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Optimization of turbidity experiments to estimate the probability of growth for individual bacterial cells

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ABSTRACT

Using turbidity measurements to quantify bacterial growth is a common and well-established practice in microbiology. Automated devices offering high throughput analyses have largely contributed to the increase of its use. A main difficulty of this method is that it detects growth only at late exponential phase, making turbidity measurements limited for studies focussing on low cell numbers. This work proposes an improved estimator for the probability of growth of individual cells using turbidity-based measurements, when the initial number of cells is low and random. We modify the currently used estimator for the expected cell number *per* well, a Poisson-parameter, and show that an optimal scenario is when *ca* 20% of the wells do not become turbid, resulting in improved accuracy and precision.

1. Introduction

One of the most important areas of quantitative microbiology is bacterial kinetics. It can be described by rates, such as the number of cell divisions or cell deaths in a unit time, or the production rate of a specified metabolite. However, for a single cell, it is difficult to interpret and measure these quantities directly. At low levels of cell concentrations, the probability of division (or death) of a single cell, as a function of time, becomes the main parameter, from which the respective population level parameters can be inferred (Renshaw, 1991). A simple example for this is the probability whether a single cell can generate an exponentially growing subpopulation.

Any system or equipment able to identify the turbidity in a culture broth can be used to detect bacterial growth (turbid/no-turbid after an experimental time). A popular example is the Bioscreen C (Oy Growth Curves Ab Ltd, Helsinki, Finland). This equipment simultaneously monitors the bacterial cultures in the wells of a microtiter plate (Löwdin et al., 1993; Lee et al., 2011). Its working principle is the assumption that the cell population of a well is homogeneous and, above the detection level, the optical density (OD) of a well is proportional to the number of cells within.

For replicate inocula , the cells are typically distributed over many wells (e.g. 50, 100, 200 or even 400, if the equipment was available in pairs). Though only a large number of cells (typically more than a million; see George et al., 2015) can make a well turbid, here we show that the probability of growth for an inoculated single cell can also be estimated by means of OD measurements. This is not straightforward since, in fact, a detectable change in the optical density is due to the subpopulation generated by an initial single cell, and not a direct measure of the cell itself. Therefore, strictly speaking, even if exactly one cell was inoculated in a well, we should speak about "individual-cell-based" (ICB), rather than "individual cell" kinetics.

To achieve our goals, we assess the number of cells *per* well before and after running the experiment, for which we assume that the micro-environment is the same for all the wells. For the first assessment, we give an *a-priori* estimator for the average number of cells in a well, based on the concentration of the diluted original culture and the applied dilution. In the second step, we make use of the fact that the number of cells in a well follows the Poisson distribution, whose parameter can be estimated by means of the proportion of the negative wells (i.e. which did not become turbid). This way, the average number of cells in a well is estimated *a-posteriori*. The ratio between the two results will be an estimation for the probability of growth for a single cell.

We will analyse the relative accuracy and precision of the above estimators (see the Nomenclature table). Recall that the accuracy is about the expected difference between the estimator and the parameter it intends to estimate (this is why it is also called the bias of the estimator), while the precision is about the scatter of the estimator, regardless of the real value of the parameter it intends to estimate. The

