

## Chapter 5

# Experimental study of the biomass distribution dynamics

### 5.1 Introduction

In previous chapters, the evolution of the biomass distribution has proved to reflect the underlying processes that affect the growth of a bacterial culture. The INDISIM simulations have shown that the study of a dynamic property of a population such as the biomass distribution provides information about the temporal evolution of the system that goes beyond the obtained information by the pure observation of the growth curve. It is a powerful tool to extract a kind of information of the bacterial system that today, given the available analytical methods, is difficult to assess.

Mathematical comparisons between distributions, such as the *product distance*, have been introduced as new approaches in the assessment of biomass evolution in microbial systems. Previous simulations have shown that models which are mainly based on biomass distributions are realistic tools for the short-term study of any microbial population.

Several of the conclusions of the precedent chapters should be amenable to experimental corroboration. Specifically, the predicted behaviour for the biomass distribution during the lag phase should be experimentally tested, since it has proved to reflect the processes that take place during the lag phase. Also, the evolution of the biomass distribution during the transition from exponential to stationary phase should be experimentally assessed, as well as its stability during the exponential and balanced growth.

IbM models and simulations are specific approaches lying between microscopic and

macroscopic models. They use the available microscopic information for building the models, and the simulation output can be compared with the macroscopic (population level) experimental data (e.g. the growth curve, the lag parameter, the doubling time and the growth rate, among others). Therefore, experimental techniques assessing non viable counts such as microscopy counts, and viable counting techniques such as plate counts or Most Probable Number, have been the major source of information for IbM.

However, none of these techniques possesses the full simulation capacity of IbM; they do not allow experimental assessment of the distribution of individual properties of a given population. Specifically, we are interested in a precise measurement of the biomass or size distribution in the population during its evolution.

Precise and satisfactory techniques to address biomass distribution are sophisticated today, with the most advanced being:

- Direct methods: light microscopy and electron microscopy combined with image analysis systems. These allow direct measurements of cell size, but they can not determine with enough precision the size of  $\sim 10^4$  cells/ml or higher concentration in a satisfactory interval of time. Moreover, the fixing techniques used modify the real size of the cells.
- Indirect methods: electric sizer, flow cytometry and laser diffraction cytometry. These methods provide indirect measurements of the cell size that must be transformed into equivalent diameters or similar units. Although these methods still have some limitations, they allow very rapid measurements of large populations.

The availability of an electric sizer and a flow cytometer at the Cytometry Unit of the Scientific-Technical Services of the *Universitat de Barcelona* (SCT-UB), as well as the collaboration with Dr. Josep Vives-Rego (*Universitat de Barcelona*, UB), have allowed us the use of these techniques for our aims.

The design and supervision of the experiments, as well as the data processing and analysis of the results, were performed by Dr. Daniel López, Dr. Josep Vives-Rego and the author of this thesis. The experimental measurements were carried out by Bàrbara Flix (*Escola Superior d'Agricultura de Barcelona*, ESAB), with the technical assistance of Jaume Comas-Riu, Ricard López, Chari González Flores (SCT-UB) and Rosa Carbó (ESAB).

To date, flow cytometry has only been used for a few applications in food predictive microbiology, i.e., to identify subpopulations or to enumerate microorganisms (Rasch, 2004). Åkerlund et al. (1995) used flow cytometry to assess the evolution of the cell size distribution of an *Escherichia coli* culture during the exponential and stationary

phases. This paper shows the stability of the cell size distribution during the steady state (exponential and balanced growth), as well as its backward shift on the subsequent transition to the stationary phase. Nevertheless, it did not tackle the initial phases of the growth.

In this chapter we use flow cytometry and electric size analysis to study the evolution of the size distribution along the growth curve: lag phase, exponential phase, and, when possible, beginning of the stationary phase. The aims of this chapter are (i) to verify the suitability of the cell size analysis techniques and the IbM models and simulations as complementary tools, (ii) to develop some mathematical tools to analyze the results, and (iii) to experimentally check some of the predicted behaviours regarding the evolution of the biomass distributions during the lag phase that were discussed in previous chapters.

