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The Origin of the Time Scale: A Crucial Issue for Predictive Microbiology

Alberto Schiraldi*

Formerly at the Department Food Environmental and Nutritional Sciences (DeFENS), University of Milan, Italy
*Corresponding author: alberto.schiraldi@unimi.it

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Abstract The collective behavior of microbial cells in a batch culture is the result of interactions among individuals and effects of the surrounding medium, which changes during the growth progress. A semi empirical model skips biological and physiological peculiarities of the microorganisms and focuses on the observed sigmoid shape of the growth curve that is a common feature of batch cultures of pro- and eukaryotic microorganisms. The model replaces the observed growth trend with the behavior of an ideal batch culture that undergoes an unperturbed duplication process. It leads one to recognize that: • the origin of the time scale for the microbes, θ, differs from that of the observer, t; • the absolute reference state for any batch culture is log (N) = 0 (no matter the log base) for $\theta = 0$; • the cell duplication occurs after an active latency gap, θ_0, that decreases with increasing inoculum population, $\log_2(N_0)$ and increasing temperature; • θ_0 substantially differs from the lag phase, λ, considered by most authors; • the use of reduced variables allows gathering different growth curves in a single master plot; • the model applies to batch cultures which undergo change of the environmental conditions and predicts the width of the intermediate latency gap just after the change; • the expression for the decay trend of the microbial population allows definition of a parameter suitable to rank the effects of bactericidal drugs. The model justifies the demand of more restricted safety limits of microbial loads.

Keywords: *predictive model, batch cultures, latency gap, time scale*

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1. Introduction

Predictive microbiology provides suggestion for a safe shelf life of many food products that can undergo microbial spoilage in various conditions of temperature, water activity, pressure, etc. Predictions mainly deal with the expected extent of the microbial growth that should remain below a safety threshold.

The early phases of the growth, including the duration of the pre-growth latency period, are therefore of major interest and have so far been the subject of many investigations [1-7]. All these authors proposed growth models that can account for the so-called growth curve, $\log(N/N_0)$-*vs*-t [9], namely, the increasing trend of the microbial population density, N, from a starting level, N_0. These models underwent adjustments by addition of fitting parameters [10,11,12]. The aim was to connect the macroscopic growth data, namely, plate counts or OD records, with the metabolic and physiologic subtleties, discovered thanks to the progress of cellular biochemistry and/or results from microbial cultures in chemostatic conditions [13]. As for the early phase of the growth, further improvements came from the search of the single-cell lag time and the construction of the onset tail of the growth curve as the result of a double gamma distribution of lag times, one for the single cell and the other for the growing population [11,12].

Consequently, new parameters and coefficients had to enter the fitting treatment, which therefore required adequately large sets of good quality experimental data.

A completely different approach sees the collective behavior of the cells as the result of simple interactions among individuals, like that of starling flocks or fish classes investigated by the group of Giorgio Parisi (2021 Nobel laureate for Physics) [14,15]. Overcoming the amazing number of variables of *a priori* unknown relevance, one should view "*microbial communities as dynamical systems and apply mathematical models to characterize community structure and dynamics, to predict responses to perturbations and to explore general dynamical properties such as stability, alternative stable states and periodicity*" [16].

The available information tells us that:

- the increase of N is the neat resultant of birth of new cells and death of others;
- no synchronism appears between single cells, and
- the medium (available substrate, production of catabolites, etc.) changes with the growth progress.

Nonetheless, the experimental evidence of the count of viable cells, *N*, or the mass of living material, *m*, produces a "growth curve" that shows a sigmoid rising trend of *N* for any batch culture of pro- and eukaryotic microorganisms [17]. This suggests that, in line with Parisi's proposal, one must envisage a simple model that skips the individual peculiarities and reproduces the observed overall trend. The model should refer to a property that prevails on the specific differences between microbial species and describe it with a suitable mathematical expression. A semi empirical model by the present author [18-23] (briefly summarized below) seems in line with such expectations.