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Nomenclature

Notation	*Meaning*
C	The number of colonies on a plate (a random variable) after appropriate dilutions
c	The expected value of C (a parameter, $E(C) = c$)
$\hat{c}$	An estimator for c (a random variable)
r	The expected number of cells inoculated in a well (a parameter)
$\hat{r}$	An estimator for r (a random variable)
w	Total number of wells (a fixed constant)
W_0	Number of wells showing no growth (a random variable)
ρ	Expected number of cells in a well (the parameter of the Poisson distribution that the number of cells per well follows)
$\hat{\rho}$	An estimator for ρ (a random variable)
$\widehat{\rho'}$	Modified estimator for ρ (a random variable)
z	Probability of a well remaining negative (i.e. not becoming turbid)
g	Probability that a single inoculated cell generates a progeny that can be detected by OD. If the maximum population density of this single-cell-generated subpopulation is above the OD detection level, then (for the sake of brevity) g is called the probability of growth for that single cell
$\hat{g}$	An estimator for g (a random variable)
$\frac{\lvert E(\hat{y}-y)\rvert}{y}$	Relative accuracy of the $\hat{y}$ estimator for the y parameter. If $E(\hat{y}) = y$, then the estimator is accurate (unbiassed)
$\frac{\sqrt{Var(\hat{y})}}{E(\hat{y})}$	Relative precision of the $\hat{y}$ estimator. The smaller it is the more efficient is the estimator

smaller this scatter the more efficient the estimator, since it requires less replicates for a given precision. Our objective is to show that a modification of the *a-posteriori* estimation leads to an improvement both in terms of accuracy and precision. The results enable us to give recommendations, at what values of ρ the experimental designs should aim, in order to obtain a sufficiently accurate and precise estimation for the expected number of cells in a well. Finally, we discuss the accuracy of the obtained estimator of the probability of growth for a single cell.

2. Material and methods

Below we summarize the statistics when assessing the distribution of the number of cells inoculated in a well before and after the Bioscreen experiment. First, we provide an *a-priori* estimator, $\hat{r}$, for the expected initial number of cells per well, following appropriate dilutions and enumeration by colony counts. Second, by means of the proportion of wells not becoming turbid during the experiment, we provide another, *a-posteriori* estimator for the same parameter, denoted by $\hat{\rho}$.

2.1. An a-priori estimation, using dilutions and colony counts

A basic assumption behind colony count methods is that one cell in the sample produces one colony. The number of colonies on a plate is a random number, C, that follows the Poisson distribution since the colonies are from a population that is orders of magnitude bigger than the sample, from which the colonies were plated. Its expected value is in the order of 100-s, so our working region is defined by $50 < c = E(C) < 200$. Due to the properties of the Poisson distribution, the variance of C is the same as its expected value. Furthermore, for the estimation of the expected number of initial cells in a well, a factor a is calculated from the used dilutions. This factor is typically around the reciprocal of c once we aim at an inoculum size of one cell *per* well. Eqn. (1) shows that $\hat{r}$ for the average number of cells in a well is an accurate (unbiassed) estimator, while its relative precision can be assessed as shown by Eq. (3).

$$E(\hat{r}) = E(a\cdot C) = a\cdot c = r \tag{1}$$

$$Var(\hat{r}) = Var(a\cdot C) = a^2\cdot c = a\cdot r \tag{2}$$

$$\frac{\sqrt{Var(\hat{r})}}{r} = \sqrt{\frac{a}{r}} \tag{3}$$

2.2. An a-posteriori estimation, using Bioscreen results

When inoculating the plate for OD measurements, a diluted culture consisting of N cells is distributed among w wells, where $w \ll N$. As described in the Introduction, the number of initial cells producing detectable turbidity follows the Poisson distribution, with the expected value ρ. An estimator for ρ can be obtained by using the proportion of "negative" wells, which do not become turbid, as follows. Let the (random) number of negative wells be denoted by W_0. The expected value of the W_0 is $w\cdot e^{-\rho}$ from which an estimator for the Poisson parameter is (Baranyi et al., 2009; George et al., 2015):

$$\hat{\rho} = -\ln(\frac{W_0}{w}) \tag{4}$$

We investigate the properties of this estimator for small values of ρ, when the occurrence of non-turbid wells is very likely.