It has to be noted that this thesis is not an experimental research project. Thus, the aim of this chapter is not to present a wide-ranging complete experimental work, but to open a new way and explore the possibilities of these techniques together with IbM simulations, as well as to validate the general conclusions obtained by previous IbM modelling and simulations. Moreover, the analysis of these initial results allows the design of further experimental tests, which is the fourth aim of this chapter.

Although the author of this thesis is not directly competent in the practical performance of experiments, the experimental results are completely valid and of the highest possible quality considering the quality of the equipments and installations, as well as the proven technical and scientific authority of the various experts that have participated in the design, execution, achievement and interpretation of them.

## 5.2 Experimental setup

### 5.2.1 Flow cytometry

Cytometry techniques are used to measure physical and/or chemical characteristics of single cells. In flow cytometry, the cells are suspended in a fluid microstream. Some flow cytometers incorporate a sorting system to separate the cells with specific properties from the population (Shapiro, 2003).

A light beam is perpendicularly directed onto the stream. Then, each suspended cell scatters the incident light. The flow cytometer captures the scattered light at different angles. One detector is located at small angles (between  $0.5^\circ$  to  $5^\circ$ ) to gather the forward scattered light (FS), as shown in Figure 5.1. The FS depends on the refractive index difference between the particle and the medium, and the cell size (Shapiro, 2003), but

the relationship between the FS amplitude and the cell size is not a monotonic function (Julià et al., 2000). Another detector is usually located at  $90^\circ$  to collect the side scattered light (SS), which is larger for cells with internal granular structure (Shapiro, 2003).

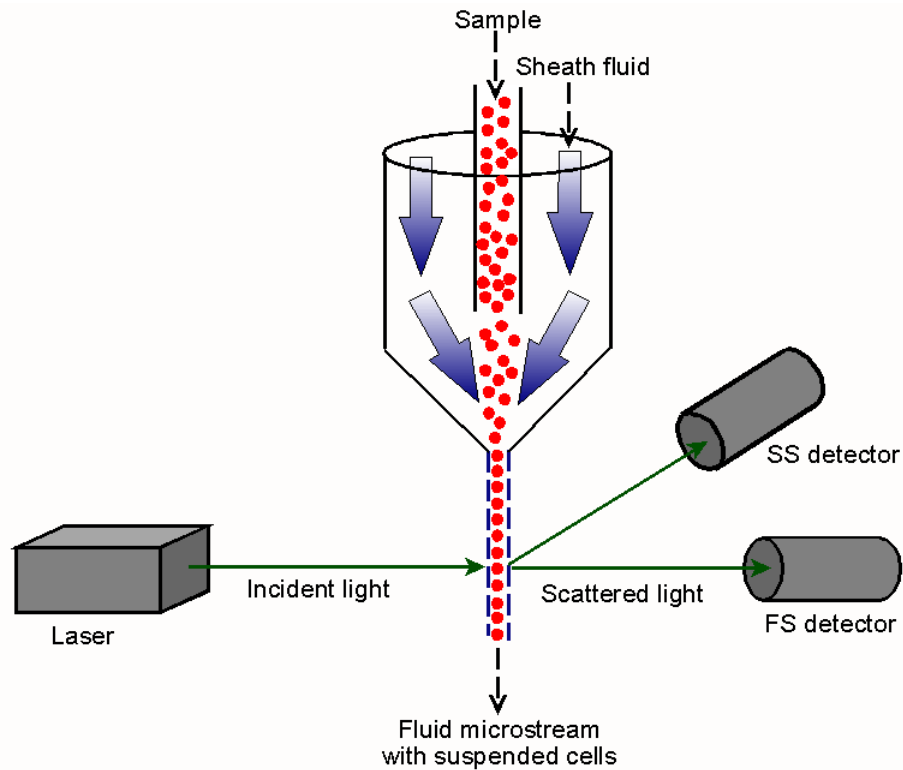


Figure 5.1: Overview of the operation of a flow cytometer. The circles symbolize the bacteria that are suspended in the fluid stream. The arrows represent the incident light beam and the scattered light that is collected at different angles by the FS and SS detectors.

