

# Task 3.3: Microscopic predictive modeling

## Step 1: Individual based models

### The theory of cell division: a valuable individual based concept to quantify lag phenomena

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In order to learn more about the basic mechanisms behind lag phenomena, in this research, the theory of cell division is studied on an individual based level. While the literature (Section 1) dates from 30 to 40 years ago, it has until now hardly been introduced in the field of predictive microbiology. Yet it provides a large amount of information on how cells would likely respond to changing environmental conditions. In Section 2, this knowledge is translated into a general theory on population behaviour in dynamic environmental conditions. In Section 3, the existing concepts on lag phenomena in predictive microbiology are critically evaluated with respect to the developed theory. In conclusion (Section 5), the theory of cell division provides a valuable concept to analyze lag behaviour.

## 1 Theory of cell division

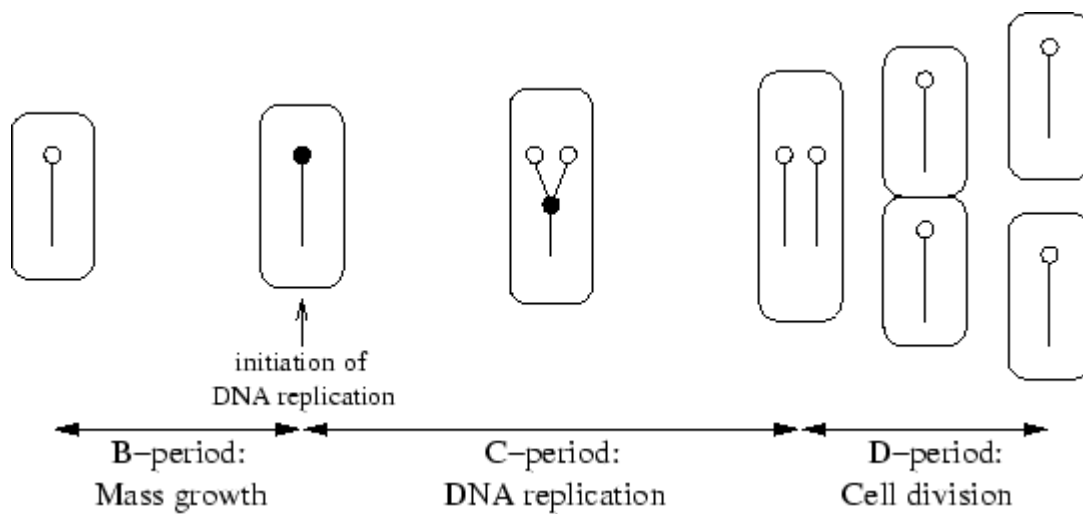
Adequately simulating cell size as a function of growth rate is an important requirement for models in microbial ecology (Kreft et al., 1998). It is a classic observation that resting, non-dividing bacterial cells are small and exponentially growing cells are larger. Hence, cells are often described as *resting* or *exponentially growing*, implicitly considering these conditions as two alternative physiological states. More generally, Schaechter et al. (1958) show that a large number of physiological states exist, each of which is characterized by a particular size and chemical composition of the cells. They observed an exponential relationship between the average cell mass  $\bar{m}$  and growth rate in different media. This relationship can be mathematically described by the following equation:

$$\bar{m} = c \exp[ \mu ] \quad (1)$$

with  $\mu$  the specific growth rate and  $c$  a positive constant. In the same way, the amount of ribonucleic acid (RNA) (Increase in size and enrichment of RNA go hand in hand (Cooper, 1991)), and the amount of deoxyribonucleic acid (DNA) can be described as exponential functions of the growth rate at a given temperature. Cooper and Helmstetter (1968) proposed a model for chromosome replication and cell division

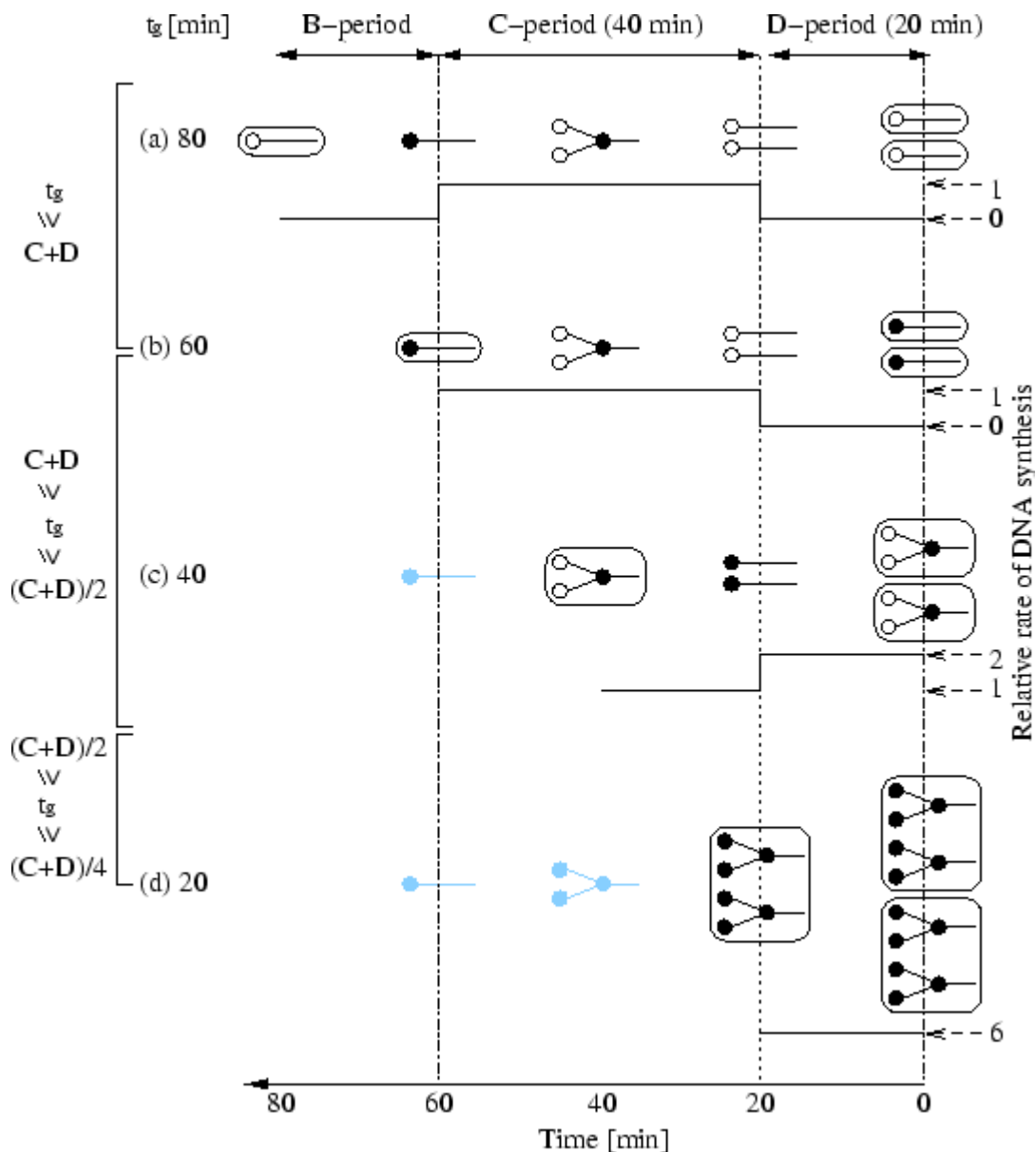
which explains the variation in DNA content in terms of variation of chromosome configurations in bacteria. This is discussed with the aid of Figures 1 and 2.

The sequence of events in the bacterial cell cycle is summarized in Figure 1.



**Figure 1:** Schematic representation of the cell cycle (after Keasling et al. (1995)). The volume of the cell grows continuously at a certain growth rate  $\mu$ . At a particular time, DNA replication is initiated. An un-initiated chromosome origin is represented by an open circle. A chromosome origin at which DNA replication is initiated is represented by a filled circle. The time required for a replication fork to proceed from one end of the genome (origin) to the other (terminus) is denoted by the *C*-period. The time needed for cell division (after replication is terminated) is denoted by the *D*-period.

The volume of the cell grows continuously at a certain growth rate. At a particular time, DNA replication is initiated. An un-initiated chromosome origin is represented by an open circle. A chromosome origin at which DNA replication is initiated is represented by a filled circle. The time required for a replication fork to proceed from one *end* of the genome (origin) to the other (terminus) is denoted by the *C*-period. The time needed for cell division (after replication is terminated) is denoted by the *D*-period. Corresponding to experimental observations, Cooper and Helmstetter (1968) assign, for a temperature of 37°C, two constant values to *C* and *D* of 40 and 20 minutes, respectively. The effect of this presumption on division cycles of cells growing at various rates is schematically illustrated in Figure 2.