Consider the two outcomes (positive/turbid or negative/non-turbid) for a well as "success and failure" of a Bernoulli trial. Then $z = e^{-\rho}$ is the probability of failure for a single trial (well) and w is the number of replicates. The W_0 number of failures out of w trials follows the binomial distribution:

$$Prob(W_0 = i) = \binom{w}{i} z^i (1-z)^{w-i} \quad (i = 0 \ldots w) \tag{5}$$

Our estimator cannot interpret the $W_0 = 0$ and $W_0 = w$ situations, which in effect means that we discard the experiments where all the wells are positive or negative. This results in a conditional distribution with b_i probabilities:

$$Prob(W_0 = i) = b_i = \frac{\binom{w}{i} z^i (1-z)^{w-i}}{1 - [(1-z)^w + z^w]} \quad (i = 1, \ldots, w\text{-}1) \tag{6}$$

The (conditional) expected value of this estimator is therefore

$$E(\hat{\rho}) = -\sum_{i=1}^{w-1} \ln(\frac{i}{w}) b_i \tag{7}$$

In the results section, we will show that, in our region of interest, this is an inaccurate (biased) estimator for ρ, because its expected value is less than ρ. Then we will modify it to improve its accuracy.

2.3. Estimating the probability of growth

Finally, recall that the above two estimators, $\hat{r}$ and $\hat{\rho}$, target the same parameter, the expected number of cells in a well. We assume that the reason why they are different (apart from their inherent variability) is that some wells, though harbouring cells, do not become turbid because the cells did not multiply. Under the condition that each cell has the same probability g to produce an OD-detectable subpopulation, an estimation for this probability can be given based on the following argument:

The number of the initial cells can be conceived a Bernoulli-sum with random, Poisson-distributed number of terms. Therefore, it follows the so-called generalized Poisson distribution (Wimmer and Altmann, 1996) and the number of initial cells in a well that would grow to a detectably turbid level is also distributed in a Poissonian way, with the $\rho = gr$ parameter (proofs on this can be found in standard textbooks, e.g. Feller, 1970). Therefore,

$$\hat{g} = \hat{\rho}/\hat{r} \tag{8}$$

estimates the probability of growth for a single cell.

For stability reasons, it is reasonable to consider its logarithm instead:

$$\ln(\hat{g}) = \ln(\hat{\rho}) - \ln(\hat{r}) \tag{9}$$

Remember that the two estimators are independent (so are their logarithm values), therefore the variance of their sum is the sum of the respective variances, i.e.

$$\mathrm{Var}(\ln(\hat{g})) = \mathrm{Var}(\ln(\hat{\rho})) + \mathrm{Var}(\ln(\hat{r})) \tag{10}$$

This will give an opportunity to estimate the precision of the $\ln(\hat{g})$ estimator based on the approximation that a small relative error of a variable is close to the error in its natural logarithm.

3. Results

Considering the pragmatic 50 to 200 colonies on a plate, obtained from a cell population of a size $> 10^6$, the relative precision of the *a-priori* estimator will be less than 10%, due to the small dilution ratio, *a* (see Equation (3)). For the *a-posteriori* estimation, if $0.5 < \rho < 3$, then the expected fraction of negative wells is between 5 and 60%, the expected value of the $\hat{\rho}$ estimator is always smaller than ρ, as can be read from Fig. 1A. Therefore, this estimator is inaccurate (biased). To improve it, we modified the estimator as below:

$$\hat{\rho}' = -\ln\left(\frac{W_0}{w-2}\right) \tag{11}$$

We show that this estimator, in a restricted, practical interval of the ρ parameter, has an improved accuracy with a precision that is locally optimal. Following the relevant definitions in the nomenclature table, the relative accuracy and precision of the estimator are:

$$acc(\widehat{\rho'}) = \frac{|E(\widehat{\rho'} - \rho)|}{\rho} \tag{12}$$

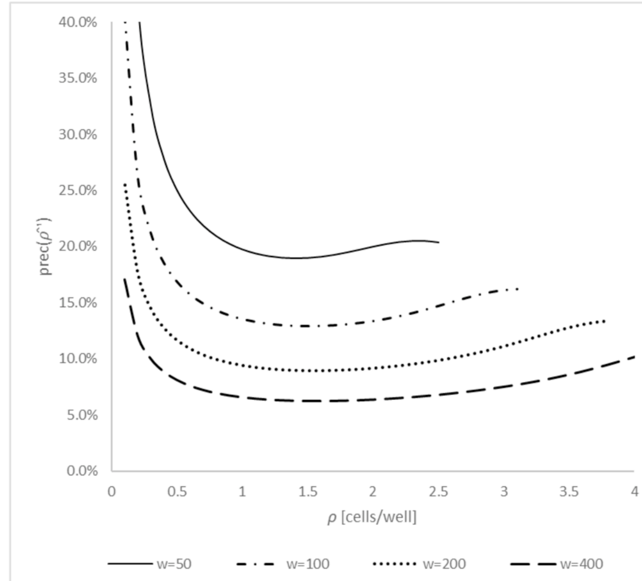

Fig. 2. Relative precision of the $\hat{\rho}'$ estimator as a function of the ρ expected number of initial cells in a well and the w total number of wells. Continuous: $w = 50$; dash-dotted: $w = 100$; dotted: $w = 200$; dashed: $w = 400$.

$$prec(\widehat{\rho'}) = \frac{\sqrt{Var(\widehat{\rho'})}}{E(\widehat{\rho'})} \tag{13}$$

Fig. 1B shows the effect of this modification. For any w number of wells, the ideal value of ρ, where the estimator is accurate, is *ca.* $\rho = 1.6$ cell per well, corresponding to *ca.* 20% negative wells ($W_0 = 0.2w$). For the studied $w = 50$, 100, 200, and 400, the expected value of the estimator nears the ρ parameter within 3% accuracy, in intervals that increase with w.

Combining the optimum efficiency with the, say, 5% accuracy region of the estimator $\hat{\rho}'$ (Figs. 1B and 2, respectively), we recommend experimental design ranges, as presented in Table 1, as a function of the total number of wells. For this, a simple criterion was established after a visual inspection of the graph: for each w, take the regions corresponding to the $acc[\hat{\rho}'] + 1.5\%$ values and take its overlap with the

