Journal of Industrial Microbiology, 12 (1993) 221–231

Published by The Macmillan Press Ltd

Mathematical models of bacterial growth, inhibition and death under combined stress conditions

Kenneth M. Pruitt[1] and David N. Kamau[2]

[1]*University of Alabama at Birmingham, UAB Station, Birmingham, AL 35294 and* [2]*Tuskegee University, Tuskegee, AL 36088, USA*

(Received 24 February 1993)

Key words: Growth models; Survival models; Bacterial growth; Bacterial inhibition; Bacterial survival; D values; Combined stress; *Listeria monocytogenes*; *Staphylococcus aureus*

SUMMARY

In this report we review the history of growth theories. We show how classical growth models may be derived as special cases of a generic growth rate equation. We show how growth models may be modified to represent survival data. We use linear combinations of growth and survival models to represent complex growth/survival curves and give practical examples utilizing nonlinear regression analysis. We show that traditional methods of estimating D values are inappropriate for complex, multiphasic growth/survival data. We show how such data may be modeled mathematically and illustrate methods for estimating true D values from such data.

INTRODUCTION

Models for bacterial growth range in complexity from simple, one-term exponentials to complex, highly non-linear functions. Practical application of the latter to analysis of experimental data has been facilitated by the development of powerful personal computers and efficient software for nonlinear regression analysis.

When bacteria are subjected to combined stress conditions, the resulting survival curves are often complex and multiphasic. Common methods used to enumerate survivors may count a population of cells recovering and growing upon long-term incubation on plating media but not growing in the actual treatment experiment.

In this report, we review the history of growth theories. We discuss the generic growth rate equation from which all of the classic growth models may be derived as special cases. We show that appropriately modified growth models may be used to represent survival data. We show how data from experiments which generate subpopulations of cells may be modeled mathematically.

Traditional methods of estimating D values (the time required to reduce a cell population 10-fold) are inappropriate for analysis of data from experiments in which growth or death are accompanied by significant lag phases or for experiments which generate subpopulations of cells differently affected by treatment. We show here how data from such experiments may be modeled mathematically, how true D values may be estimated for such data, and we illustrate the methods with several practical examples.

Correspondence to: K.M. Pruitt, University of Alabama, UAB Station, Birmingham, AL 35294, USA.

DEVELOPMENT OF MATHEMATICAL MODELS FOR GROWTH AND DEATH

The history of growth theories dates back nearly 200 years to Malthus' 'Law of geometric growth' [4]. This model assumes that the population grows at a rate which is proportional to its size at any given time. Just a few years later, Verhulst [11] developed the logistic equation. The logistic model assumes that growth is limited by the availability of a single essential factor. The rich history of subsequent literature of growth theories has been reviewed by Turner et al. [10] and by Turner and Pruitt [9].

Turner et al. [10] showed that all of the classic growth models are interrelated. They may be derived from a single parent: the generic growth rate equation:

$$\frac{dx}{dt} = \frac{\beta}{k^n} x^{1-np}(k^n - x^n)^{1+p} \tag{1}$$

where x is the population size at time t. This rate equation contains four parameters (β, k, n, p) which determine the shape of the curve.

The generic growth model may be obtained by integrating Eqn (1):

$$x = \frac{k}{(1+[1+\beta np(t-\tau)]^{-\frac{1}{p}})^{\frac{1}{n}}} \tag{2}$$

where τ is the integration constant.

A good model should use the minimum number of parameters required to give predicted values which are consistent with the experimental data. These parameters should be directly related to significant biological properties of the system under study. Although the generic growth model has only four parameters it may be utilized effectively in the study of many different types of systems. The parameters are directly related to significant properties of the systems [7]. The parameter β is the maximum specific growth rate which the population could attain in the absence of any limitations to growth. The maximum size which the population could attain under limited growth is given by the parameter k. The parameter τ is related to the time at which the maximum specific growth rate is attained. The parameters n and p are related to metabolic efficiency.

Growth models which are special cases of the generic model include the hyper-Gompertz, hyper-logistic, Bertalanffy–Richards, Gompertz, logistic and geometric increase curves. These models are obtained by placing appropriate limits or conditions on selected parameters in the generic curve. For example, if the population grows at a rate which is always directly proportional to its size, and there are no limiting factors, the generic curve reduces to the simple exponential curve. If, in addition, growth is limited by the availability of a single factor, the generic curve reduces to the logistic.

Turner and Pruitt [9] showed that the generic growth model can be extended to survival curves and autocatalytic phenomena. Special cases of the generic growth model have been used to analyze a variety of experimental data [6] from bacterial agglutination (hyper-Gompertz), complement-mediated hemolysis (hyperlogistic), and transplantable tumor growth (logistic).

Mathematically, the difference between growth and death rates is a matter of sign. Consequently, growth curves may be converted to death curves by changing the sign in the growth rate equation. We illustrate the procedure using the simple exponential growth rate:

$$\frac{dx}{dt} = \beta x \tag{3}$$

Integrating between the limits $(0, x_0)$ and (t, x):

$$x = x_0 e^{\beta t} \tag{4}$$

where x is the population size at any time t, x_0 is the initial size and β is the specific growth rate. Changing the sign gives a death rate:

$$-\frac{dx}{dt} = \beta x \tag{5}$$

$$x = x_0 e^{-\beta t} \tag{6}$$

where x is the population surviving at any time t, x_0 is the initial population and β is the specific death rate. The generic growth curve and any of the family members may be changed into death curves using this same procedure.

The rate equation for simple exponential growth shows that the specific rate of increase is equal to a constant, β. In this simple case there is no limit on growth. For death curves, β becomes the specific death rate when there is no limit and the initial value of x declines exponentially to zero.

If the population growth rate is proportional to the population size and is also limited by the availability of a single growth factor, then the generic curve reduces to the logistic curve.