**Figure 2:** Schematic illustration of the model of Cooper and Helmstetter (1968) (after Margolis and Cooper (1971)). Two constant values (40 and 20 minutes respectively) are assigned to the C and D-periods, for an environmental temperature of 37°C. The effect of this presumption on the postulated chromosome configurations during the cell cycle is represented for different generation times  $t_g$ . The drawings in gray do not belong to the actual cell cycle but depict the outgrowth of a hypothetical cell containing a single chromosome. The time of termination of cell division has been given the same position in each case (time 0 at the right). The abscissa indicates minutes prior to termination of cell division. The relative rate of DNA synthesis (~ number of replication forks) is represented on the axis on the right.

For a number of generation times  $t_g$ , a schematic representation of the cell cycle (with the postulated chromosome configurations) is given. The relative rate of DNA synthesis (~ number of replication forks) is represented underneath. The time of termination of cell division has been given the same position in each case (time 0). The abscissa indicates minutes prior to termination of cell division. Because of the constant values for C and D, ( $C + D = 40 + 20$  minutes), initiation of DNA replication

coincides at 60 minutes before termination of cell division. Beneath, a some possible cases are discussed.

(a)

$t_g > C + D$  : when the generation time is larger than  $C + D$ , the first part of the cell division cycle is entirely devoted to cell growth ( $B$ -period). 60 minutes before the end of the cycle, DNA replication is initiated (a filled circle at the chromosome origin) and the  $C$  and  $D$  periods are commenced. The relative rate of DNA synthesis varies between 0 and 1.  $C$  minutes later (at time 20), chromosome replication is finished. Then it takes a further 20 minutes until cell division is terminated.

(b)

$t_g = C + D$  : when the generation time is equal to  $C + D$ , the start of the division cycle and the initiation of DNA replication coincide.

(c)

$t_g < C + D$  : when the generation time is smaller than  $C + D$ , DNA replication for the next cell division must already have been initiated when a new cell cycle begins. For a generation time of 40 minutes, for example, 20 minutes before the start of a new division cycle, DNA replication is already initiated. This leads to a larger DNA content per cell as well as multiple active replication forks in one cell.

(d)

For the case of  $t_g = 20$  minutes, the bacteria contain chromosomes with multiple replication points. Every 20 minutes, new replication forks are established.

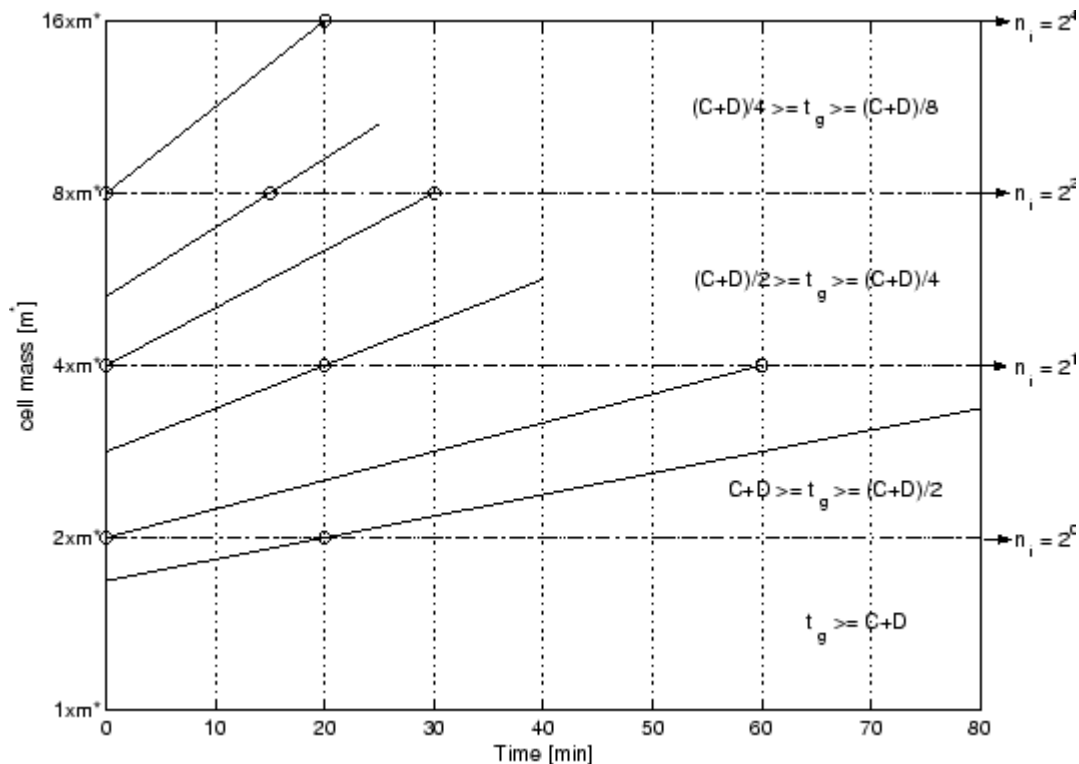
The relative rate of DNA synthesis is shown in the same figure, underneath the respective chromosome configurations. In cells growing slower than 40 minutes per doubling ( $t_g > C$ ), there is a period devoid of DNA synthesis during the division cycle, because a round of chromosome replication ( $C$ ) is completed in less time than it takes to complete a division cycle ( $t_g$ ). In faster growing cells, ( $t_g < C$ ), rounds of DNA replication overlap, i.e., a new round of replication begins before the previous one has finished. Periods with multiple replication forks are indicated with high relative DNA synthesis rates. It can clearly be observed that this rate is higher when generation times are lower.

Over a range of growth rates from 20 to 60 minutes generation time, shapes of experimental DNA production rate curves (Helmstetter and Cooper, 1968; Helmstetter, 1967) are in good agreement with the theoretical curves obtained from the model, except for dispersion in the results (the experimental curves are more *rounded*) which is probably due to variability between the cells. In the same paper, Cooper and Helmstetter (1968) obtained experimental values for  $C$  and  $D$  for cells growing at different rates. Both parameters are nearly constant and  $C$  and  $D$  are about 41 and 22 minutes, respectively. From this theory, also a calculation of the average DNA content in cells growing at various rates was made:

$$\begin{aligned} \bar{G} &= \frac{t_g}{C \ln 2} \left( 2^{(C+D)/t_g} - 2^{D/t_g} \right) \\ &= \frac{1}{\mu C} \left( \exp[ \mu(C + D) ] - \exp[ \mu D ] \right) \end{aligned} \quad (2)$$

with  $\bar{G}$  the average number of genome equivalents of DNA per cell. A *genome equivalent of DNA* is defined by Cooper and Helmstetter (1968) as the mass of DNA corresponding to a single, non-replicating chromosome. They experimentally determined the average DNA content per cell in cultures of *E. coli* B/r growing exponentially in glucose medium. From comparison to the number of genome equivalents calculated from the model theory (Equation (2)), a DNA content per genome equivalent of  $4.22 \times 10^{-15}$  g is obtained. This is in good agreement with a value calculated from a genome length of 1100 to 1400  $\mu\text{m}$  for *E. coli*. The theoretical calculations of the average DNA content per cell for different growth rates were then compared to the data of Schaechter et al. (1958) who measured DNA content in cells of *Salmonella typhimurium* as function of growth rate. These are also in good agreement.

Cooper and Helmstetter (1968) assumed that there was a constant interval ( $C + D$ ) of 60 minutes (for a temperature of  $37^\circ\text{C}$ ) between the initiation of a round of DNA replication and a subsequent division of the cell. However, no explanation was suggested as to why replication of DNA was initiated at a particular time. Donachie (1968) combined the observations of Cooper and Helmstetter (1968) with those of Schaechter et al. (1958) on the average size of cells growing at different rates (1). The result is illustrated graphically in Figure 3.



**Figure 3:** Graphic representation of the course of increase in mass of individual cells with different generation times (after (Donachie, 1968)). The initial mass at time zero is taken to be proportional to the average mass of a population of cells growing at the same growth rate (Equation (1) with  $\mu = \ln 2/t_g$ ). Cell mass is assumed to grow exponentially. With a constant time of 60 minutes ( $C + D = 20 + 40$  Cooper and Helmstetter (1968)) between initiation of DNA replication and cell division, the time of initiation of DNA replication relative to cell division can be derived for each growth curve. These times are marked as open circles on the corresponding curves of mass increase. It can thereby be seen that the masses at which initiation takes place are the

same or multiples of the same cell mass ( $m^*$ ) for cells growing at all growth rates.