The main scope of the present paper is to show that this model is of interest for Predictive Microbiology investigations. It identifies the latency gap preceding the start of the cell duplication process in an alternative way with respect to the traditional [7] lag phase, λ, and suggests a time scale of the batch culture that differs from the time scale of the experimenter. This is a key issue to predict a correlation between the width of the latency gap and starting population density and the forthcoming specific duplication rate. The model applies also to the intermediate latency gap observed on abruptly changing the environment (temperature, water activity, pH, etc.) of the culture. Finally, the extension of the model to the decay of the cell population allows definition of a parameter suitable to rank the effects of bactericidal drugs.

2. The Model

The model comes from the unquestionable assumption that cell growth of prokaryotic microbes occurs via duplication of single cells, starting from the initial level N_0, namely,

$$N = N_0 2^{t/\tau} \tag{1}$$

where $(1/\tau)$ is the duplication rate and t is the elapsed time.

The proposed approach replaces reality with a virtual situation: an ideal batch culture mimics the observed growth trend through an unperturbed duplication process. The ideal culture hosts synchronic generation lines from the N_0 cells of the inoculum, which have the same age. No cell dies during the progress of the growth. To reproduce the observed sigmoid trend, τ varies during the growth process, namely $\tau = \tau(t)$. The function $\tau(t)$ replaces the discontinuous succession of generation steps and implicitly accounts. for the collective effects of many different contributions, like crowding of the cell population, quorum sensing, presence of adverse metabolic catabolites, etc., which come into play during and because of the growth progress of real batch cultures. $\tau(t)$ has to comply with the constraints that the duplication rate, $1/\tau$, must be null for $t \to 0$ and $t \to \infty$. It was proven [23] that a simple and suitable expression for $\tau(t)$ is:

$$\tau(t) = \frac{\alpha}{t} + \frac{t}{\beta} \tag{2}$$

where the parameters α and β are defined through the best fit of the experimental data (plate counts or OD records) of the real batch culture. This makes the model semi empirical and suitable to account for the interactions between cells and for changes of the medium during the growth progress.

A continuous function can therefore describe the growing trend of the microbial population

$$\log_2(N) = \log_2(N_0) + \frac{\beta t^2}{\alpha\beta + t^2} \tag{3}$$

where *N* stands for either population density (CFU/volume unit) or overall number of viable cells (CFU).

The parameters α and β have a physical meaning: β is the number of duplication steps undergone along each generation line (with $N_{max} = N_0\, 2^{\beta}$), while the ratio (β/α) reflects the maximum value, μ_e, of the specific duplication rate, $\dot{N}/N = \frac{d\log_e(N)}{dt}$ (with the Newton notation for time derivatives).

$$\mu = \frac{\mu_e}{\log_e(2)} = \frac{3\sqrt{3}}{8}\sqrt{\frac{\beta}{\alpha}} \quad (in\ the\ log_2\ scale). \tag{4}$$

$$\log_2\left(\frac{N}{N_0}\right) = \frac{3\sqrt{3}}{8}\sqrt{\frac{\beta}{\alpha}}t - \frac{\beta}{8}$$

is the corresponding expression for the straight-line tangent to the growth curve, $\log_2(N/N_0)$ *-vs -t*, at the inflection point,

$$t = t^* = \sqrt{\frac{\alpha\beta}{3}} \tag{5}$$

which crosses the *t*-axis at $t = t(0) = t^*/3$ and reaches the value $N_{max} = N_0\, 2^{\beta}$ at $t = t_{end} = 3t^*$. The reader can find in [23] the algebraic details and the suggested the best fit routine for the experimental data, which must be significantly (in the statistical sense) above the $\log_2(N_0)$ level. Almost identical expressions hold for eukaryotic microorganisms that grow with different mechanisms: one has simply to replace *N* with an analog quantity, like the living biomass, *m*, and use an adequate base of the logarithm (*e.g.*, 10 or *e*) to treat the experimental data.