The logistic growth rate is:

$$\frac{dx}{dt} = \frac{\beta}{k} x(k-x) \tag{7}$$

integrating:

$$x = \frac{k}{1+e^{-\beta(t-\tau)}} \tag{8}$$

where x is the size at time t, β is the maximum specific growth rate, k is the maximum attainable size, and τ is the time at which $x = k/2$. Changing the sign gives a logistic death rate:

$$-\frac{dx}{dt} = \frac{\beta}{k} x(k-x) \tag{9}$$

$$x = \frac{k}{1+e^{\beta(t-\tau)}} \tag{10}$$

where x is the population surviving at time t, β is the maximum specific death rate, and τ is the time at which $x = k/2$. The parameter k is related to the initial size. When $t = 0$:

$$x_0 = \frac{k}{1+e^{-\beta\tau}} \tag{11}$$

Thus, $k = x_0 (1+e^{-\beta\tau})$. In this case death is limited by the amount of some stress factor or by the amount of damage to the cell. The maximum size in this case is the initial size of the cell population. Thus, the logistic curve contains two additional parameters (k, τ) compared to the simple exponential curve.

We report here practical applications developed using the exponential and logistic models and/or combinations of both together with the assumption of subpopulations which behave differently and independently. In principle, the strategies which we use may be applied to any other members of the growth/death family of models.

PRACTICAL APPLICATIONS

Simple exponential model for destruction of bacteria

Assume a homogeneous cell population in which the cells are all dying at a rate proportional to their concentration. Eqn (6) will describe the time course of changes in the cell population. If the surviving cell number (x) is divided by initial number (x_0), a very simple model is obtained. The natural log of this model yields a linear equation, and a plot of the natural log of the surviving fraction is a straight line of slope $-\beta$ and intercept 0.

$$\ln\left(\frac{x}{x_0}\right) = -\beta t \tag{12}$$

The experimental data for survival of *Listeria monocytogenes* in milk heated at 57.8 °C provide a good illustration of the application of this simple model. This experiment was part of a series [1] in which the heat sensitivity of *L. monocytogenes* in milk was evaluated for different pre-heat treatments and process temperatures. The exponential model was applied to the data and the natural logarithm (ln) of the surviving fraction plotted as a function of time as shown in Fig. 1, panel A.

Although the plot appears to give a good fit, there is clearly a systematic deviation of the data from the predicted line. Instead of the data being randomly distributed about the predicted line, the second group of four points are below the predicted line while the last two points are both above. To demonstrate the reasoning which we used in developing an improved model, it is necessary to point out some common features of bacterial death/growth experiments.

The usual procedure for such experiments consists of inoculation of bacteria from a stock culture into some medium which is exposed to treatment after the inoculation. Samples are withdrawn from the treatment medium and assayed for the number of viable cells. If no complications occur, a homogeneous cell population can be assumed throughout the course of the experiment. In the event of complications, two or more subpopulations responding differently to the treatment may be encountered. One subpopulation may be inhibited during the time course of the treatment, but subsequently grow during the assay. For example if the assay is done by spread plating aliquots from the treatment solution and culturing over a time period significantly different from the treatment time period, the subpopulation which was inhibited during treatment may recover and grow during enumeration. This would result in an overestimation of the number of growing bacteria in the treatment solution. A similar situation may occur in heating studies, whereby injured cells that do not grow in the test sample may recover and grow in the viable cell assay medium.

Two-term exponential model for mixed cell population

A simple extension of the exponential model would be to assume that there are two populations of bacteria which differ in their sensitivity to heat and therefore die at different rates. The model is illustrated as follows:

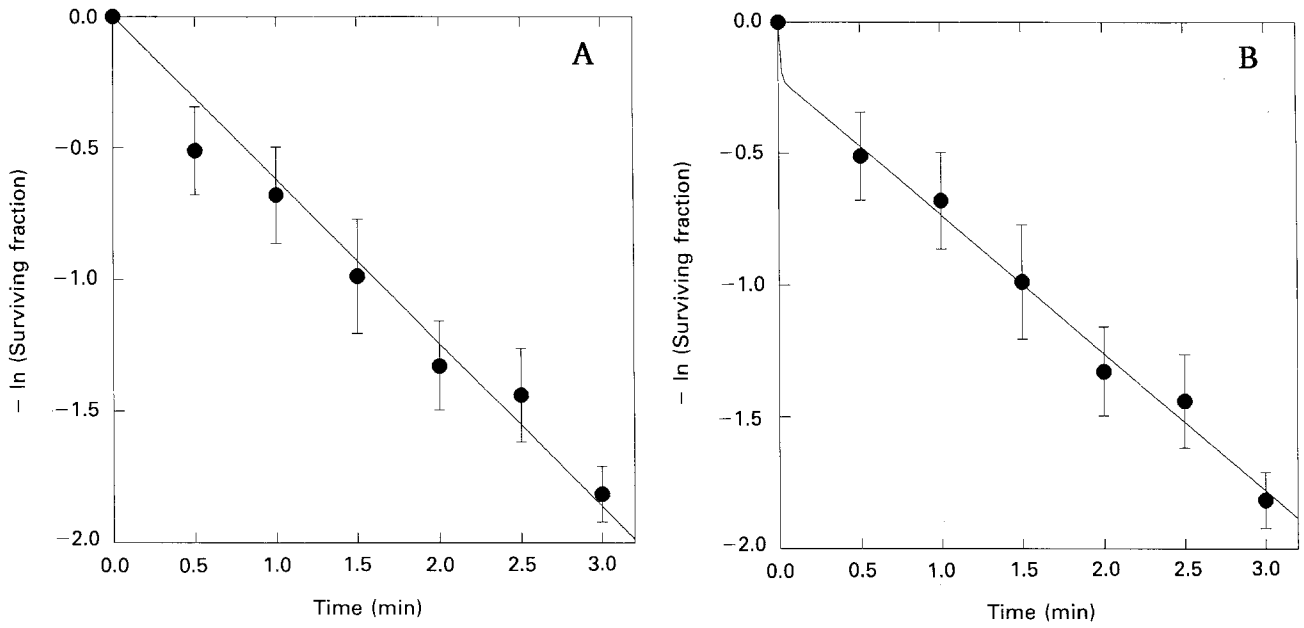

Fig. 1. Survival curves for *L. monocytogenes* heated in bovine milk at 57.8 °C. Aliquots were pipetted into sterile ampules and placed in a 57.8 °C bath. Samples were removed at the indicated times and serially diluted. Survivors were enumerated by spread plating on tryptic soy-yeast extract agar and incubating for 48 h at 35 °C. The plotted points are means of at least 14 observations. Vertical bars represent plus or minus one standard deviation. The line in panel A is the predicted curve based on fitting the data to Eqn (6) and in panel B to Eqn (14) using nonlinear regression [8]. Adapted from [1].

$$S = fe^{-\beta_1 t} + (1-f)e^{-\beta_2 t} \quad (13)$$

where:

S = fraction of total survivors (x/x_0) at time = t,
f = fraction of survivors in population 1 (heat-sensitized),
$(1-f)$ = fraction of survivors in fraction 2 (less heat-sensitized),
β_1 = specific death rate of subpopulation 1, and
β_2 = specific death rate of subpopulation 2.

Taking the natural logarithm of both sides of Eqn (13):

$$\ln(S) = \ln[fe^{-\beta_1 t}+(1-f)e^{-\beta_2 t}] \quad (14)$$

The surviving fraction S is made up of two subpopulations. The fraction dying at specific rate β_1 is represented by f and the fraction dying at rate β_2 is represented by $(1-f)$. In Eqn (14), the simplicity of the single-term exponential equation is lost and linear regression can no longer be applied to the model. However, nonlinear regression can be easily applied using commercially available PC programs, such as BMDP software packages. The data obtained from thermal destruction studies of *L. monocytogenes* heated at 57.8 °C were fitted to this model. The results of ln vs time presented in Fig. 1, panel B, were obtained by nonlinear regression using the BMDP program.

These are the same data which were analyzed by the one-term exponential model. There are two features of Fig. 1B which are worth noting. First, the predicted curve drops very sharply from the initial data point, reflecting an initial rapid death of a more heat-sensitive subpopulation. The second feature is that the observed data points are very close to the predicted line and are randomly distributed on either side. This is in contrast to the apparent systematic deviation of observed mean values from the predicted line using the one-term exponential model in Fig. 1A. In the latter case, the first four points were all below the predicted line and the last two were above. The parameter estimates for these two models are compared in Table 1. Clearly the two-term exponential gives a better fit to the data. The residual sum of squares is significantly lower for the two-term model as would be expected. Also the pseudo r^2 is significantly higher for the two-term model. The β_1 estimates are very interesting. The one-term model predicts a specific death rate of 0.621 for the entire population. The β_1 for the two-term model could not be estimated by linear regression since the routine estimated very high values. In order to get convergence, a value of 100 was inserted. Since β_1 occurs as an exponential, raising 'e' to a power of $(-100\ t)$ gives a result which is essentially zero for any value of t other than zero. This implies that the more heat-sensitive fraction is killed instantly. If this is true, then all of the data at points other than $t = 0$ should be consistent with the following equation:

$$\ln(S) = \ln[(1-f)e^{-\beta_2 t}] \quad (14A)$$

Eqn (14A) is identical to Eqn (14) except that the $fe^{-\beta_1 t}$ term has been omitted. Regression analysis with Eqn (14A) gave $f = 0.193$ (SD 0.031) and $\beta_2 = 0.523$ (SD 0.031). These values are not significantly different from those (Table 1) obtained from regression with Eqn (14) with $\beta_1 = 100$.

The surviving, less heat-sensitive fraction dies at a specific rate of 0.522 which is significantly less than the value predicted by the one-term model. The death of the heat-sensitive fraction (0.19) results in an instant reduction of the inoculum in the untreated milk from 1×10^6 CFU ml^{-1} to 0.8×10^6 CFU ml^{-1}. The difference in these two numbers is of no practical consequence. However, if some additional stress is introduced, the heat-sensitive fraction may be significantly increased.

The effect of an additional stress is demonstrated by the results obtained after exposure of bacteria to the activated lactoperoxidase system followed by heating. The lactoperoxidase (LP) system is a natural defense mechanism found in bovine milk and other exocrine secretions review [5]. The lactoperoxidase enzyme is a normal component of bovine milk. It will catalyze the peroxidation of thiocyanate (SCN^-) to generate oxidized forms of SCN^- which are toxic to many species of microorganisms. The LP system may be activated by supplementing milk with small amounts of SCN^- and hydrogen peroxide [1–3].

TABLE 1

Survival parameter estimates[a] for *L. monocytogenes* min milk at 57.8 °C

Pretreatment	Model	Residual sum of squares	Pseudo r^2	β_1 (SD) min^{-1}	β_2 (SD) min^{-1}	Fraction with β_1 (SD)
None	one-term equation (12)	0.0699	0.970	0.621 (0.023)	—	1(—)
None	two-term equation (14)	0.0169	0.993	>100	0.522 (0.028)	0.193 (0.044)
Activated LP system	two-term equation (14)	0.0491	0.998	>100	3.75 (0.14)	0.899 (0.010)

[a]Determined by nonlinear regression [8]. Adapted from [1].