First of all, the exponential relationship between cell mass and growth rate (Equation (1)) is represented on the ordinate. Then, the growth rate of the cells is represented by the slope of the lines. As an example, a cell with 60 minute generation time doubles its mass in 60 minutes. The time of initiation of DNA replication was then calculated using the rule of Cooper and Helmstetter (1968), that a constant interval of  $C + D = 60$  minutes elapses between initiation and cell division. This results in a certain mass of the cells at the time of initiation of DNA replication, indicated by the open circles. It is appears that this mass  $m_i$  is a constant multiple of a fixed mass  $m^*$ .

The rules of Cooper and Helmstetter (1968) also allow one to predict the number of chromosome copies per cell at the time of initiation of DNA replication. Because of the constant time ( $C = 40$  minutes) required to complete the replication of a single chromosome, the number of chromosome origins at the time of initiation varies discontinuously with the rate of growth. The number of chromosome origins  $n_i$  at initiation of replication, as read from Figure 2, is

$$\begin{aligned}
 n_i &= 1 \quad \text{for} \quad t_g \geq C + D \\
 n_i &= 2 \quad \text{for} \quad C + D \geq t_g \geq \frac{C + D}{2} \\
 n_i &= 4 \quad \text{for} \quad \frac{C + D}{2} \geq t_g \geq \frac{C + D}{4} \\
 &\dots
 \end{aligned} \tag{3}$$

This is also indicated in Figure 3, at the right axis. This is also indicated in Figure 3, at the right axis. Thus, the mass at initiation of DNA replication  $m_i$  and the number of chromosome origins at which replication is initiated  $n_i$  both change discontinuously in such a way that the ratio  $m_i/n_i$  is constant for cells of any size growing at any rate. Note that

$$\begin{aligned}
 \frac{m_i}{n_i} &= 2m^* \\
 &\Downarrow \\
 m_i &= 2m^* \cdot n_i \\
 &= 2m^* \cdot 2^j \quad \text{with } j = 0, 1, 2, \dots
 \end{aligned} \tag{4}$$

From this theory, for which, after initiation of chromosome replication the cells continue to grow exponentially for  $C + D$  minutes at their specific growth rate  $\mu$ , Donachie (1968) derives an exponential relationship between the mass at division and the growth rate  $\mu$ :

$$\begin{aligned}
m_d &= m_i/n_i \exp[ \mu(C + D) ] \\
&= 2m^* \exp[ \mu(C + D) ]
\end{aligned}
\tag{5}$$

In the above equations, the value  $2m^*$  corresponds to the *minimum cell mass at division*, for cells with a growth rate approaching zero. Therefore,  $m^*$  corresponds to the *minimum mass at birth*. To make the connection between these individual cell parameters and population level parameters, like the average cell mass  $\bar{m}$ , Kreft et al. (1998) obtained the ratio of the median cell *volume* to the birth *volume* to be  $b = 1.433$ . Considering the dry mass density  $d$  of the cells to be constant (Cooper, 1991), this factor can also be used to link the median cell mass  $\bar{m}$  to the cell mass at birth  $m_d/2$ :

$$b = \frac{\text{median cell volume}}{\text{birth volume}} = \frac{\text{median cell mass}}{\text{mass at birth}} = 1.433 \tag{6}$$

Therefore,

$$\begin{aligned}
\bar{m} &= b m^* \exp[ \mu(C + D) ] \\
\bar{V} &= \frac{b m^*}{d} \exp[ \mu(C + D) ]
\end{aligned}
\tag{7}$$

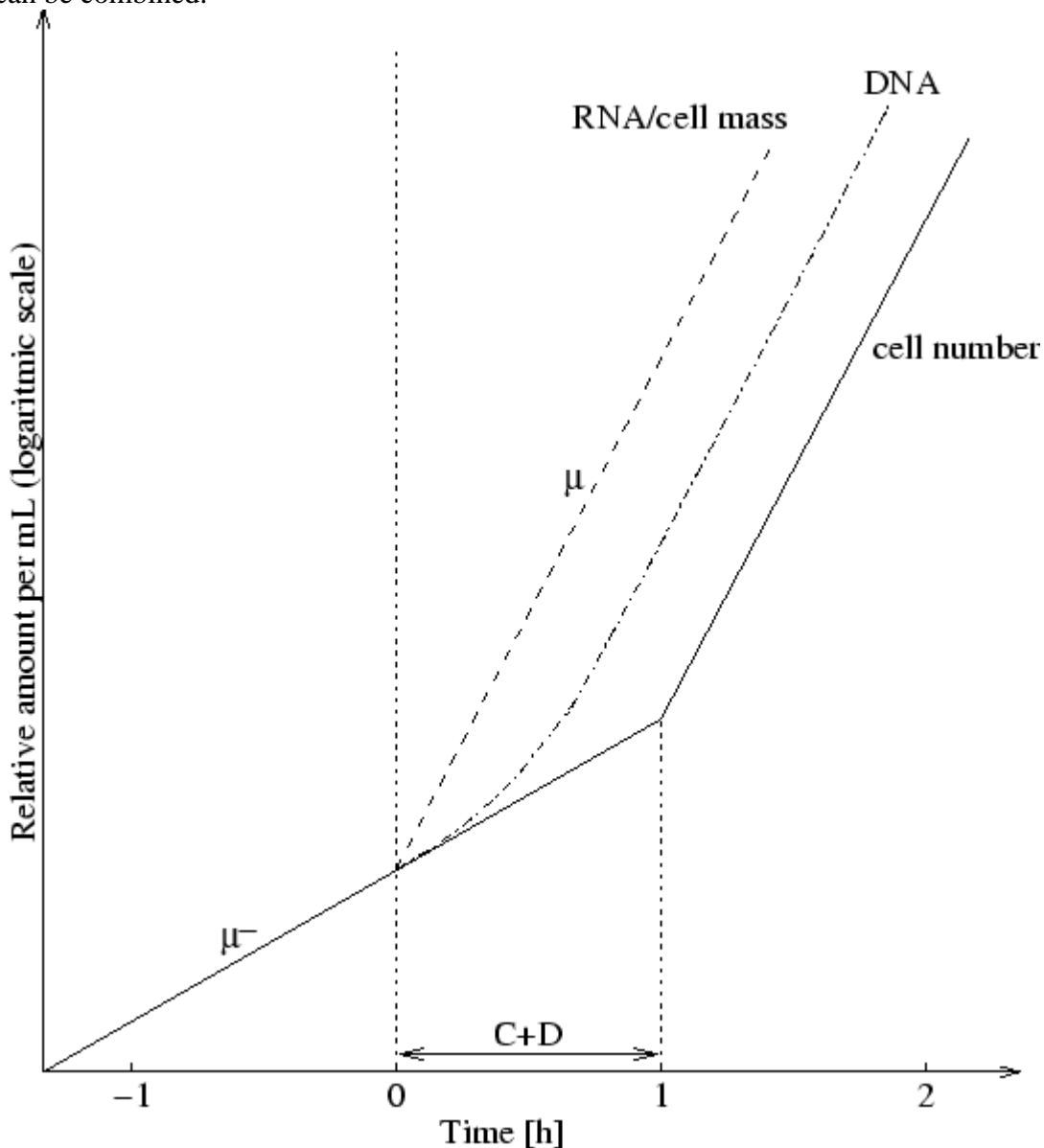
with  $\bar{V}$  the median cell volume. This is a restatement of Equation (1) which was derived from the observations of Schaechter et al. (1958).

The observation that a constant mass/chromosome origin is required for initiation of DNA replication may also suggest possible mechanisms whereby the timing of DNA initiation is controlled. For example, the synthesis of a certain amount of some cellular initiator substance is required per chromosome origin and this material is synthesized at a rate proportional to the overall increase in mass. This way of implementing the theory into a model has been followed by Cooper (1969) and Margolis and Cooper (1971). Pritchard et al. (1969) suggested an alternative mechanism, in which an inhibitor of DNA initiation is diluted by cell growth until it drops below a critical threshold. Actually, these two mechanisms are equivalent, since both initiator production and inhibitor dilution are considered to be proportional to cell mass growth.

