-dispersion) lead to higher values of the LOD (Figures 2(b) and 2(c)).

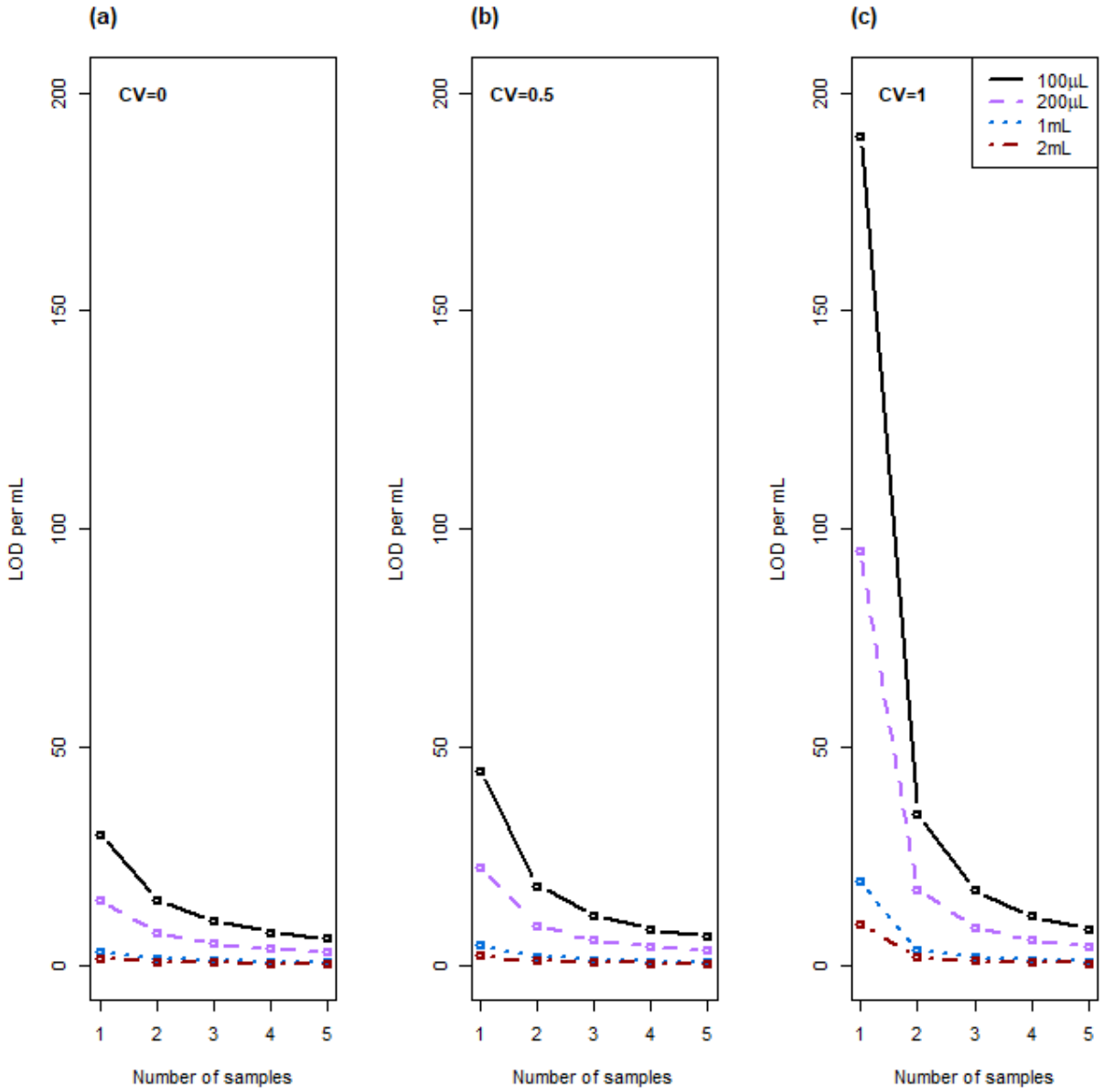

Figure 2. The LOD per mL as a function of the number of replicate samples and the volume plated (u = 100µL, 200µL, 1mL, 2mL) at the f=0th dilution with β=0.05. (a) CFUs follow a Poisson distribution ($CV = 0$); (b) CFUs follow a negative binomial distribution with $CV = 0.5$ (i.e., moderate over-dispersion); (c) CFUs follow a negative binomial distribution with $CV = 1$ (i.e., high over-dispersion).