The effects of the activated LP system on the survival of *L. monocytogenes* in heated milk are shown in Fig. 2. In presence of the LP system, the fraction of survivors dropped by three log cycles within the first 0.2 min. This was a dramatic difference from the control where the LP system was *not* activated. In the control, there was less than one log cycle drop within the first two minutes, whereas the drop was more than 6 log cycles in presence of the LP system. The reasons for these differences are apparent from a comparison of the parameter estimates shown in Table 1. The residual sum of squares and the pseudo r^2 values show that Eqn (14) fits both data sets very well. (Estimates of f and of β_2 obtained from using Eqn (14A) were not significantly different from those listed in Table 1.) In both cases the heat-sensitive fraction is killed almost instantly. Furthermore, the survivors in presence of the activated LP system died at a specific rate which was nearly 8-fold higher than that observed in the control.

The two-term exponential model clearly reveals that the activated LP system increased the more heat-sensitive fraction to 90% compared to 19% in the control. The death of the heat-sensitive fraction (0.90) results in an instant reduction of the inoculum from 1×10^6 CFU ml^{-1} to 1×10^5 CFU ml^{-1}. If the data from the latter experiment had been analyzed using a one-term exponential, the results would have applied to only 10% of the inoculum. Similar effects are observed when other combined methods of food preservation are used. Examples include the use of irradiation and heat (thermoirradiation), acidification and heat, and other food additives followed by secondary processes.

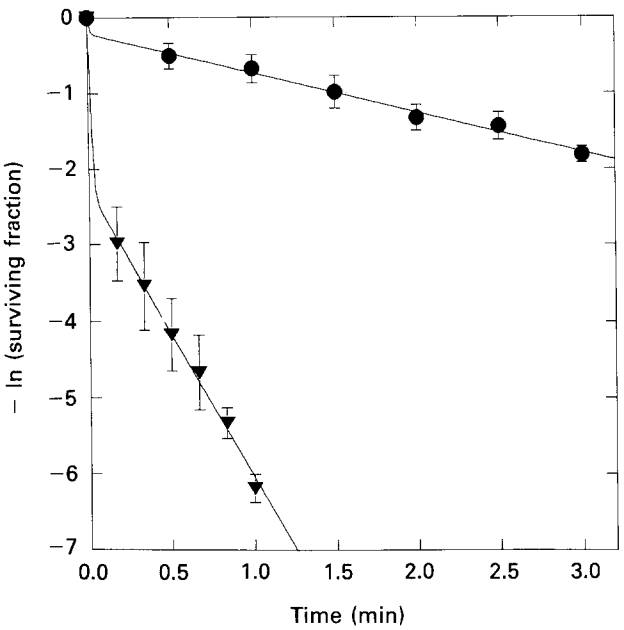

Fig. 2. Survival curves for *L. monocytogenes* heated in bovine milk at 57.8 °C (filled circles) and in bovine milk with the activated lactoperoxidase system (filled triangles). Samples were removed at the indicated times and serially diluted. Survivors were enumerated by spread plating on tryptic soy-yeast extract agar and incubating for 48 h at 35 °C. The plotted points are means of at least 14 observations. Vertical bars represent plus or minus one standard deviation. The lines are the predicted curves based on fitting the data to Eqn (14) using nonlinear regression [8]. Adapted from [1].

Logistic models for destruction of bacteria

The results described above illustrate a practical application of the simple exponential decay curve. In the exponential models, each subpopulation of cells dies at a rate which is proportional to the subpopulation size at any time. There is no apparent limiting factor for killing and no initial lag in killing. If there is a lag in killing, then the survival curves are more complex, and the simple exponential model will give a poor fit to the data. Logistic models may be used to analyze data from these complex survival curves for homogeneous as well as heterogeneous cell populations. In terms of the surviving fraction S for a homogeneous cell population, Eqn (11) may be written as:

$$S = \frac{1+e^{-\beta\tau}}{1+e^{\beta(t-\tau)}} \tag{15}$$

where $S = x/x_0$ = fraction surviving at any time t, β = maximum specific death rate, and τ is a measure of lag in killing. An example of data consistent with Eqn (15) is given in Table 2 for *L. monocytogenes* heated in milk at 55.2 °C. The LP system reduced the value of τ from 16.6 to 3.2 min.

If there is no lag, $\tau = 0$ and Eqn (15) may be written:

$$S = \frac{2}{1+e^{\beta t}} \tag{16}$$

An example is given in Table 2 for *L. monocytogenes* heated in milk at 52.2 °C. Activation of the LP system abolished the lag ($\tau = 0$) in killing.

If there are two cell populations, and each subpopulation responds independently:

$$S = \frac{f[1+e^{-\beta_1\tau_1}]}{1+e^{\beta_1(t-\tau_1)}} + \frac{(1-f)[1+e^{-\beta_2\tau_2}]}{1+e^{\beta_2(t-\tau_2)}} \tag{17}$$

where f = fraction of population with $\beta = \beta_1$ and $(1-f)$ = fraction of population with $\beta = \beta_2$.

If there are no lags, τ_1 and $\tau_2 = 0$ and Eqn (17) may be written:

$$S = \frac{2f}{1+e^{\beta_1(t)}} + \frac{2(1-f)}{1+e^{\beta_2(t)}} \tag{18}$$

Parameter estimates (Table 3) based on data for *Staphylococcus aureus* heated in milk at 55.2 °C after activation of the LP system were consistent with Eqn (18). The more heat-sensitive fraction (0.92) dies almost instantly.