## 2 Critical evaluation of cell behaviour in dynamic environmental conditions

In the previous section, the theory of cell division was explored. It appears that initiation of DNA replication is regulated by the increase in cell mass (Equation (4)) and that a constant time is needed for DNA replication and cell division ( $C$  and  $D$

period respectively). As a consequence, cell mass at division (and also average cell mass) is an exponential function of growth rate and ( $C + D$ ) period (Equations (5) and (7)). In this section, the consequences of this theory on the behaviour of cell populations in dynamic environmental conditions are investigated. A distinction is made between *medium* and *temperature* changes. Note that the medium is primarily defined by the substrate concentration available in the growth medium. In the first paragraph on medium shifts, the existing theory on *rate maintenance* of cell number is considered. Based on published experimental evidence and the theory of Donachie (1968) and Cooper and Helmstetter (1968) on DNA replication and cell division, we extend this theory to the behaviour of cell populations at a temperature shift. These two aspects are then combined into a general theory on population behaviour in dynamic environmental conditions, in which both medium and temperature changes can be combined.



**Figure 4:** Schematic representation of the evolution of RNA, DNA, cell mass and cell number per mL after a shift-up in medium conditions (after Cooper (1991)). There is an essentially immediate change in the rate increase in cell mass and RNA and a slower change in the rate of increase of DNA. Most specifically, the rate of cell division continues at the pre-shift rate for approximately 60 minutes and only then

abruptly shifts to the new growth rate. This phenomenon is called *rate maintenance*.

## 2.1 Medium shifts.

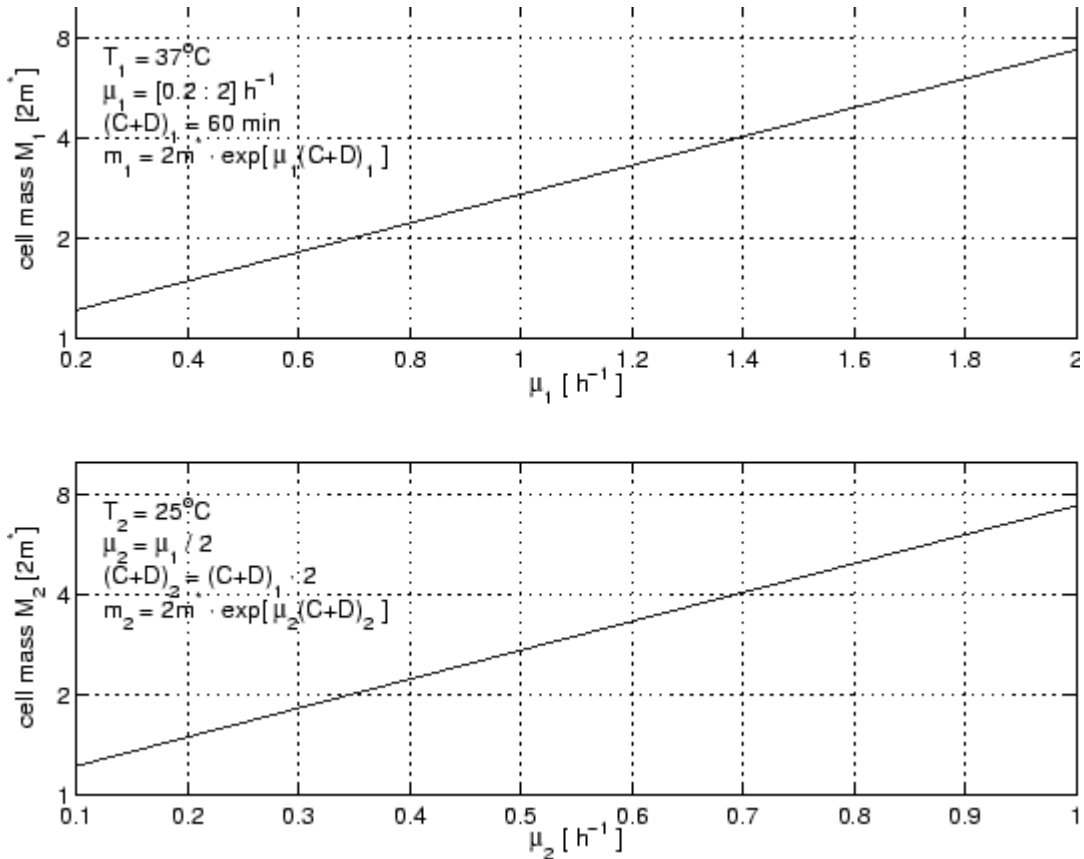
Kjeldgaard et al. (1958) investigated nutritional shift-up (from a poorer to a richer medium) for cultures of *Salmonella typhimurium*. Shifts from a low to a high growth rate result in a strict succession of events. There is an essentially immediate change in the rate increase in cell mass and RNA and a slower change in the rate of increase of DNA. Most specifically, the rate of cell division continues at the pre-shift rate for approximately 60 minutes and only then abruptly shifts to the new growth rate. This phenomenon is called *rate maintenance* and is graphically illustrated in Figure 4. These shift-up results are understandable when considering the theory of Donachie (1968) on cell division (Equations (4) and (5)). Upon shift-up, first of all, the specific rate of biomass growth increases from  $\mu^-$  to  $\mu$  (see Figure 4). Therefore, the critical mass  $m_i$  for initiation of DNA replication will be attained in a faster way and from then on, new acts of initiation will occur at a higher rate (each  $t_g = \ln 2/\mu$  minutes). However, since a constant interval of  $C + D = 60$  minutes separates initiation and the subsequent cell division, an increased rate of cell division does not appear until  $C + D$  minutes after the increase in rate of replication initiation. In other words, the new replication points which are initiated *prematurely* after a shift (i.e., replication points which appear earlier than those in an unshifted culture) will not be related to a division until at least  $C + D$  minutes after the shift. This explains the *rate maintenance* in the change in cell number for  $C + D$  minutes, like was experimentally observed by Kjeldgaard et al. (1958).

## 2.2 Temperature shifts.

In the case of a shift from poorer to richer medium conditions, *rate maintenance* was observed for the cell number, which is a repercussion of the fact that  $C$  and  $D$  periods are constant for different medium conditions *at a given temperature*. This results in an exponential relation between cell mass, RNA and DNA content per cell and the growth rates determined by the different media at a given temperature. This is indicated by experimental evidence (Schaechter et al., 1958). On the contrary, the size and chemical composition of the cells characteristic of a given medium were found *not* to be influenced by *temperature* of cultivation (Kjeldgaard et al., 1958). Also, the pattern of DNA replication in a given medium is the same when cells are grown at different absolute growth rates by varying the temperature (Pierucci, 1972; Lark and Maaløe, 1956). When Equation (5) is considered to hold true in changing temperature conditions -which is plausible, since this equation is derived from the theory of Cooper and Helmstetter (1968) which describes the general way of initiation of DNA replication and cell division- the fact that cell mass does *not* change with changing temperature conditions implies that the product  $\mu \cdot (C + D)$  must be constant, independent of temperature. This conclusion is supported in a number of literature sources:

- First of all, the statement of Cooper (1991) that *with changing temperature every aspect of the cell increases in the same proportion*, points in the direction of a shorter time for DNA replication ( $C$ ) as well as cell division ( $D$ ) with higher temperatures (higher  $\mu$ ).
- Schaechter et al. (1958) carried out steady state experiments at both 37°C and 25°C. In all media, the growth rate at 25°C was about half that at 37°C.

Nevertheless, mass and DNA remained nearly constant for a given medium at different temperatures. They suggest that at various fixed temperatures, exponential relationships between mass (or DNA) and growth rate in different media are identical, except for a change in scale. This is illustrated in Figure 5 for 25°C and 37°C. The curves for 37 and 25°C only differ in the scale of the abscissa (i.e.,  $\mu_2 = \mu_1/2$ ). The implication is that  $(C + D)_2$  is twice  $(C + D)_1$ , i.e., the product of  $C+D$  and  $\mu$  must be constant, independent of temperature. For these conditions,  $m_1 = 2m^* \cdot \exp[\mu_1 \cdot (C + D)_1] = m_2 = 2m^* \cdot \exp[\mu_2 \cdot (C + D)_2]$ . Moreover, Kjeldgaard et al. (1958) observed the rate maintenance period ( $\sim(C + D)_1$ ) at 37°C to be about half of this period observed at 25°C ( $\sim(C + D)_2$ ).



**Figure 5:** Exponential relationship between cell biomass and growth rate for two different temperatures (37 and 25°C). As postulated by Schaechter et al. (1958), the curves are identical, except for a change in scale. This is obtained by keeping the product  $\mu \cdot (C + D)$  constant, i.e., for  $\mu_2 = \mu_1 / 2$ ,  $(C + D)_2$  is taken to be  $(C + D)_1 \cdot 2$ .

