

that enable microbes to flourish in the coldest places on the planet.

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Letter

Rocking the curve

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Quantifying microbial growth is of primary interest in microbiology and is routinely involved in a diversity of studies, such as comparing and/or contrasting strains and/or species, competition associated with co-infections, assessments of risks related to food contamination, testing drug efficacy, or the development of candidate vaccines [1–4]. The typical types of questions asked can be summarized as ‘does strain A grow faster than strain B?’ or ‘will species A reach larger population size than species B?’. Despite the development of new and powerful statistical tools [5] many studies on microbe growth curves still rely on poor and old-fashioned methods. Beyond their low statistical power and high risk for incorrect conclusions, such analyses often fail to optimise the information contained in the datasets, thus limiting the range of biological questions that can be addressed by a single experiment.

The study of microbe growth basically involves three successive steps: (i) experimental establishment of growth curves, (ii) estimation of the parameters of a growth model on the experimental dataset, and (iii) statistical tests on the growth parameters estimations. The second step is a key one because it links the information contained in the raw dataset (step i) to the biological questions under investigation (step iii). This step implies not only the choice of a mathematical model of growth but also the characterisation of the variation in the data to correctly estimate the model parameters from the data. A review of the literature reveals that a large proportion of analyses of

kinetic growth are based on the classical linear regression of log-transformed data. This method is certainly the simplest one but suffers from two major drawbacks. First, in considering an exponential growth model, it restricts the exploitation of data to the initial phase of the growth curve, missing out on potentially interesting biological phenomena. Second, the use of a linear regression implicitly assumes that the random variation in log-transformed data follows a normal distribution, which is not always the case. Our computer simulations show that the use of miss-specified models and/or probability distributions can produce imprecise and/or biased parameter estimations (Figure 1a). Imprecise estimations lower the power of statistical tests in step (iii). Biased parameter estimations simply render the outcome of any statistical test unreliable.

We suggest that a modern quantification of microbe growth should be carried out using the powerful likelihood framework. In this context, parameters of any growth model – whatever its complexity and the distribution of the data variation – can be estimated without data transformation [5]. The choice of the model is dictated by both the form of the data mean and the question under investigation [5]. Thus, in continuous cultures, such as chemostats, one might want to study the size that the microbe population eventually reaches after its initial exponential expansion. There are a variety of models accounting for such S-shape growth curves, including the classic logistic one. In the more common closed-culture environment (i.e. culture flasks), the accumulation of toxins or the depletion of nutrients eventually makes

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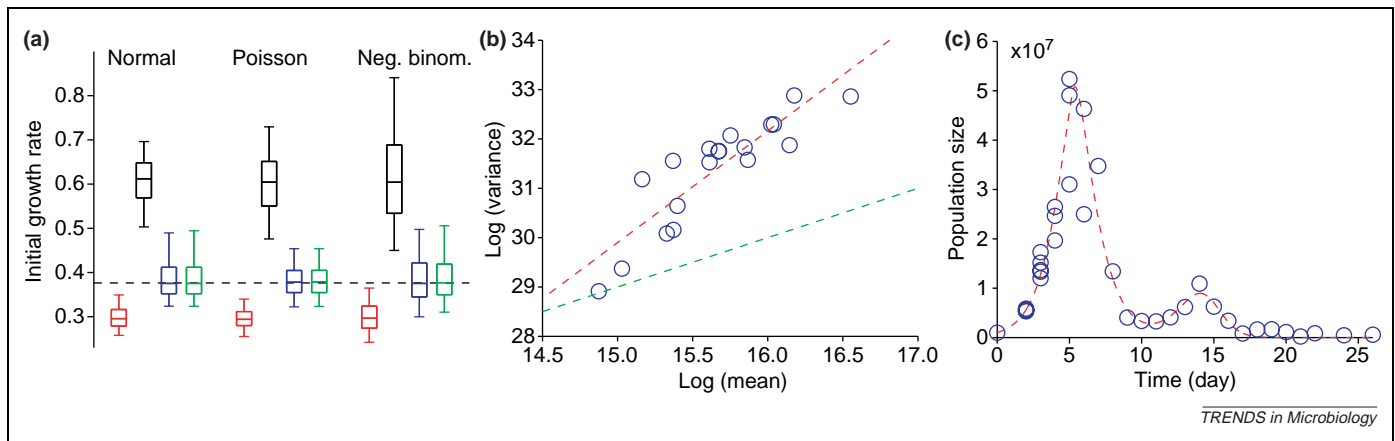