D value calculation for logistic survival curves

A parameter commonly used by microbiologists to measure heat sensitivity is the decimal reduction time (D value)

TABLE 2

Survival parameter estimates[a] for *L. monocytogenes* in milk

Pretreatment	Temp. °C	τ (SD) min	β (SD) min^{-1}	Logistic D value	'Decimal Reduction Time'[d]
None	52.2	42.0 (2.1)	0.0690 (0.008)	74.7[b]	52.2
Activated LP system	52.2	0	0.188 (0.008)	15.7[c]	19.1
None	55.2	16.6 (1.2)	0.244 (0.032)	25.7[b]	14.8
Activated LP system	55.2	3.2 (0.3)	1.22 (0.14)	5.0[b]	3.0

[a]Determined by fitting the data to Eqn (15) by nonlinear regression [8]. Adapted from [1].
[b]Calculated from Eqn (19).
[c]Calculated from $D = 2.94/\beta$.
[d]Calculated from $D = 3.6/\beta$.

TABLE 3

Survival parameter estimates[a] for *S. aureus* heated in milk

Pretreatment	Temp. °C	$(1-f)$ least heat-sensitive fraction	β_2 (SD) – least heat-sensitive fraction min^{-1}	β_1 – most heat-sensitive fraction min^{-1}	Logistic D_2 value[c] min	'Decimal Reduction Time'[d]
None	52.2	1.0	0.0600 (0.0012)	—	49.1	60.0
Activated LP system	52.2	1.0	0.920 (0.029)	—	3.2	3.9
None	55.2	1.0	0.264 (0.089)	—	11.1	13.6
Activated LP system	55.2	0.0800	1.840 (0.360)	>100[b]	1.60	1.96

[a]Determined by fitting the data to Eqn (18) using nonlinear regression [8]. Adapted from [1].
[b]The value of β_1 was too high to be estimated. This means that the most sensitive fraction, 0.92 was killed almost instantly, or $D_2 = 0$.
[c]Calculated as $\ln(19)/\beta_2$.
[d]Calculated as $3.6/\beta_2$.

defined as the time required to reduce a given cell population 10-fold under specified conditions.

The D value is usually reported as the absolute value of the reciprocal of the slope of a plot of $Log_{10}S$ vs time. If the plot is not linear, D is measured from the 'linear' portion of the curve. The 'linear' portion of the logistic curve occurs at τ, which is the point of inflection. The slope at this point is given by $-\beta/4$. Since the D value is the time to reduce the survivors 10-fold, then:

Slope at $t-\tau = \Delta S/\Delta t = -\beta/4$

If $\Delta S = 0.9$, then $\Delta t = D$ and
$D = (0.9 \times 4)/\beta = 3.6/\beta$

Calculated 'Decimal Reduction Times' are listed in Table 2 for *L. monocytogenes* survival curves in heated milk.

'Decimal Reduction Times' estimated in this way do not take into account any lag in killing or any heterogeneity in cell response. Therefore, for survival data which show a lag or are heterogeneous, these D values do *not* give a true estimate of the time required to effect a 10-fold reduction in cell population. A better estimate of the D value for

logistic survival curves can be obtained by using the parameter estimates to calculate the time at which $S = 0.1$. By definition this time is the true D value.

For a population of identical cells dying at the same rate, with no initial lag in death, the D value may be obtained by substituting $S = 0.1$ and $t = D$ in Eqn (16) and solving for D. The result is $D = 2.94/\beta$. The 'Decimal Reduction Time' as calculated above is given by $D = 3.6/\beta$. Therefore, for the same survival curve when $\tau = 0$, logistic D values will always be less than the 'Decimal Reduction Time'. An example is given in Table 2 for heating *L. monocytogenes* in milk at 52.2 °C with the activated LP system. The logistic D value was 15.7 min and the 'Decimal Reduction Time' was 19.1 min.

For a population of identical cells with an initial lag ($\tau > 0$) in death, the D value can be calculated by substituting $S = 0.1$ and $t = D$ in Eqn (15) and solving for D. The result is:

$$D = \tau + \frac{\ln(9+10e^{-\beta t})}{\beta} \tag{19}$$

These calculations are illustrated in Table 2 for *L. monocytogenes* heated in milk at 52.2 °C and 55.2 °C and with activation of the LP system at 55.2 °C. In each instance, the logistic D value is significantly greater than the 'Decimal Reduction Time' because the latter does not take into account the lag in killing.

When there are two subpopulations of cells dying independently at different rates, with no lag in death ($\tau = 0$), D values for the two subpopulations can be estimated by substituting $\tau = 0$ and the appropriate β values into Eqn (19). The results are:

$$D_1 = \ln(19)/\beta_1$$
$$D_2 = \ln(19)/\beta_2$$

The data for thermal destruction of *S. aureus* at 55.2 °C, in the presence of the LP system demonstrated this behavior and were consistent with Eqn (18). Parameter estimates are listed in Table 3. Most of the cell population ($f = 0.92$) was killed almost instantly ($D_1 = 0$). The remaining fraction (0.080) had a D_2 value of 1.6 min. However, the latter value is not representative of the cell population as a whole because it contains such a small fraction of the total population.

Mixed cell response: lag, death, recovery and growth

It may happen that a cell culture is only partially damaged and/or recovers from damage during the course of sample storage and resumes growth. This mixed response may be observed when using antibacterial substances that are both bactericidal and bacteriostatic. Cell populations treated with such substances may show subsequent cell recovery and growth of surviving cells. An appropriate model must take into account growth, inhibition (lag), and death.

For a homogeneous population of cells growing at a rate which is jointly proportional to cell concentration and to the concentration of a single growth-limiting factor, the logistic model, Eqn (8), is appropriate. The cells continue to grow until the growth factor supply is exhausted and they reach a maximum population k. The parameter β is the maximum specific growth rate at which growth would be observed with excess growth factor. The time at which growth reaches $k/2$ is τ. As an example of the application of this equation, data which were collected for the growth of *S. aureus* in raw milk at 37 °C are used [2]. The data were clearly consistent with Eqn (8) as shown in Fig. 3.