All the above equations concern just the cell duplication, with no account for any preceding phase, where no cell duplication takes place. Consequently, the start of the cell duplication process does not coincide with the time origin for the experimenter. The corresponding time gap, t_0, is to subtract from the time elapsed since the actual start of the experiment. Figure 1 shows the main peculiarities of such ideal growth curve.

Figure 1 clearly shows that the traditional sigmoid shape of the growth curve actually concerns the increase of the cell population, determined from the plate count or OD data. It may not include any preliminary non-proliferative process. Its upward onset simply reflects longer duplication times. The true pre-growth phase occurs during the t_0 time span that precedes the growth onset. This is a main difference with respect to the lag-phase so far described by other authors [7].

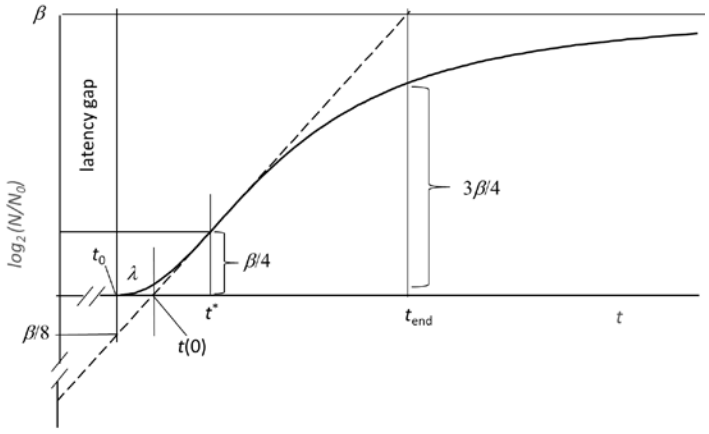

Figure 1. Sketch of the ideal growth curve according to the proposed model. The latency gap of the present model substantially differs from the lag phase, λ, considered in preexisting models [7]

The introduction of reduced variables, $\xi = \log_2(N/N_0)/\beta$ and $t_R = (t\text{-}t_0)/(t^*\text{-}t_0)$, allows one to gather in a single master plot (Figure 2) the growth curves of any duplicating microbial strain, no matter the experimental conditions [18,19,20]. The corresponding equation holds for pro- and eukaryotic microorganisms [18].

$$\xi = \frac{t_R^2}{3+t_R^2} \qquad (6)$$

The data reported in Figure 2 come from the quoted literature: treated according to the present model, each data set provided the relevant t^*, t_0, α and β. These parameters allowed the evaluation of the related reduced variables and the insertion in the (ξ, t_R) plot.

Now one can define the shift between the time origin of the scale of the experimenter, t, and the time scale of the batch culture, θ. Using the Eq.4 adjusted taking into account the t_0 shift and Eq.s 3 and 5, one gets

$$\log_2\left(\frac{N}{N_0}\right) = \frac{3\beta}{8}\frac{(t-t_0)}{(t^*-t_0)} - \frac{\beta}{8} \qquad (7)$$

The natural origin of time scale of the experimenter, $t = 0$, coincides with the beginning of the experiment. It is soon evident that eq.7 implies an unlikely negative value of $\log_2(N/N_0)$ for $t = 0$. Looking at such incongruence with the eyes of the microbial culture, an ideal start condition, $\theta = 0$, suitable for any batch culture (and any log scale), is $\log(N) = 0$ (namely, $N = 1$ CFU (or 1 CFU/unit volume, or 1 CFU/unit mass). The corresponding value in the scale of the experimenter is t_{start} (Figure 3), which is a virtual value with no effect on the fitting treatment and related conclusions (even when $t_{start} < 0$). This choice leads to

$$\log_2\left(\frac{N}{N_0}\right) = \frac{3\beta}{8}\frac{(\theta-\theta_0)}{(\theta^*-\theta_0)} - \frac{\beta}{8} = \mu(\theta-\theta_0) - \frac{\beta}{8} \qquad (8)$$