- Chohji et al. (1976) investigated the kinetic behaviour of the macromolecule synthesis (cell mass, DNA content, RNA content and protein content per cell) of *Escherichia coli* cells during balanced growth in various media at different temperatures. Like Schaechter et al. (1958) (Equation (1)), they observed exponential relationships between macromolecule contents per cell and the specific growth rate at a given temperature. More specifically, for cell mass, the following relationship was proposed

$$\bar{m} = c \exp[\mu \cdot \phi(T)] \quad (8)$$

with  $c$  and  $\phi$  two positive parameters and parameter  $\phi$  dependent of temperature. This equation is completely analogous to Equation (7). It was observed that the content per cell at zero growth rate ( $c$  in the case of cell mass) was constant in each macromolecule component, irrespective of growth temperature. With Equation (7) in mind, this means that the critical mass  $m_i/n_i = 2m^*$  per chromosome origin required to initiate DNA replication is independent of temperature. This is confirmed by Pierucci (1972). Another conclusion which can be drawn from the analogy between the two equations is that  $\phi$  represents the sum of the replication time  $C$  and cell division time  $D$ . Chohji et al. (1976) give a representation of  $\phi$  as a function of temperature, from which we can read the values for  $(C + D)$  in function of temperature. Also, the values of  $(C + D)$  as function of temperature correspond well to the values obtained by Kjeldgaard et al. (1958). The dependence of  $1/\phi$  on temperature is found to be completely analogous to the Arrhenius dependency of  $\mu$  on temperature which leads to the fact that the product

$$\begin{aligned} \mu \cdot \phi &= K \\ \mu \cdot (C + D) &= K \end{aligned} \tag{9}$$

is independent of the growth temperature. As temperature rises ( $\mu \uparrow$ ),  $C + D$  decreases in a proportional way. This is again confirmed by Pierucci (1972). He observed that the temperature coefficients for  $(C + D)$  and the generation time are the same.

- Pierucci (1972) also found the ratio's  $a = C/(C + D)$  and  $1 - a = D/(C + D)$  to be independent of both media and temperature, with  $a \sim 2/3$ . The length of the  $C$ -period was thus always twice the length of the  $D$ -period. Implementing these facts into Equation (2) for the average DNA content per cell, derived by Cooper and Helmstetter (1968), it is rewritten as follows:

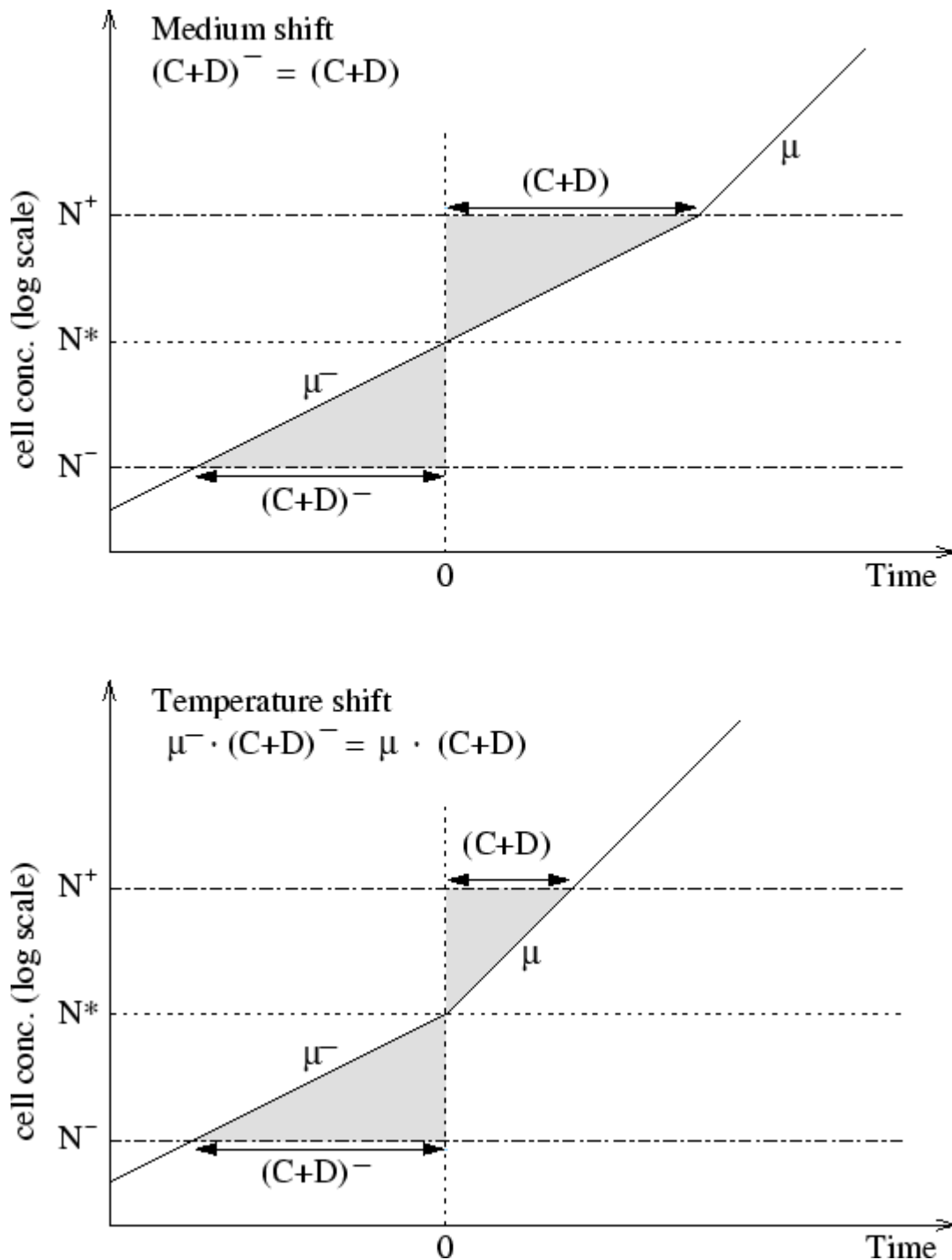
$$\begin{aligned} \mu \cdot \phi &= K \\ \mu \cdot (C + D) &= K \end{aligned} \tag{10}$$

This result demonstrates once again that if the average DNA content in the cells at different temperatures is supposed to stay constant, the product of  $\mu$  and  $C + D$  must be constant.

We may therefore decide that, while for a change in medium conditions,  $(C + D)$  stayed constant while  $\mu$  increased, for a change in temperature conditions,  $(C + D)$  decreases proportional to the increase in specific growth rate, such that the product  $\mu \cdot (C + D)$  stays constant. This is thus a repercussion of the fact that *the rate of DNA replication and cell division process  $(C + D)$  is only influenced by temperature, and not by medium conditions.*

A consequence of the fact that the product  $\mu \cdot (C + D)$  is constant for a given medium, independent of temperature, is that the size and chemical composition of the cells are not influenced by temperature of cultivation, and that no lag or rate maintenance in

cell number is expected when the temperature is shifted. This is illustrated in Figure 6, in which the difference between a medium shift and a temperature shift is summarized.



**Figure 6:** In this figure, the difference between a medium shift and temperature shift is summarized. For a medium shift, the sum  $(C + D)$  stays constant and thus there is a rate maintenance period of  $(C + D)$  minutes, or until the level  $N^+$ , for which  $(N^+ - N = N^+ - N^*)$ , is reached. At a temperature shift, the product of  $\mu \cdot (C + D)$  is constant and no lag in the rate of change in cell number is observed.