When the LP system was activated in milk, there was an initial decline in cell number followed by recovery and growth to the same final level. It seems that there is some initial killing of cells by the LP system. Therefore, we used the following model:

$$x = \frac{k}{1+e^{-\beta(t-\tau)}} + x_{dying}e^{-\alpha t} \tag{20}$$

where x_{dying} is the part of the subpopulation fatally damaged by the activated LP system, α is the specific death rate constant, and the other parameters have the same meaning as before. The first part of the model assumes logistic

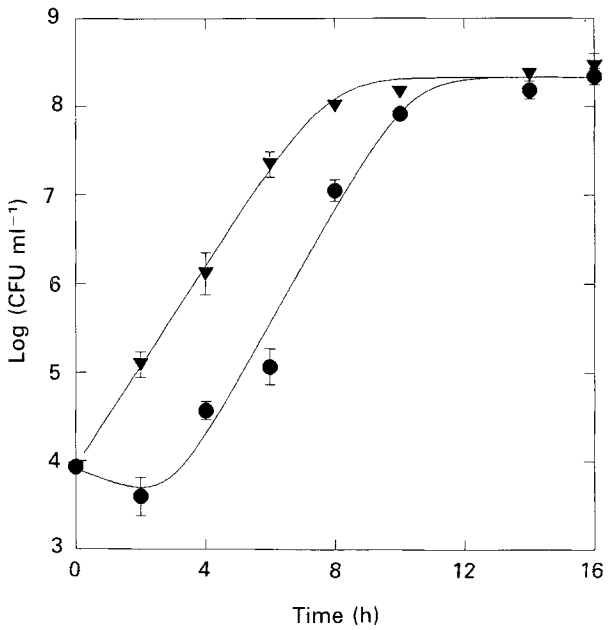

Fig. 3. Growth of *S. aureus* in bovine milk at 37 °C (filled triangles) and in milk with the activated lactoperoxidase system (filled circles). Samples were removed at the indicated times and serially diluted. Survivors were enumerated by spread plating on tryptic soy-yeast extract agar and incubating for 48 h at 32 °C. The plotted points are means of at least four observations. Vertical bars represent plus or minus one standard deviation. The lines are the predicted curves based on fitting the data to Eqn (8) for milk and Eqn (20) for the activated LP system using nonlinear regression [8]. Adapted from [2].

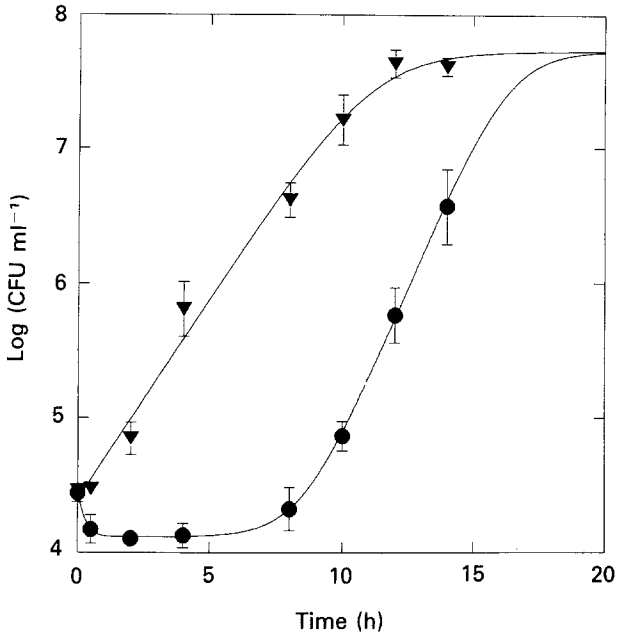

Fig. 4. Growth of *L. monocytogenes* at 35 °C in bovine milk (filled triangles) and in milk with the activated lactoperoxidase system (filled circles). Samples were removed at the indicated times and serially diluted. Survivors were enumerated by spread plating on tryptic soy-yeast extract agar and incubating for 48 h at 32 °C. The plotted points are means of at least four observations. Vertical bars represent plus or minus one standard deviation. The lines are the predicted curves based on fitting the data to Eqn (8) for milk and Eqn (21) for the activated LP system using nonlinear regression [8]. Adapted from [2].

growth. The data were consistent with this model, as shown in Fig. 3.

Nonlinear least-squares parameter estimates for the data plotted in Fig. 4 are listed in Table 4. The dying fraction represents 94% of the cells initially present, which is not obvious from the chart. The specific death rate is 0.34 h^{-1}. However, the standard deviation is quite large indicating a lot of 'floppiness' in this number. This is due to the relatively

TABLE 4

Parameter estimates[a] for *S. aureus* milk at 37 °C

Pretreatment	Dying fraction (SD)	Specific death rate, α (SD) h^{-1}	Maximum specific growth rate, (SD) h^{-1}	Time at which $x = k/2$, τ (SD) h
Control	0	0	1.30 (0.06)	8.22 (0.06)
Activated peroxidase	0.94[b] (0.29)	0.34 (0.57)	1.49 (0.21)	10.3 (0.6)

[a]Adapted from data reported in [2]. Data were fitted to Eqn (20) by nonlinear regression [8].

[b]Calculated by dividing the inoculum size into the population of dying cells, x_{dying}, as estimated from fitting Eqn (20) to the data.

few observations available in the early part of the curve where dying cells are making the greatest contribution to changes in cell population. Although 94% of the inoculum is so damaged by the LP system that these cells ultimately die, the survivors recover and grow at a rate comparable to that of the control. The maximum specific growth rates for survivor and control populations are not significantly different. We call them 'maximum' because they represent the specific growth rate in the absence of any limiting factors. The data show that the cells which survived the LP system were unimpaired. The LP system increases the value of τ by 2 h. However, this effect is primarily due to the initial reduction in the numbers of viable cells.