No matter the origin of the time scales, $(t\text{-}t_0) = (\theta\text{-}\theta_0)$, $(t^*\text{-}\ t_0) = (\theta^*\text{-}\theta_0)$ and $\theta_0 = (t_0 - t_{start})$. For $t_0 > t > t_{start}$ (or within the θ_0 interval in the time scale of the culture), the culture would undergo the preliminary metabolic and physiological steps that prepare the cell duplication. One may dub this time gap "active latency gap", which substantially differs from the so-called lag phase, λ, of the other models, which simply corresponds the onset tail of the sigmoid trend of the growth curve [7]. For the present model, this tail is a consequence of the variable duplication time, $\tau(t)$, and deals with a duplication progress that is slower in its earlier steps (*e.g.*, cells prefer to grow in size [24] enriching in ribosomes [25] rather than in number).

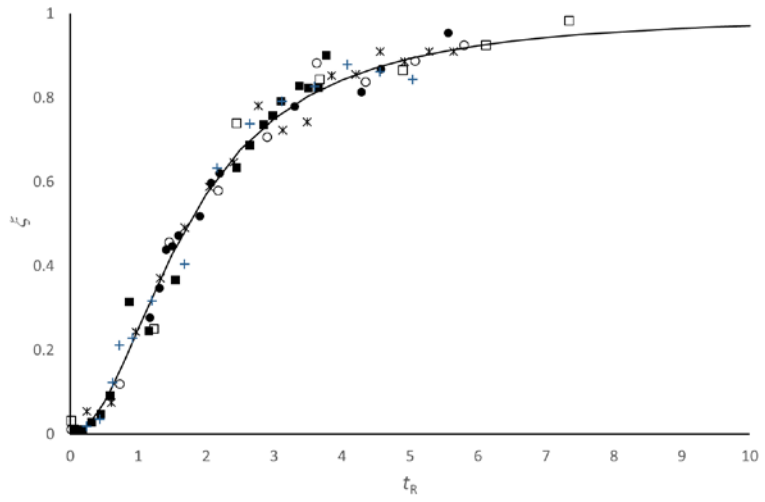

Figure 2. Gathered growth curves from a variety of microbes (Lactobacillus delbrueckii subsp. bulgaricus, Streptococcus thermophilus, Salmonellae, C. jejuni, Pseudomonas fluorescens and Candida sake) adjusted from [18]. For further data, see also Figure 2 in [21]

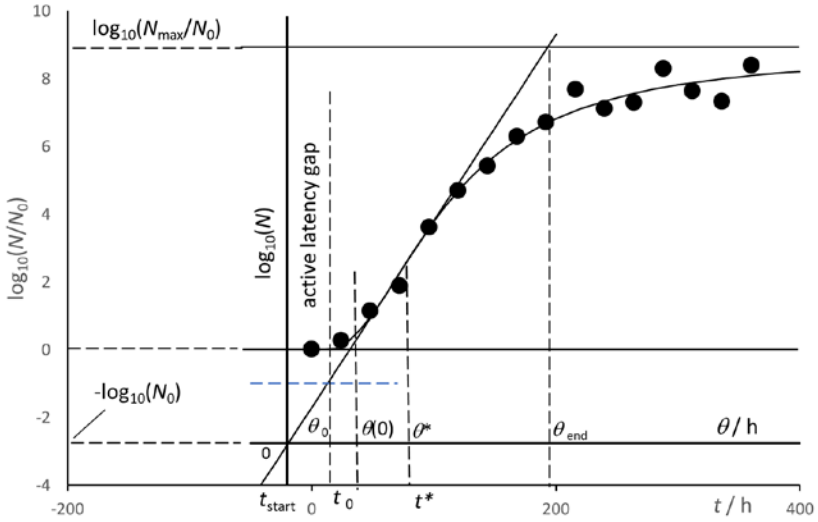

Figure 3. The origin of the time scale of the microbial culture, θ, differs from that of the experimenter, t. The dashed line that allows calculation of θ_0 corresponds to the level –β/8. For $0 < \theta < \theta_0$, the microbial culture experiences activities related to the incoming duplication (active latency gap). Plate count data come from a batch culture of Pseudomonas aeruginosa at 5°C [26]. Calculated values: t_{start} = -20 h; t_0 = 12 h; θ_0 = 32 h

For $\theta < 0$ (no matter the corresponding value of t), the activity of the culture would not directly concern the preparation of the forthcoming duplication process [23]. This holds also if $t_{start} < 0$ in the time scale of the experimenter: the negative value of t_{start} would simply suggest that the used inoculum was already in the active latency gap at the beginning of the experiment ($t = 0$). Figure 3 reports an example.