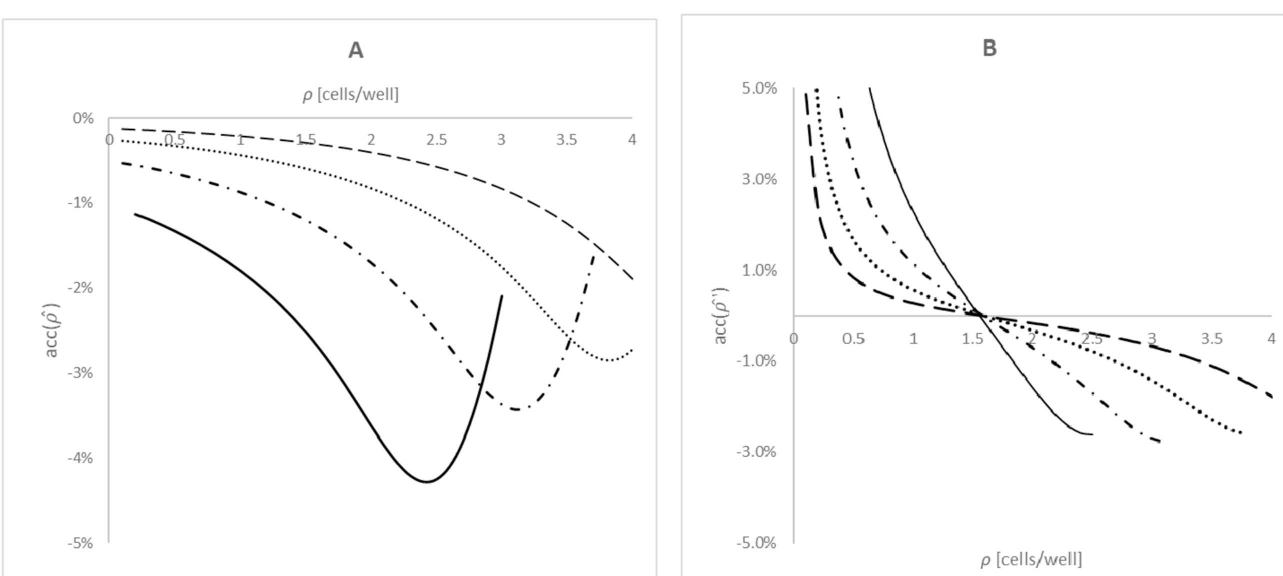

Fig. 1. Relative accuracy of the estimator $\hat{\rho}$ (A) and its modification $\hat{\rho}'$ (B), as a function of the total number of wells. Generally, the first one underestimates the ρ parameter, while $\hat{\rho}'$ is sufficiently accurate in regions centered around $\rho = 1.6$. Continuous: $w = 50$; dash-dotted: $w = 100$; dotted: $w = 200$; dashed: $w = 400$.

Table 1
Recommendations on target optimal value of ρ. At these values, both the accuracy coefficient and efficiency of the $\hat{\rho}'$ estimator are close to optimum.

w [number of wells]	Ideal range of ρ [cells/well]
50	0.9–2.0
100	0.9–2.2
200	0.9–3.0
400	0.8–3.0

regions where $prec[\hat{\rho}']$ is around its optimum (Fig. 2). By and large, the ideal scenario turned out to be the case when *ca.* 10–40% of the wells do not become turbid.

4. Discussion

Previous studies (Guillier and Augustin, 2006) have recommended to obtain a number lower than our finding for the *cell-per-well* inoculum level. However, those authors aimed to analyse individual cell lag times, which is out of the scope of the present study. Note that Métris et al. (2003), while also investigating single-cell lag times, prepared the inoculation so that the fraction of negative wells was between 12.5% and 37.5%. Based on the Poisson distribution and assuming that all cells produced growing subpopulations, this means that the mean number of initial cells in a well, to grow to detectably turbid level, was between 1 and 2 cells per well in all experiments, in accord with the recommendations given in this paper.

As we could see, it can easily be achieved that the efficiency of the unbiased ***a-priori*** estimator is less than 10% in the studied region. However, the ***a-posteriori*** estimator has 10% or less efficiency only for $w > 200$ (Fig. 2). In that case, based on Eq. (12), the scatter of the $\ln(\hat{g})$ estimator is about 0.1–0.2, which means that the method offers a way to measure the probability of growth with one digit accuracy and, for this, all the 200 wells of the Bioscreen plate are desirable. A consequence is that it is not feasible to identify changes in the probability of growth, if it is close to 1 or 0, and only changes in probability greater than about 10% are detectable. This level, however, can be still useful, considering that the probability of growth rapidly changes with environmental factors like temperature or water activity; that is, relatively small changes in the conditions can induce detectable changes in the single cell probability of growth provided by our method.

Notice that, for $w = 50$, there is a reasonable chance that all wells will be positive (which case is omitted by our estimator). Also, many wells will have one single cell, which is ideal to estimate the probability of growth. It is natural to aim at the region of 1–3 for the average number of inoculated cells per well if the anticipation is that the probability of growth will be noticeably less than 1.

Remember that, strictly speaking, by the "probability of growth for a single cell", we mean the probability that an inoculated single cell generates a progeny that grows over the optical density detection level. In stress conditions (e.g. at low water activity), it is possible that a single cell produces a growing subpopulation which however does not grow over the detection level. This possibility needs to be considered when interpreting the results.

For individual cells, it is difficult to acquire sufficiently accurate data. The proposed recommendations provide a means to deal with this challenge. They can be used to optimize experimental designs when assessing the probability of growth for single cells using turbidity measurements on populations generated by low and random initial number of cells.

Conflicts of interest

The authors declare no conflict of interest.

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