In this work, flow cytometric experiments were carried out using a Cytomics FC 500 MPL flow cytometer (Beckman Coulter, Fullerton, CA, USA) (Fig. 5.2). Excitation of the sample is brought about using a standard  $488\text{ nm}$  air-cooled argon-ion laser at  $20\text{ mW}$  power. The instrument was set up with the standard configuration. The FS sensor is a photodiode that collects the forward scatter. When light reaches it, the FS sensor generates voltage pulse signals proportional to the amount of light the sensor receives. The cytometer is sufficiently sensitive to detect particles with a diameter between  $0.5\ \mu\text{m}$  and  $40\ \mu\text{m}$ . The SS detector is a photo diode sensor that collects the amount of laser light

(488 nm) scattered at an approximately  $90^\circ$  angle to the axis of the laser beam. In our experiments, optical alignment was based on an optimized signal from 10 nm fluorescent beads (Flowcheck, Coulter Corporation, Miami, Florida, USA). Time is used as a control of instrument stability.

Forward light scatter signal intensity is strongly affected by the wavelength of light used and by the precise range of angles over which light is collected, the latter being determined by focal lengths and the numerical apertures of the collecting lenses, including the size, shape and position of irises, slits and obscuration bars in the optical system. Since no two manufacturers of flow cytometers use the same optical design for forward scatter measurements, it is unlikely that exactly the same results will be obtained from measuring the same cells with different instruments.

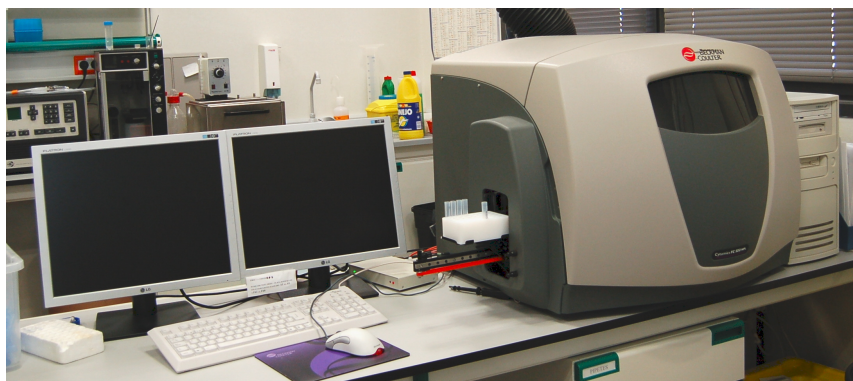


Figure 5.2: *Cytomics FC 500 MPL flow cytometer (Beckman Coulter, Fullerton, CA, USA). Scientific-Technical Services UB (SCT-UB, Serveis Científicotècnics UB).*

Data are loaded with Summit<sup>®</sup> V3.1 software (Cytomation, Inc.), and exported to be analyzed with Microsoft Excel 2002 (© Microsoft Corporation 1985-2001) and Matlab<sup>®</sup> R2006b (© The Mathworks, Inc. 1984-2006).

### 5.2.2 Particle size analysis

Particle size analysis is a technique to assess the cell size distribution of a bacterial population. It can be achieved through electrical or light scattering measurements. In this work, particle size analysis is carried out with a Multisizer II electronic particle analyzer (Coulter Corporation<sup>®</sup>) (Fig. 5.3). Particles suspended in a weak electrolyte solution are drawn through a small aperture (30  $\mu\text{m}$  in diameter), separating two electrodes between which an electric current flows. The voltage applied across the aperture creates a *sensing*

*zone*. As particles pass through the aperture (or *sensing zone*), they displace their own volume of electrolyte, momentarily increasing the impedance of the aperture (Beckam Coulter, 2007).