3. Predictive Consequences

This choice ($N = 1$ for $\theta = 0$) leads to relationships of some interest for predictive microbiology studies. With reference to Eq. 8 and putting the condition $\theta = 0$ and $N = 1$, one obtains

$$\log_2(N_0) = \mu\theta_0 + \beta/8 \qquad (9)$$

From the α and β values (obtained from the best fit of experimental data), one can calculate the relevant μ through Eq.3 and θ_0 from Eq.9 for any value of N_0. Some authors [15,26] suggest that, at given temperature, water activity, pH, etc., μ is an intrinsic property of the system, namely, cells and surrounding medium, and reflects the condition that allows all the underlying metabolic and physiologic steps to occur at the same pace - the so-called "*balanced growth*"-, no matter the starting level of the batch culture, N_0. According to this vision, for a given microbial culture at a given T, μ is a constant. One therefore may align the observed growth trends starting from different N_0 levels along a single straight line tangent to all the trends at the respective θ^* and going through the same origin [$\theta = 0$, $\log_2(N) = 0$] [22,23]. Figure 4 shows a sketched picture that shows how the active latency gap increases with increasing N_0, while the traditional lag phase, λ, shows an opposite behavior.

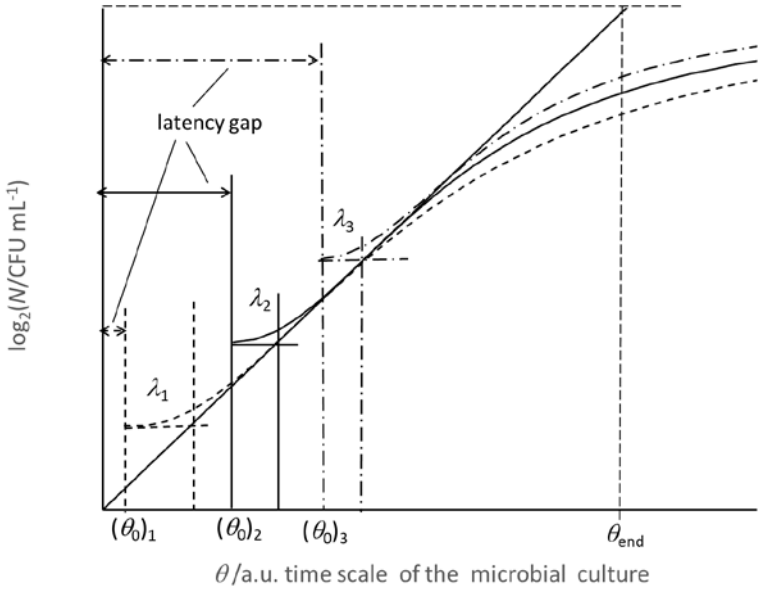

Figure 4. Growth curves from different N_0 inoculums, at the same temperature, aligned along the same straight line that corresponds to the same maximum specific duplication rate, μ.

This can be of interest for predictive studies, allowing an immediate and direct comparison of growth trends starting from different N_0 inoculums. An advantage of using the θ scale, is that one can easily recognize (see Figure 3) that

$$\frac{\theta_{end}}{\theta(0)} = \frac{\log_2(N_{\max})}{\log_2(N_0)} \quad (10)$$

$$\mu = \frac{\log_2(N_{\max})}{\theta_{end}} = \frac{\log_2(N_0)}{\theta(0)} \quad (11)$$

Equations 10 and 11 can be of help to perfect the fitting treatment of the experimental data, improving the reliability of calculated values of μ and $\theta(0)$.