3.2 Case Study for Estimating the Coefficient of Variation

P. aeruginosa biofilms with high density of about 10^9 CFU/sample were grown in a high shear environment (Goeres et al. 2019). Six treatments were applied to these dense biofilms over 3 experiments, and the CFUs per sample after treatment were recorded. We used data from a single lab to estimate the Poisson rate (i.e., the mean CFU, see Methods section) for each experiment and treatment combination from which we estimated the rate mean ($\hat{\mu}$), rate standard deviation ($\hat{\sigma}$) and CV as $\widehat{CV} = \hat{\sigma}/\hat{\mu}$ (Table 2). For example, the means (Poisson rates) for the three high level bleach experiments were 8.74×10^4 CFU/sample, 6.1×10^6 CFU/sample, and 1.16×10^4 CFU/sample. The mean of these three values is $\hat{\mu}= 2.07\times10^6$ and the standard deviation of these three values is $\hat{\sigma} = 3.49\times10^6$ which gives $\widehat{CV}=2.07\times10^6/3.49\times10^6 = 1.69$ (Table 2). This $\widehat{CV}$ was used to estimate the LOD per plated volume when there are either n=1 or n=3 replicate samples in a study.

The $\widehat{CV}$ values for the six treatments ranged between 0.14 and 1.69 for this data set. The $\widehat{CV}$ and LOD values were largest for the high level of bleach and $\widehat{CV}$ and LOD generally decreased as the number of CFUs after treatment either decreased or increased (i.e., as antimicrobial efficacy deviated from a $\log_{10}$ reduction around 3). Such a 'frown-shaped' relationship is similar to that found by Parker et al. (2018) when studying biofilm, dried surface, and sporicide tests. Interestingly, even though a higher concentration of Phenol happened to be more efficacious, on average, against these biofilms compared to the high concentration of bleach, there were always CFUs after treatment recovered from the plated volumes after the phenol treatment. Hence, the high efficacy quat-alcohol and bleach treatments for which there were many zero counts (6/9 samples (66%) and 4/9 samples (44%), respectively) are most

pertinent to calculating the LOD. The associated $\widehat{CV}$ values were $\widehat{CV}_{quat-alcohol} = 0.88$ and $\widehat{CV}_{bleach} = 1.69$ from which the LOD per plated volume was $L_{\text{plate}} = 11.6$ CFUs and 1830 CFUs in a single sample, respectively. When the plated volume is u = 200µL (as occurs when there's 100µL in each of two plates as occurred in the biofilm case study) and when the original volume that contained the biofilm sample is V=40mL (as occurred in the biofilm case study), then the LOD per sample is $L_{\text{original}} = \frac{L_{\text{plate}}}{k} = \frac{11.63}{.2/40} = 2{,}326$ CFUs and $L_{\text{original}} = \frac{L_{\text{plate}}}{k} = \frac{1830.10}{.2/40} = 366{,}020$ CFUs, respectively (Equation (5) and Equation (9)).

Table 2. For six treatments against biofilms (Goeres et al. 2019), percentage of the 9 samples for which CFU=0 was observed, estimates of the CFU mean, standard deviation (SD), coefficient of variation (CV), and LOD per plated volume (L_{plate}) when for n=1 and n=3 and β=0.05.

Treatment		**CFUs per sample**				**LOD**	
Level	**Antimicrobial**	**% Samples with CFU=0**	**Mean ($\hat{\mu}$)**	**SD ($\hat{\sigma}$)**	$\widehat{CV}$	***n*=1**	***n*=3**
High	Quat-alcohol	66%	6,854	5,997	0.88	11.63	1.50
	Phenol	0%	320,054	254,928	0.80	8.97	1.39
	Bleach	44%	2,066,354	3,493,446	1.69	1830.10	5.72
Low	Quat-alcohol	0%	10,170,009	15,771,823	1.55	559.21	4.17
	Phenol	0%	3,638,667	4,087,610	1.12	33.95	2.00
	Bleach	0%	7,735,015	7,229,797	0.94	14.53	1.59
Untreated	Control	0%	1,574,285,714	531,039,284	0.34	3.57	1.06
	Control	0%	2,020,000,000	289,367,126	0.14	3.09	1.01

4. Discussion

Although we focused on counting CFUs, the techniques presented here can be applied to any counting process in any field of science. For example, when imaging microbes using high magnification microscopy, one may not observe any microbes in the small field of view observed. In this scenario, the Poisson model indicates that the LOD is 3 microbes per field of view (Table 1). An LOD = 3 has been reported by others when applying the Poisson distribution to define the LOD for counting processes (AOAC 2006, ISO 2016, Koenig 2021). If the field of view is 250um x 250um and the surface area of the sample is $1cm^2$, then a calculation similar to equation (5) shows that LOD = $3 \times \frac{1}{0.025^2} = 4800$ microbes spread randomly over the entire $1cm^2$ surface of the original sample leads to a small likelihood of observing no microbes in the one field of view (with probability β=0.05). As we have seen, if there is extra-Poisson variability in the distribution of microbes over the surface, then the LOD can be much higher.