As a final example, the effects of the LP system on the growth of *L. monocytogenes* in milk at 35 °C are presented in Fig. 4. The period of delayed growth when the LP system was activated was more extended for these organisms compared to *S. aureus*. The model which was used for the *S. aureus* would not fit these data. The addition of exponential killing did not satisfactorily explain the extended period of no growth. In this case an assumption was made that there was a subpopulation of cells which were inhibited by the LP system and showed no growth during the experiment, but they were able to recover and be counted during the 48-h plating assay. The model assumed was as follows:

$$x = x_{recover} + \frac{k - x_{recover}}{1 + e^{-\beta(t-\tau)}} + x_{dying} e^{-\alpha t} \qquad (21)$$

The $x_{recover}$ is the population of cells which show no growth during the experiment but do recover and grow during the plating assay. The effect of this term is to add a constant number to the colony counts observed during plating. The second term represents the surviving, non-inhibited subpopulation which grows during the experiment. The numerator, $k - x_{recover}$, in the second term insures that k represents the ultimate cell population which will be reached. The last term represents the subpopulation which is so damaged by the peroxidase system that it dies exponentially during the treatment period. The other parameters have the same meanings as before. The data for samples with activated LP system were analyzed using nonlinear regression of this model and the results are shown in Fig. 4 and Table 5.

(The data plotted in Fig. 4 are means of four or more observations for each time point. However, the data were collected at only eight time points. Eqn (21) has five adjustable parameters ($x_{recover}$, β, τ, x_{dying}, and α). With so many parameters for so few time points, the question arises as to whether or not fitting Eqn (21) to these data is merely an exercise in curve-fitting rather than modeling. We address this question in the Appendix.)

The predicted line in Fig. 4 agrees precisely with the data. The sharp initial drop is predicted by the model as well as the extended period of little or no change in cell population. A comparison of the parameter estimates for the control and the treated samples (Table 5) reveals some interesting relationships. The recovering fraction (that fraction which is inhibited during the experiment but is able

TABLE 5

Parameter estimates[a] for *L. monocytogenes* milk at 35 °C

Pretreatment	Recovering fraction[b] (SD)	Dying fraction[c] (SD)	Specific death rate, α (SD) h^{-1}	Maximum specific growth rate, (SD) h^{-1}	Time at which $x = k/2$, τ (SD) h
Activated LP system	0.47 (0.02)	0.53 (0.08)	4.06 (1.35)	1.05 (0.03)	16.4 (0.1)
Control	0	0	0	0.69 (0.05)	11.1 (1.0)

[a]Adapted from data reported in [2]. Data were fitted to Eqn (21) by nonlinear regression [8].

[b]Calculated by dividing the inoculum size into the population of recovering cells, $x_{recover}$, as estimated from fitting Eqn (21) to the data.

[c]Calculated by dividing the inoculum size into the population of dying cells, x_{dying}, as estimated from fitting Eqn (21) to the data.

to recover and be counted during the plating assay) and the dying fraction together constitute nearly all of the initial population of cells. The specific death rate (α) for the damaged population is very high. It is interesting that the maximum specific growth rate of the few unaffected survivors in the activated peroxidase sample is significantly greater than that of the control population. In other words, the few cells which survive the peroxidase system and go on to grow do so more vigorously than the untreated controls. Why this should be so is not clear at the present time. The time, τ, required for the population to reach half of the maximum level is extended by 4 h in the activated peroxidase sample. This extension is due primarily to the very small number of cells which are able to recover and grow. This number may be calculated as follows.

The second term in Eqn (21) represents the population of unaffected cells in the sample where the LP system was activated. 'Unaffected' means that the cells were neither inhibited nor fatally damaged by the activated LP system. The measured cell population at $t = 0$ in the LP activated experiment was 28000 CFU. This population grew to a final level of 5.36×10^7 CFU. The latter number is the value of k in Eqn (21). Using this number and setting $t = 0$, $\beta = 1.05$, $\tau = 16.4$ (from Table 5) we may calculate from the second term in Eqn (21) the population of unaffected cells initially present. The result shows that of the initial 28000 CFU, there were only 1.8 unaffected CFU remaining. Thus, the activated LP system essentially, but not quite, wiped out the inoculum. However, even though only a few viable cells remained, they are able to recover and to grow vigorously. This analysis reveals that for this organism, under these experimental conditions, a complete extinction of the inoculum is required to prevent subsequent growth.

CONCLUSION

The time course of complex changes in cell populations can be modeled effectively using the strategies presented here. Growth and death may both be modeled separately or in combination. The division of the total cell population into subpopulations responding differently and independently to the treatment seems to be a useful approach. Commercially available software implemented on a personal computer may be used effectively to carry out nonlinear regression with these models. The values of parameter estimates obtained from this method may yield insights into destructive mechanisms which are not immediately apparent from inspection of the raw data or from linear regression analyses of log CFU vs time plots. Although the examples given here used the simple exponential and logistic models, the same strategy may be used with more complex members of the growth/death family.

REFERENCES

1 Kamau, D.N., S. Doores and K.M. Pruitt. 1990a. Enhanced thermal destruction of *Listeria monocytogenes* and *Staphylococcus aureus* by the lactoperoxidase system. Appl. Environ. Microbiol. 56: 2711–2716.

2 Kamau, D.N., S. Doores and K.M. Pruitt. 1990b. Antibacterial activity of the lactoperoxidase system against *Listeria monocytogenes* and *Staphylococcus aureus* milk. J. Food Protect. 53: 1010–1014.

3 Kamau, D.N., S. Doores and K.M. Pruitt. 1991. Activation of the lactoperoxidase system prior to pasteurization for shelf-life extension of milk. Milswissenschaft 46: 213–214.