Furthermore, Eq.9 indicates a straight line correlation between θ_0 and $\log_2(N_0)$, at a given temperature. Since $\beta = \log_2(N_{max}) - \log_2(N_0)$, such correlation implies that

$$\lim_{\log_2(N_0)\to\beta/8}(\theta_0) = \lim_{\log_2(N_0)\to\log_2(N_{\max})/9}(\theta_0) = 0 \quad (12)$$

This means that the width of the active latency gap of low N_0 inoculums tends to vanish. The above relationships also lead to

$$\lim_{\beta\to 0}(\theta_0) = \log_2(N_{\max})/\mu \quad (13)$$

This means that when the cell population of the starting inoculum is not far from the maximal capacity of the system, N_{max}, the active latency gap tends to a maximum width.

Experimental evidence generally confirms the expectation that μ increases with increasing T (below pasteurizing thresholds). Since $\log_2(N_{max})$ too changes, but to a much smaller extent than μ (see below), Eq. 11 states that θ_0, as well its upper limit, $\log_2(N_{max})/\mu$, has to decrease with increasing T (Figure 5).

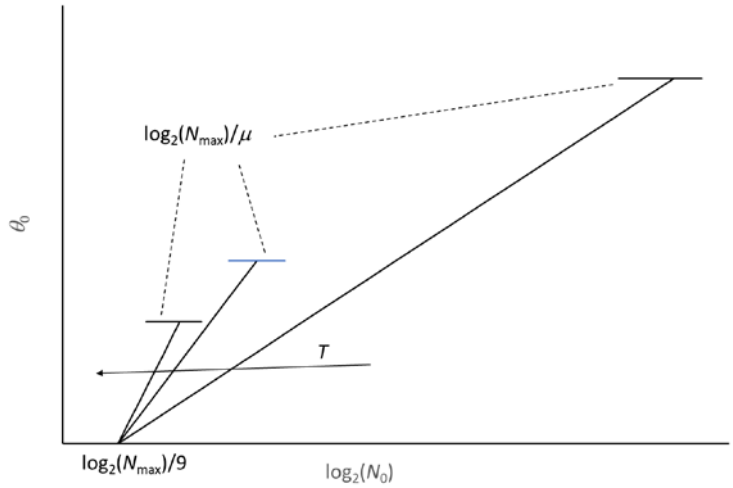

Figure 5. The width of the active latency gap, θ_0, vanishes for $\log_2(N_0) = \log_2(N_{max})/9$. At a given T, θ_0 increases on increasing $\log_2(N_0)$, but decreases on increasing T. Its upper limit, $\log_2(N_{max})/\mu$, decreases on increasing T

For a given N_0, the change of μ with T still implies that the corresponding straight-line tangent to the growth curve at θ^* must go through the origin of the time scale of the microbial culture ($\theta = 0$, $\log(N) = 0$), as shown in Figure 6. This allows a direct comparison of growth curves recorded at various temperatures and prediction of the effect of temperature on the duplication rate in a way that is much more reliable than the abused (too narrow temperature range) Arrhenius expression.