Figure 1. (a) Humped growth curves such as the one in (c) have been simulated with three different distributions of the variation in the data: normal, Poisson, and negative binomial (as indicated above the box-plots). For each of these three distributions, 500 datasets were simulated. On each of these 3×500 datasets, the initial growth rate was estimated by the least square method on an exponential growth model with a log-transformation of data (classic method, red), without transformation of data (black), the likelihood method on a model accounting for the dynamics of both the microbe and the culture medium with a Poisson distribution of errors (blue) and a negative binomial one (green). Naturally, the first two methods considered only the data points in the initial exponential growth, whereas the likelihood methods considered the whole dataset. Each box-plot displays the median together with the 50% and 90% percentiles of the 500 estimations. The horizontal dashed line indicates the true value of the initial growth rate. Note the biases associated with the first two methods even when the variation in the data is actually normal. This is due to the unavoidable imprecision in the identification of the initial exponential growth phase. The seemingly precise estimation with the first method is an effect of the log-transformation. Likelihood methods are performing well even when the variation in the data is normally distributed. (b) Log-log plot of the variance against the mean of 19 *in vitro Leishmania* growth curves (blue circles). The red dashed line is the linear regression with a slope $(2.2642 \pm 0.2902, 1 \text{ s.e.}, n=19)$ indicative of dispersed data as expected from a negative binomial distribution. For comparison, the green dotted line has slope equal to 1, as would be expected from a Poisson distribution. (c) A model (red dashed line) adjusted to one of the 19 *Leishmania* kinetics (blue circles) by maximisation of the likelihood. The model accounts for the dynamics of both the promastigote population and the culture medium and considers a negative binomial distribution of errors as suggested by the log-log mean-variance relationship (b). The likelihood function of the model expresses the probability of the observed data for a particular set of the model parameter values. The maximum likelihood estimations (MLE) of the model parameters are the values of the parameters that maximise the likelihood function.

population size decline towards extinction (such as on Figure 1c). *Ad hoc* models that account for the dynamics of both the microbe population and the culture medium help the analysis of these frequently observed humped growth curves. The choice of a probability distribution for the variation in the data is generally inspired from the data structure [5]. With regard to the mean-variance relationship, three probability distributions are commonly considered. The normal distribution has a constant variance, independent of the mean. The Poisson distribution is more adapted to count processes and has a variance proportional to the mean. The negative binomial distribution is a generalisation of the Poisson distribution and is characterized by a power relationship between the mean and the variance with a power factor ranging between 1 and 2. Also, in providing a likelihood value, these methods render possible the test of hypotheses about both the growth model and the distribution of the underlying variation in the data [6].

As an example we have recently developed a model to study the *in vitro* growth of *Leishmania* promastigotes (M. Hide, PhD thesis, University of Montpellier, 2004). Within their arthropod vector, *Leishmania* exist in two main forms. The first one – procyclic promastigote (PP) – divides intensively in the insect's gut. The PP then migrates to the stomodeal valve where they transform into a dispersion form that does not divide: the metacyclic promastigote (MP). Cultures of PP in flasks produce typical humped growth curves (Figure 1c). *A priori*, two kinds of hypotheses can be formulated to explain the decrease of the population size that follows the phase of exponential growth. The first one is a resource hypothesis and assumes that the depletion of nutrients in the medium is responsible for an excess of deaths relative to

divisions. The second one is a pH hypothesis. Indeed, there are good experimental evidences suggesting that the PP to MP transformation is pH dependent and the low pH inside the stomodeal valve is suspected to trigger the transformation [7–9]. Additionally, it is a common observation that promastigotes release protons into the medium. In a closed experimental system, such as a culture flask, the metabolism of promastigotes tends to decrease the pH, thus possibly inducing the PP to MP transformation. Because only PP divide, this PP to MP transformation can explain the decay of the population size. We can therefore consider three different models: one for each hypothesis and a third one for the two hypotheses at the same time. As regards variation in the data, we might consider the three types of probability distribution presented above, even if the datasets strongly suggest a negative binomial distribution (Figure 1b). These three models and three probability distributions give $3 \times 3 = 9$ combinations. Comparing the maximum likelihood of each of these combinations leads to the conclusion that the best model (Figure 1c) is the one that considers the resource and the pH hypotheses at the same time and confirms the negative binomial distribution for variation in the data. The advantage of such likelihood methods compared with the classic linear regression of log-transformed data is that, in addition to the initial growth rate, it allows estimation (with low bias and good precision) of other parameters of potential biological interest, such as the pH at which the PP to MP transformation occurs, the speed of the PP to MP transformation, and the death rate of the MP.

Complex biological questions can be answered by complex experiments. However, such experiments often require heavy setups to control confounding effects and might be expensive in time and money. The message of

this letter is that it becomes possible to explore complex questions with very simple experiments when an elaborated data analysis is performed. The approach presented here is very general and can easily be adapted to any system where one wants to test hypotheses that integrate growth and other biological parameters of interest, such as temperature, antibiotic concentration, and so on.

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