*The sum  $C + D$  is only influenced by temperature, not by medium conditions.*  
 Therefore,  $C + D$  stays constant at a medium shift when no temperature change takes

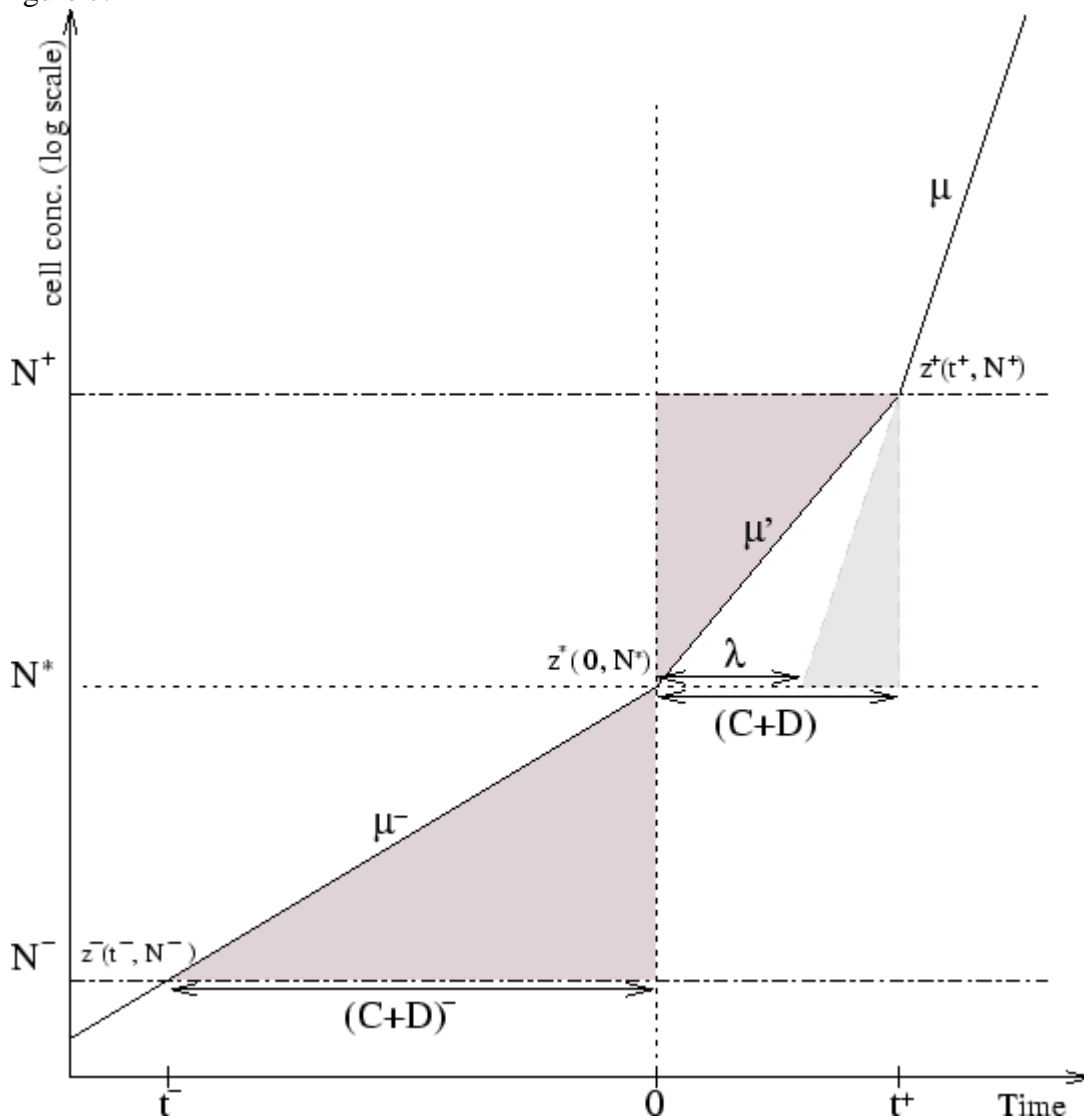
place. This results in a rate maintenance period of  $C + D$  minutes. *At a temperature shift, the sum  $C + D$  decreases, proportional to the increase in specific growth rate  $\mu$ . Therefore, the product  $\mu \cdot (C + D)$  stays constant and no lag in the rate of change in cell number is observed.* In conclusion:

**Medium shift:** the sum  $C + D$  is a characteristic for a given temperature, independent of the medium.

**Temperature shift:** the product  $\mu \cdot (C + D)$  is a characteristic for a given medium, independent of temperature.

### 2.3 Combination of medium and temperature shift.

The difference between the effect of medium and temperature changes on the behavior of a cell population was illustrated in Figure 6. Correspondingly, the more general case of a combination of medium and temperature change is illustrated in Figure 7.



**Figure 7:** The evolution of the cell concentration with time after a combination of changes in medium and temperature conditions is represented by the solid line. The shift occurs at time 0. The number of cells ( $N^* - N^- = N^+ - N^*$ ) which had already initiated DNA replication before the shift but did not yet reach cell division at the

moment of the shift is entirely defined by the product  $\mu^- \cdot (C + D)^-$ . The rate at which these cells will divide after the shift is defined by  $(C + D)^+$ . The lag time  $\lambda$ , defined in predictive microbiology as the time obtained by extrapolating the tangent at the exponential part of the growth curve back to the initial level  $N^*$ , depends also on the growth rate  $\mu$ , dictated by the post-shift conditions.

Because of the temperature increase,  $(C + D)^+$  is smaller than  $(C + D)^-$ . However, since also a change in medium conditions occurs, the growth rate increases relatively more than  $(C + D)$  decreases. Consequently, the product  $\mu \cdot (C + D)$  increases, which induces a lag phase. The lag behaviour of a bacterial population is entirely defined when the point  $z^+(t^+, N^+)$  is known at which the cells start to grow at the rate  $\mu$  (see Figure 7). Relative to the moment of the shift ( $z^*(0, N^*)$ ),  $z^+$  is characterized by two quantities:

- first of all the number of cells ( $N^* - N = N^+ - N^*$ ) which had already initiated DNA replication before the shift but did not yet reach cell division at the moment of the shift. This quantity is perfectly defined by the product  $\mu^- \cdot (C + D)^-$  which is independent of temperature and thus only influenced by the **pre-shift medium**.
- second, the time it takes for these cells to divide, defined by  $(C + D)^+$ . This depends solely on the **temperature in the post-shift environment**.

It is remarkable that the magnitude of the jump from pre-shift to post-shift medium has no influence on the rate maintenance period. This is because, the larger the jump, the faster the mass and DNA of the cells will accumulate. This conclusion is only true for the assumption that biomass growth responds instantly to a shift-up, i.e., that there is no lag in biomass growth. While in the previous paragraphs, this assumption has always been made, we know that it is not generally valid. It depends on whether the cells go *out of balance* because of the environmental change. This will depend on the magnitude of the change between the two environmental conditions, but also on the rate of change between the two conditions, and even on the value of the previous and present condition itself. A lag in biomass growth can for example be caused by a delayed biosynthesis of some essential growth factor (e.g., enzymes). In that case, this lag may also depend on the **magnitude of the jump**, or on the **pre-shift temperature** for example.

## 3 Connection with existing theories on lag in predictive microbiology

### 3.1 The lag parameter $\lambda$ .

In the domain of predictive microbiology, the duration of the lag phase is determined by the lag parameter  $\lambda$ , which is defined as the time obtained by extrapolating the tangent at the exponential part of the growth curve, back to the initial level  $N^*$  at the moment of the environment shift. From Figure 7, the lag time  $\lambda$  can be derived geometrically in function of  $\mu$  and  $(C + D)$  at the pre- and post-shift conditions:

$$\begin{aligned}\mu^- \cdot (C + D)^- &= \mu \cdot ((C + D) - \lambda) \\ \text{or} \quad \mu \cdot \lambda &= \mu \cdot (C + D) - \mu^- \cdot (C + D)^-\end{aligned}\tag{11}$$

While in the previous section, we learned from the same figure that only the pre-shift medium (i.e., the product  $\mu^- \cdot (C + D)^-$ ) and the post-shift temperature (i.e., the sum  $(C + D)$ ) have an influence on the lag behaviour of a population, this analysis reveals that the lag parameter  $\lambda$  is also influenced by the growth rate in the new environment  $\mu$ . It is very important to remark that this effect is solely due to the definition of  $\lambda$ , but does not add extra information on the lag behaviour of the population.

### 3.2 The physiological state of the cells.

While the term *physiological state of the cells* is frequently used to denote the effect of a population's history on its lag behaviour, this property has not yet been clearly defined.

Baranyi and Roberts (1994) assume that the lag phase is caused by the delayed synthesis of a critical substance  $E(t)$ , which is vital to ensure growth. Growth would then depend on this substance following the Micaelis-Menten kinetics:

$$\frac{\mu'(t)}{\mu} = \frac{E(t)}{K_e + E(t)} = \frac{E(t)/K_e}{1 + E(t)/K_e} = \frac{Q(t)}{1 + Q(t)}\tag{12}$$

in which  $\mu'(t)$  represents the *actual* specific growth rate, while  $\mu$  represents the *potential of maximum* specific growth rate, proper to the current medium and temperature conditions. In this theory, the physiological state of the cells  $Q(t)$  is defined as the fraction  $E(t)/K_e$ , with  $K_e$  the half-saturation constant. It is further assumed that the critical substance  $E(t)$  is present in a negligible small amount before the change in environment takes place, and that it grows exponentially in the new environment at the rate  $\mu$ . There are however a number of drawbacks related to this definition for the physiological state of the cells. First of all, since it is not indicated what the critical substance  $E(t)$  could be, this property cannot be measured directly. It can only *a posteriori* be estimated from a growth curve, by making use of the following relationship

$$\lambda \cdot \mu = \ln(1 + 1/Q(0))\tag{13}$$

with  $Q(0)$  the initial physiological state of the cells. Also in their model,  $Q(t)$  is assumed to grow exponentially and unbounded. This means that the physiological state of the cells would become infinite, which is physically not acceptable.