In 1996, Currie proposed precise mathematical definitions for LODs (Equation (1)). Currie also provided equations for "the very special circumstances where the distribution of $\hat{L}$ can be taken as Normal." Currie left readers to decide what distribution is best for their purposes indicating how the definition of the detection limit is dependent on expert opinion concerning the probability model. Because of the severe right skew in the CFU distribution and the hard lower limit at CFUs=0, normal distribution theory does not apply to CFU counting. The Poisson and negative binomial distributions accommodate these distributional constraints when modeling CFU data (as we did in equation (6)). We plotted these distributions for three different CV values and $\beta = 0.05$ in Figure 1. Currie and others have presented analogous figures for normally distributed data (Currie 1996, Thompson and Ellison 2013).

449 It is expected that the coefficient of variation in the negative binomial model will be zero
450 if the microbes are disaggregated and perfectly mixed in the initial density. No clustering and no
451 variation in pipette volumes suggest that there would be no additional variability and thus in this
452 scenario the CFUs will follow a Poisson distribution. If there is extra-Poisson variability, we
453 have assumed that the Poisson rates (Λ) vary from experiment to experiment according to a
454 gamma distribution which results in the CFU counts following a negative binomial distribution
455 and larger LODs. It is conventional to use the negative binomial distribution to represent extra-
456 Poisson variation in microbiology (Jones et al. 1948, Gonzalez-Barron et al. 2010).
457 Others have modeled dilution series count data using non-Poisson distributions. Ben-
458 David & Davidson (2014), Garres et al. (2019) used a binomial distribution. Jongenburger et al.
459 (2010) proposed modeling extra-Poisson variability with a normal distribution. Polese et al.
460 (2021) used a generalized-Poisson distribution and Garres et al. (2022) used a hierarchical
461 Poisson-normal distribution. Gonzalez-Barron et al. (2010) modeled count data using the
462 negative binomial distribution as we do here, in addition to using zero-inflated Poisson and zero-
463 inflated negative binomial models. These models could be used to calculate LODs while
464 accounting for extra-Poisson variability, although we are not aware that this has been reported in
465 the literature. Two notable exceptions include Christen & Parker (2020) who used a hierarchical
466 binomial-log-normal model to model count data and calculate LODs and Duarte et al. (2015)
467 who used a Poisson-zero-inflated-log-normal model to model counts and calculate LODs.
468 However, both Christen & Parker (2020) and Duarte et al. (2015) used LOD definitions different
469 than that espoused by Currie (1996) as we do here. Interestingly, Duarte et al (2015) and Garre et
470 al (2022) advocate for the use of the negative binomial model for analyzing microbial
471 enumeration data. We used the negative binomial model to calculate LODs.

There are several methods for estimating the coefficient of variation (CV) in practice when modelling extra-Poisson variability using the negative binomial model. We proposed a method with a possible extension using bootstrapping. This approach provides a straightforward method for computing the $\widehat{CV}$ using simple means and standard deviations without the need for applying specialized software. Other approaches to estimating the CV would be to directly model the CFU data using a negative binomial or a zero-inflated negative binomial model. In the former case the CV is estimated as the square root of the inverse of the shape parameter ($CV = 1/\sqrt{d}$). In our experience, small samples sizes and zero CFUs may adversely impact the accuracy and precision of the estimates and model convergence. The best approach to estimating the CV is a topic for future research. Some processes have been run for many years and so the CV may be estimated using the approach we describe. Given little information on the experimental study conditions (e.g., for a new microbe under study), experimentalists can choose a smaller value of the CV in experiments where there is little over-dispersion expected (i.e., random microbe distribution that may occur when studying planktonic bacteria) or a greater value of the CV when more over-dispersion is expected (i.e., when the microbe distribution is expected to have more clumps that may occur when studying biofilm bacteria). Using CFU data from a biofilm study (Goeres et al. 2019) of six different treatments, estimates for the CV ranged from 0.14 to 1.69 for efficacious treatments. For the highly effective treatments applied to these biofilms that resulted in CFU=0 in many samples, the CV estimates were large, at 0.88 and 1.69. That is, overdispersion was high when counting CFUs from biofilm assays. Based on previous observations that biofilm assays are more variable (Parker et al. (2018)), we conjecture that overdispersion is lower (i.e., CV will be smaller) for assays that study planktonic bacteria and bacteria dried onto surface.

5. Conclusions

We propose an approach, based on the negative binomial distribution, for determining the LOD for any quantitative method that counts microbes, whether from plates, filters, or microscopy. This approach accounts for extra-Poisson variation that could occur as a result of technical or microbiological variations. The extra-Poisson variation is quantified by a coefficient (*CV*) of variation that we illustrate how to calculate using real data.

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