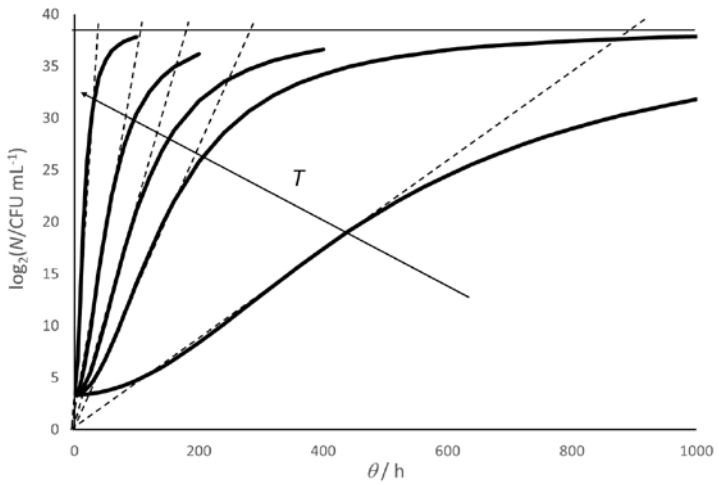

Figure 6. The maximum specific duplication rate increases with increasing temperature, while no significant changes concern the maximum population density, N_{max}. Such a behavior occurs for the batch cultures of psychrotrophic bacteria [21].

At fixed N_0, the trends of μ and θ_0 with increasing temperature do not counterbalance each other [21]. This means that the product ($\mu\theta_0$) can increase or decrease on increasing T. Since $\beta = \log_2(N_{max}) - \log_2(N_0)$, one can rewrite Eq.9,

$$\mu\theta_0 = \left[9\log_2(N_0) - \log_2(N_{max})\right]/8 \qquad (14)$$

This implies that $\log_2(N_{max})$ and the product ($\mu\theta_0$) have opposite trend with increasing T and that the variation of $\log_2(N_{max})$ is smaller than that of μ (see above).

When $\log_2(N_{max})$ does not change too much (within the experimental error; see, for example, [21,28]), α is the parameter most affected by the variation of T [22].

In a predictive perspective, the main conclusion to draw is that small microbial loads can quickly grow up to noxious levels, because of their very short latency gap, especially if the environment temperature rises above a given safe threshold. Since the above relationships hold for either pro- or eukaryotic microorganisms (as previously mentioned), it seems advisable to review the safety limits of the microbial loads for many microorganisms, on the base of evaluations through the present model.

4. Predicted Response to Environment Changes

The model of the ideal batch culture implies only changes of the culture medium related to the growth progress and implicitly accounted for by the function $\tau(t)$, because of the empirical origin of α and β.

Changes of temperature, pH, water activity, or concentration of some critical substrate, intentionally induced by the experimenter or related to an external perturbation, modify the growth trend [25]. Applying the same model to the trends observed before and after an environment change, the description of the overall behavior of the batch culture implies a discontinuity between two "standard" duplication processes, relevant to environment 1 and environment 2, respectively. Such discontinuity corresponds to an intermediate latency gap [27,28]. Assuming that the straight line with slope μ reflects a peculiar balance between cell population and surrounding environment [25], the abrupt change of the latter (change of temperature, pH, water activity, or ionic strength, extra microbial strains, etc.) should imply an instantaneous change of μ. If the change occurs at θ_{change}, this instant becomes the new origin of the time scale for the microbial culture. Figure 7 reports the sketched picture of an abrupt change of temperature: increase, $T_1 \rightarrow T_2$, or decrease, $T_1 \rightarrow T_3$, and related intermediate latency gaps.

It is worth noticing that the width of the intermediate latency gap depends both on the difference between μ values of the two environments and on the value of $\log_2(N_{change})$. However, quenching the culture to lower temperature implies a wider intermediate latency gap than heating to higher T. This conclusion is in partial agreement with some experimental evidence [29]. Such a behavior may have several biological and physiological reasons: the simplest one is the change of the rate of biochemical reactions underlying cell duplication [30,31], but one may not exclude the unbalanced crowding/mechanical interaction perceived by the cells [32]. Similar considerations hold for environment changes dealing with other physical, chemical and biological variables, like water activity, pH, medium solvent, extra microbial contamination, etc. [33,34,35], as far as they imply a change of μ. The quasi-chemical model by Ross and his coworkers [34,35] leads to similar conclusions, at the price of *a priori* assumptions and many adjustable parameters, and does not distinguish the time scale of the culture, θ, from that of the experimenter, t.