As was indicated by Schaechter et al. (1958), a large number of physiological states exist, each of which is characterized by a particular *size* and *chemical composition* of the cells. Since all the properties of the cells (cell mass, RNA, DNA, proteins, ...) have an exponential relationship with the growth rate  $\mu$  (Chohji et al., 1976):

$$\begin{aligned}
\bar{m} &= c_m \exp[ \mu \cdot \phi_m(T) ] \\
\overline{\text{DNA}} &= c_d \exp[ \mu \cdot \phi_d(T) ] \\
\overline{\text{RNA}} &= c_r \exp[ \mu \cdot \phi_r(T) ] \\
\overline{\text{protein}} &= c_p \exp[ \mu \cdot \phi_p(T) ]
\end{aligned}
\tag{14}$$

and the dependency of the parameters  $\phi$  on temperature is identical for each property, the proportion between these properties is constant for any medium or temperature condition. Consequently, the so-called *physiological state of a cell* can be defined by any of those properties. In our discussion, we prefer the average cell mass as the representation of the physiological state of the cells. According to Equation (7), which describes the dependence of the average cell mass on the growth rate  $\mu$  and the time of DNA replication and cell division ( $C + D$ ), the physiological state can be defined as

$$\bar{m} = \bar{m}_0 \exp[ \mu \cdot (C + D) ]
\tag{15}$$

with  $\bar{m}_0 = bm^*$ .

### 3.3 The general working hypothesis.

An important general working hypothesis on lag in predictive microbiology states that lag is determined by two hypothetical quantities, namely, (i) the amount of work that a cell has to perform to adapt to new conditions and (ii) the rate at which it can perform that work, and this in the following way (e.g., Robinson et al. (1998)):

$$\langle \text{lag} \rangle \cdot \langle \text{rate} \rangle = \langle \text{work} \rangle
\tag{16}$$

In this hypothesis,  $\langle \text{lag} \rangle$  is identified with the lag parameter  $\lambda$ , The  $\langle \text{rate} \rangle$  is mostly identified with the specific growth rate  $\mu$  of the organisms dictated by the present environmental conditions. The  $\langle \text{work} \rangle$  to be done by the cells upon change from one environmental condition to another would therefore be given by the product  $\lambda \cdot \mu$  and is generally accepted to be constant for different growth curves, provided that the physiological state of the cells at inoculation is identical. This is only the case if the subculturing procedure is carefully standardized.

Corresponding to Equation (15), for a shift-up from the previous environmental conditions (with  $\mu^-$  and  $(C + D)^-$ ) to the current environmental conditions (with  $\mu$  and  $(C + D)$ ), the physiological state of the cells changes from

$$\begin{aligned}
\bar{m}_- &= \bar{m}_0 \exp[ \mu^- \cdot (C + D)^- ] \\
\text{to } \bar{m}_+ &= \bar{m}_0 \exp[ \mu \cdot (C + D) ]
\end{aligned}
\tag{17}$$

Therefore, starting from equation (11), the product  $\lambda \cdot \mu$  can also be defined as the difference in logarithm of the physiological state from the previous to the current environment:

$$\langle \text{work} \rangle = \lambda \cdot \mu = \mu \cdot (C + D) - \mu^- \cdot (C + D)^- = \Delta \ln \frac{\bar{m}}{\bar{m}_0} = \ln \frac{\bar{m}_+}{\bar{m}_-} \quad (18)$$

This equation indicates that the  $\langle \text{work} \rangle$  to be performed, as defined by Equation (16), is actually related to the *change* in physiological state, and not only depends on the pre-inoculation conditions.

### 3.4 The model of Hills and Wright (1994).

Hills and Wright (1994) tried to incorporate the theory of cell division, as described in Section 1 into a population based model to describe lag behaviour. Therefore, they distinguish between the total cell biomass of a population  $M$  and cell number  $N$ . These two quantities are related by the total biomass per cell  $\bar{m}$  which is the sum of the minimum biomass per cell  $\bar{m}_{\min}$  and the excess biomass per cell  $\bar{m}_{exc}$ . Defining the minimum biomass per cell as the *unit biomass* results in:

$$\frac{M}{N} = \bar{m} = (1 + \bar{m}_{exc}) \quad (19)$$

Since total biomass grows exponentially in balanced growth and shows negligible lag in step-up experiments, it is postulated that, at any time,

$$\frac{dM}{dt} = \mu M \quad (20)$$

In contrast, the increase in cell number can show lag behaviour and this lag behaviour appears to be synchronized with the excess biomass per cell  $\bar{m}_{exc}$ , therefore,

$$\frac{dN}{dt} = k_n \bar{m}_{exc} N \quad (21)$$

The model is composed of Equations (19) to (21). The evolution of the excess biomass with time can be deduced from these equations to be

$$\frac{d \bar{m}_{exc}}{dt} = (\mu - k_n \bar{m}_{exc})(1 + \bar{m}_{exc}) \quad (22)$$

From this equation can be seen that  $\bar{m}_{exc}$  reaches a steady state at the value  $\mu/k_n$ , and thus depends on the growth rate. Replacing this value into equation (19), and comparison to Equation (15) with  $m_0$  also taken as the *unit biomass*, leads to the following relationship:

$$1 + \frac{\mu}{k_n} = \exp[ \mu \cdot (C + D) ] \quad (23)$$

When  $\mu$  is known, Equation (23) represents a relation between the parameters  $k_n$  and  $(C+D)$ . In (Hills and Wright, 1994) it was noted that  $k_n$  would be proportional to the rate constant for DNA synthesis per cell. Since no exact physical meaning could be

attributed to this parameter, it merely remained a fitting parameter. Through Relation (23), an exact relation between the parameter  $k_n$  and the physically meaningful and measurable parameters  $C$  and  $D$  is obtained.

### 3.5 The model of Augustin et al. (2000).

Augustin et al. (2000) investigated the influence of the pre-inoculation temperature and pre-inoculation duration (growth stage of the cells) on the product  $\lambda \cdot \mu$  for regrowth of *Listeria monocytogenes* at low temperature (i.e.,  $T^+ = 6^\circ\text{C}$ ). Since the growth rate is assumed not to be influenced by the pre-incubation conditions, the product  $\lambda \cdot \mu$  provides direct information on the lag parameter itself. From observations, they conclude that a similar evolution of  $\lambda \cdot \mu$  with duration of pre-incubation was observed for different temperatures. From a relatively high value (long lag time for cells that are inoculated from the lag phase), the product  $\lambda \cdot \mu$  decreases during the lag phase of growth of the inoculum to a value of approximately 0 (no lag phase for cells inoculated from the exponential growth phase). During the exponential growth phase of the inoculum, the product stays at this minimum value and gradually increases during the stationary or death phase. They correlate these observations to the theory of cell division as described in Section 1, in the sense that they also assume that lag is controlled by a factor which must attain a critical concentration before cell division occurs and this factor was assumed to be correlated to the cell biomass. Consequently, a new lag time was then defined as the time necessary to increase from the initial biomass to the critical one. During lag and stationary phase, cell biomass is smaller than during exponential growth, and therefore, lag time will be larger when inoculation is done from these growth stages. To mathematically describe the observed evolution of  $\lambda \cdot \mu$ , they proposed the following model:

- For inoculation at time  $t_j$ , when the cells are still in the lag phase ( $t_j \leq \lambda_j$ ), with pre-inoculation temperature  $T_j$ , corresponding growth rate  $\mu_j$  and lag time  $\lambda_j$ :

$$\lambda \cdot \mu = \mu_j \cdot (\lambda_j - t_j) \quad (24)$$

which expresses the fact that the cells still have to increase their biomass to the critical value, before they can start dividing.