4 Malthus T.R. 1798. An Essay on the Principle of Population, (1st edn). J. Johnson, London.

5 Pruitt, K.M. and D.N. Kamau. 1991. The lactoperoxidase

systems of bovine and human milk. In: Oxidative Enzymes in Foods (Robinson, D.S. and N.A.M. Eskin, eds), pp. 257–265, Academic Press, New York.
6 Pruitt, K.M. and M.E. Turner, Jr. 1978. A kinetic theory for analysis of complex systems. In: Biomolecular Structure and Function (Agris, P.F., ed.), pp. 257–265, Academic Press, New York.
7 Pruitt, K.M., R.E. DeMuth and M.E. Turner, Jr. 1979. Practical applications of generic growth theory and the significance of the growth curve parameters. Growth 43: 19–35.
8 Ralston, M. 1990. Derivative-free nonlinear regression. In: BMDP Statistical Software Manual, Vol. 1, (Dixon, W.J., ed.), pp. 395–424, University of California Press.
9 Turner, M.E., Jr and K.M. Pruitt. 1978. A common basis for survival, growth, and autocatalysis. Math. Biosci. 39: 113–123.
10 Turner, M.E., Jr, E.L. Bradley, Jr, K.A. Kirk and K.M. Pruitt. 1976. A theory of growth. Math. Biosci. 29: 367–373.
11 Verhulst, P.F. 1838. Notice sur la loi que la population suit dans son accroissement. Corr. Math. Phys. 10: 113.

APPENDICES

Modeling or curve-fitting?

We consider 'modeling' to be the fitting of experimental data to a mathematical expression whose parameters have plausible relationships to the biological properties of the system upon which the data are based. We consider 'curve-fitting' to be the fitting of experimental data to a mathematical expression whose parameters have no meaningful relationships to the biological properties of the system under study. Since the biological significance of the five adjustable parameters in Eqn (21) is plausible, we consider the fitting of the data to this equation as modeling.

Although we have not carried out the exercise, the data could probably be accurately represented by a polynomial such as:

$$x = a_0 + a_1 t + a_2 t^2 + a_3 t^3 + a_4 t^4 \qquad (1A)$$

where the parameters a_0, a_1, a_2, a_3, a_4 may be positive, negative, or zero. With the exception of a_0 (the value of x at $t = 0$), these parameters have no obvious relationships to the biological properties of the system. In our view, fitting Eqn (1A) to the data would be 'curve-fitting'.

However, fitting Eqn (21), with its five parameters, to a data set composed of only eight time points raises the question of uniqueness for this particular model. Would another model with five parameters having plausible relationships to biological properties fit the data equally as well? To explore this issue, we used the following model:

$$x = \frac{k}{1+e^{-\beta(t-\tau_\beta)}} + \frac{x_{dying}}{1+e^{\alpha(t-\tau_\alpha)}} \qquad (2A)$$

This model assumes that the cell population consists of two fractions behaving differently and independently. The first term in Eqn (2A) describes the behavior of that fraction which is growing logistically at a specific growth rate β and which will reach $\frac{1}{2}$ of its maximum size (k) when $t = \tau_\beta$. The second term describes the behavior of that fraction (x_{dying}) which is dying logistically at a specific rate α. For this fraction, $\frac{1}{2}$ of the cells will have died when $t = \tau_\alpha$.

We fitted the data plotted in Fig. 4 to Eqn (2A) using nonlinear regression [8]. For comparative purposes, the relevant statistics are:

Model	Mean squared error	r^2
Eqn (21)	0.001012	0.9995
Eqn (2A)	0.001984	0.9990

The mean squared error was nearly twice as great for Eqn (2A) as for Eqn (21). However, the r^2 values for the two models were not significantly different. Parameter estimates are compared below:

Model	Parameter	Estimate	CV[b]
Eqn (21)	$x_{recover}$[a]	0.471	5.0
	x_{dying}[a]	0.529	14.5
	α	4.06	33.3
	β	1.05	2.5
	τ	16.4	0.7
Eqn (2A)	x_{dying}[a]	0.420	783
	α	0.266	404
	τ_α	17.0	653
	β	1.18	21.0
	τ_β	16.7	26.7

[a]Fraction of the inoculum
[b]Coefficient of variation = 100 × asymptotic standard deviation/parameter estimate.

The coefficients of variation for the parameters x_{dying}, α, and τ_α of Eqn (2A) are many fold greater than the parameter estimates themselves. Thus, there is great uncertainty about these parameter estimates. For the parameters β and τ_β of Eqn (2A), the coefficients of variation are an order of magnitude greater than those for the corresponding parameters β and τ of Eqn (21). Therefore, although regressions using either equation give acceptable values of r^2, the parameter estimates for Eqn (21) are much more sharply defined than are those for Eqn (2A).

The uncertainty associated with parameter estimates from Eqn (2A) may be attributed to the high correlations among the parameters. Asymptotic correlation matrices for both models are given below.

EQUATION (21)

	x_{recover}	x_{dying}	α	β	τ
x_{recover}	1				
x_{dying}	−0.303	1			
α	0.448	−0.095	1	1	
β	0.283	−0.0867	0.130	−0.929	1
τ	−0.205	0.060	−0.0911		

EQUATION (2A)

	x_{dying}	α	τ_α	β	τ_β
x_{dying}	1				
α	0.977	1			
τ_α	0.992	0.942	1		
β	−0.984	−0.925	−0.998	1	
τ_β	−0.905	−0.974	−0.843	0.815	1

These matrices show that for Eqn (21) only the parameter estimates for β and τ are highly correlated, but that for Eqn (2A) *all* of the parameter estimates are highly correlated. Therefore, we conclude that Eqn (21) is the better model.

We have no additional, independent experimental observations to confirm the assumptions leading to Eqn (21). Although the above analyses do not prove that Eqn (21) represents a true, unique model for the biological events underlying the observations, they do establish that fitting the data to Eqn (21) is an exercise more akin to modeling than to curve-fitting.