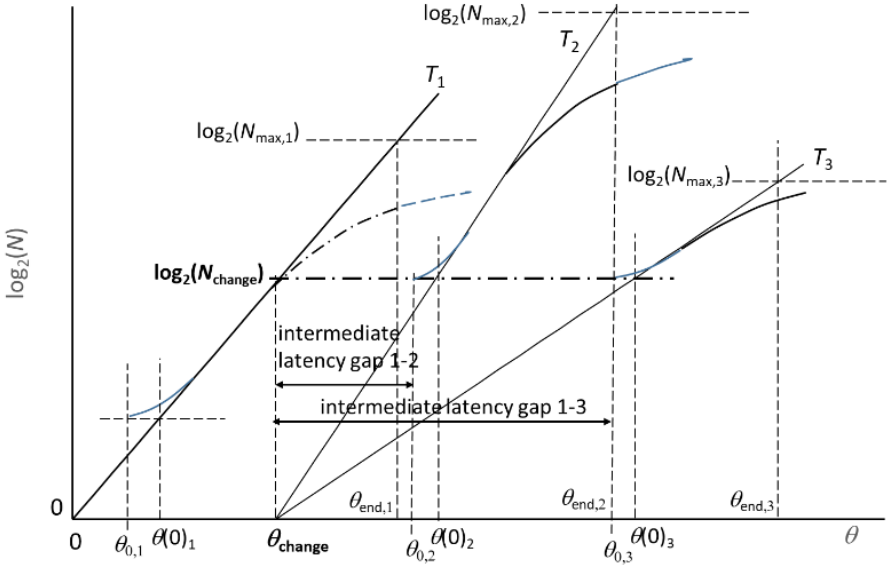

Figure 7. The change of the temperature from T_1 to T_2 or T_3 ($T_2 > T_1 > T_3$) modifies the growth trend. θ_{change}, is the new origin of the time scale for the culture and the corresponding level of the cell population is the new $\log_2(N_0)$. ($\theta_{0,2} - \theta_{change}$) and ($\theta_{0,3} - \theta_{change}$) are the intermediate latency gaps for the $T_1 \rightarrow T_2$ and $T_1 \rightarrow T_3$ temperature changes

When the environment 2 is strongly adverse to the growth progress and implies the death of the cells, as for the inoculation of a bactericidal drug, one should observe the decline of the microbial population. The growth trend first relent because of the death of the old (weakest) cells, but, once the younger generations are involved, the decay of the population density takes place with a cascade trend very similar to the "natural" decay so far described and tested for aged unperturbed batch cultures of non-sporulating and non-phenotypically adapting bacteria [20,23],

$$N = N_{change} \exp\left(-\frac{\theta^2}{d}\right) \qquad (12)$$

where $\theta = (t - t_{inoculation})$. An experimental evidence concerns, for example, the inoculation of a 20 γ (γ = 1 μg mL^{-1}) dose of doxorubicin in a batch culture of *Lactobacillus helveticus* [(Figure 12 in 23]). The parameter *d* accounts for the effect of the drug: a large *d* corresponds to a weak and delayed bactericidal action. One can therefore use the value of *d* to compare the efficacy of different drugs and related doses. With respect to other mathematical description of the population decay [34,35] the present model uses a single parameter, without compromising the fitting reliability [23].

5. Conclusion

A semi-empirical model describes the collective behavior of cells and medium in a batch culture and allows one to recognize that:

- the origin of the time scale for the microbes, θ, differs from that of the observer, *t*;
- the absolute reference state for any batch culture is log (*N*) = 0 (no matter the log base) for $\theta = 0$;
- the cell duplication occurs after an active latency gap, θ_0, which decreases with increasing inoculum population, $\log_2(N_0)$, and increasing temperature;
- θ_0 substantially differs from the lag phase, λ, considered by most authors;
- the use of reduced variables allows gathering different growth curves in a single master plot;
- the model applies also to batch cultures perturbed by environment changes;
- the expression for the decay trend of the microbial population allows definition of a parameter suitable to rank the effects of bactericidal drugs.

The model applies to both pro- and eukaryotic microorganisms and suggests a restriction of the safety limits of the allowed microbial load for many microorganisms.

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