- For inoculation from cells in the exponential growth phase ( $t_j > \lambda_j$ )

$$\lambda \cdot \mu = 0 \quad (25)$$

- When the inoculum enters the stationary phase ( $t_j \geq t_s$ ), the biomass decreases and thus the product  $\lambda \cdot \mu$  increases. A square root relationship was fitted to the data,

$$\lambda \cdot \mu = \sqrt{\nu(t_j - t_s)} \quad (26)$$

with  $t_s$  the time at which the cells go into stationary phase and  $\nu$  a function of the maximum specific growth rate ( $\nu = f(\mu_j)$ ) since the rate at which the cells will decrease their biomass depends on the rate at which the environment gets depleted and this depends on the growth rate  $\mu_j$

This theory can be compared to our theory as presented in the previous paragraphs, and more particularly to Equation (18), which also presents a clear dependence of the product  $\lambda \cdot \mu$  on the history of a population.

- When the cells are still in the lag phase, they are augmenting their cell mass exponentially according to the following equation:

$$\bar{m}_j = \bar{m}_0 \exp[ \mu_j \cdot t_j ] \quad (27)$$

With a lag phase duration  $\lambda_j$ , the remaining *work to be done* until exponential growth is reached can then be calculated from Equation (18):

$$\langle \text{work} \rangle = \lambda \cdot \mu = \Delta \ln \frac{\bar{m}_j}{\bar{m}_0} = \mu_j \cdot (\lambda_j - t_j) \quad (28)$$

which confirms the statement of Augustin et al. (2000).

- For cells taken from the exponential growth phase, and if only a temperature jump (no changes in medium conditions) occurs (i.e.,  $\mu^+ \cdot (C + D)^+ = \mu \cdot (C + D)$ ), Equation (18) also reduces to

$$\lambda \cdot \mu = 0 \quad (29)$$

- For cells from the stationary state, they applied a fitting function (Equation (26)), with no explicit mechanistic meaning. From Equation (18), and assuming that the stationary phase is caused by medium depletion, we suggest

$$\lambda \cdot \mu = \mu \cdot (C + D) - \mu^- \frac{S(t)}{K_S + S(t)} \cdot (C + D)^- \quad (30)$$

with  $S$  the substrate concentration and  $K_S$  the half saturation constant of the Monod kinetics. When only a change in temperature would occur,  $\mu^+ \cdot (C + D)$  would be equal to  $\mu^- \cdot (C + D)$ . Consequently,

$$\lambda \cdot \mu = \mu \cdot (C + D) \left( 1 - \frac{S(t)}{K_S + S(t)} \right) \quad (31)$$

In this way, also a gradually increasing  $\lambda \cdot \mu$  product is obtained, since the term  $S(t)/K_S + S(t)$  gradually decreases from one to zero, as the substrate is consumed. When the cells are still in the exponential phase, the term approximates one, and  $\lambda$  approximates zero. When the substrate is completely exhausted, the term equals zero, and  $\lambda$  equals  $(C + D)$ .

## 4 How to obtain the essential parameters to predict lag behaviour

In the previous section, we learned from Equation (18) that the lag parameter  $\lambda$  could be estimated from the change in the product  $\mu \cdot (C + D)$  or from the change in

physiological state of the cells. Therefore, if data are available on the average cell biomass, RNA, DNA, or protein content in the population at the previous and current environment,  $\lambda$  can be calculated. In general, however, these data are not available. In that case, the lag behaviour can also be estimated from the change in the product  $\mu \cdot (C + D)$  from the previous to the current environment. When we assume the change in medium conditions to be induced by a change in nutrient conditions (nutrient concentration  $S$ ), we can relate the dependence of the growth rate on the medium and temperature conditions in the following way:

$$\mu = b^2 (T - T_{min})^2 \cdot \frac{S}{K_S + S} \quad (32)$$

which is a combination of the square root model of Ratkowsky et al. (1982) and the monod relationship to describe the dependence of growth rate on nutrient conditions. The parameters  $b$ ,  $T_{min}$  and  $K_S$  are characteristics for a specific organism and specific medium conditions and must be estimated beforehand.

On the estimation of  $(C + D)$  for different organisms, less information is available. The authors are only aware of data for *Escherichia coli* populations. Helmstetter and Pierucci (1976) monitored the rate of [ $^{14}C$ ] thymidine incorporation in different strains of *E. coli* cells, as a measure for the relative rate of DNA synthesis during the division cycle. From the resulting radioactivity curves, the  $C$  and  $D$  periods can be estimated. They concluded that the  $C$  and  $D$  parameters are not likely to be the same in all strains of *E. coli*. Keasling et al. (1995) note that, while in some *E. coli* strains, the average rates of replication and cell division are relatively invariant with growth rate, other strains exhibit decreasing rates (increasing  $C$  and  $D$  periods) for doubling times greater than 60 min -like was also observed by Helmstetter and Pierucci (1976)-. According to Keasling et al. (1995), this could be due to a decrease in the flux of deoxyribonucleotide triphosphates to DNA polymerase with decreasing growth rate. They fitted an exponential function to the experimental data for the length of the  $C$  and  $D$  period as a function of the doubling time:

$$\begin{aligned} C &= C_{min} [1 + \alpha \cdot \exp(-\beta/t_g)] \\ D &= (D_{min} + D_{1/2}) [1 + \alpha \cdot \exp(-\beta/t_g)] \end{aligned} \quad (33)$$

The values for the parameters  $C_{min}$ ,  $\alpha$  and  $\beta$  were estimated to be 43.2 min, 4.86 and 4.50 respectively. The value for  $D_{min}$  was taken to be 17 min. with a half-life of the exponential decrease  $D_{1/2}$  of 4.5 min. Since there are very few data available on the values for  $C$  and  $D$  for different organisms than *E. coli*, and because of the rather sophisticated experimental techniques to measure these two parameters, it might be more obvious to estimate the parameters indirectly from biomass measurements in combination with Relation (15), or protein, RNA or DNA measurements in combination with Relations (14).

## 5 Conclusions

In this research, the theory of cell division as it was published by Cooper and Helmstetter (1968) and Donachie (1968) has been critically evaluated with respect to its potential to describe microbial population behaviour in dynamic environmental conditions. On the basis of this theory, a general theory on the behaviour of microbial populations exposed to a combination of medium and/or temperature shifts has been developed. The proposed theory is then the basis for a critical evaluation of the existing modelling concepts on lag in predictive microbiology.

It can be concluded that the theory of cell division on an individual based level provides a valuable framework to analyze lag behaviour. A first important conclusion is that the lag parameter  $\lambda$  depends on the growth rate in the new environment, only because of its definition.  $\lambda$  is defined as the time obtained by extrapolating the tangent at the exponential part of the growth curve ( $\sim$  the maximum specific growth rate in the new environment), back to the initial cell number level at the moment of the environmental shift. However, it is emphasized that the lag behaviour of a population can entirely be defined by two values, namely the product  $\mu \cdot (C + D)$  which is defined by the pre-shift medium, and the sum  $(C + D)$  which is defined by the post shift temperature. It must be accentuated that this conclusion is only true when biomass growth responds instantly to a shift-up, i.e., that there is no lag in biomass growth. This assumption has been made throughout this research. We are aware of the fact that this assumption is not generally acceptable. Lag in biomass can be caused, for example, by a delayed synthesis of some essential growth factor, which could be an enzyme or a group of enzymes (Hills and Mackey, 1995).

Another important conclusion is that the previously very vaguely defined *physiological state of the cells*, which is very important in the prediction of lag behaviour, can now be identified with measurable cell parameters like cell mass, DNA, RNA and protein content per cell. In our discussion, we worked with the average cell mass within a population as a measure for the physiological state of the cells. The physiological state of the cells evolves exponentially as a function of the growth rate  $\mu_{\max}$  and interval between initiation of DNA replication and cell division  $(C + D)$ . With the aid of this definition, also the *work* to be done by the cells upon change in environmental conditions could be rigorously defined as the difference in logarithm of the physiological state of the cells from the previous to the current environment. In this way, the assumption that the *work* (defined as the product  $\lambda \cdot \mu$ ) does only depend on the pre-inoculation conditions, was contradicted.

Then also the models of Hills and Wright (1994) and Augustin et al. (2000) -a *primary* and *secondary* predictive model to describe lag, respectively- are evaluated within the same framework. In the case of the model of Hills and Wright (1994), the fitting parameter  $k_n$  could be related to the physically meaningful and measurable parameters  $C$  and  $D$ . The modelling approach of Augustin et al. (2000) was validated within the cell division theory framework, and in addition, for the dependence of the product  $\lambda \cdot \mu$  on the pre-inoculation duration of stationary phase cells, a more mechanistically acceptable model structure was proposed. In this way, the cell division theory applied to microbial populations in dynamic medium and/or

temperature environments provides a useful framework to discuss lag behaviour in general.

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