This change in impedance produces a pulse that is digitally processed in real time. The Coulter Principle states that the pulse is directly proportional to the volume of the particle that produced it (Beckam Coulter, 2007). Three types of size measurements are obtained after the transformation of the electric pulses generated by the counter: diameter, volume and revolution surface. In addition, a metering device is used to draw a known volume of the particle suspension through the aperture. A count of the number of pulses can then yield the concentration of particles in the sample.



Figure 5.3: *Multisizer II* electronic particle analyzer (Coulter Corporation ®). Scientific-Technical Services UB (SCT-UB, Serveis Científicotècnics UB).

This Multisizer II has the capacity to process  $100\ \mu\text{l}$  of cell suspension in 0.9% NaCl previously filtered through  $0.2\ \mu\text{m}$ . Data are firstly analyzed with Coulter Multisizer AccuComp software version 1.15 (Coulter Corporation). Files generated by the particle size analyzer (Multisizer II) are exported to ASCII (tab delimited) format and analyzed with Microsoft Excel 2002 (© Microsoft Corporation 1985-2001) and Matlab ® R2006b (© The Mathworks, Inc. 1984-2006).

### 5.2.3 Comparative performance

The flow cytometer allows rapid, numerous and precise measurements of the FS distribution among the population with small samples ( $0.5\ \text{ml}$ ). Therefore, it may produce a huge amount of detailed experimental data about the studied culture. The relationship with the biomass distribution is not monotonic, so it requires prior work in order to interpret the obtained results. The cell concentrations can also be assessed by using a larger

volume of culture sample and an internal calibrator. The optimal cell concentration for FC measurements is over  $10^5$  *cell/ml*, but we can work with lower densities by means of extending the acquisitions. The inferior limit for cell density is  $10^4$  *cell/ml*.

The multisizer allows the simultaneous assessment of the size distributions and the cell concentrations. The sizes are given in terms of equivalent spheres. That is, the output gives the diameter of a sphere that has the same volume as the bacterium. The required volume of culture sample to carry out each measurement is  $10$  *ml*, and it works with cell densities over  $10^4$  *cell/ml*. The main handicap is the truncation of the distributions under  $0.7$   $\mu\text{m}$ , so a part of the population is not represented in the results. The cell concentration counting is very efficient, since it does not require a special volume of sample or a specific measurement.

In this work, cell concentration was measured with the multisizer and the size distribution assessed with the cytometer. Later, it was seen that this is not correct because the fraction of the cells that is lost under the  $0.7$   $\mu\text{m}$  threshold is significant. The multisizer measurements are also used to verify the processing of the cytometer counts into equivalent diameters.

#### 5.2.4 Bacterial strain and growth conditions

Experiments were performed using *Escherichia coli* CECT 101 (*Colección Española de Cultivos Tipo*, Spain). Bacteria were grown in M9 medium consisting of part A) per liter,  $\text{Na}_2\text{HPO}_4$ ,  $6$  *g*,  $\text{KH}_2\text{PO}_4$ ,  $3^{\circ}$  *g*,  $\text{NaCl}$ ,  $0.5$  *g*,  $\text{NH}_4\text{Cl}$ ,  $1$  *g*; part B) a  $1$  *M* solution of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; and part C) a  $0.01$  *M* solution of  $\text{CaCl}_2$ . Parts A, B and C were autoclaved separately.  $1$  *ml* per liter of part B and  $10$  *ml* of part C were added to part A. Glucose sterilized by filtration through  $0.2$   $\mu\text{m}$  was added at  $0.5$  *g* per liter. Final pH was  $7.5$ . Cultures were incubated at  $35^{\circ}\text{C}$  with shaking at  $150$  *r.p.m.* Inocula of about  $0.5\%$  in volume were sampled from overnight cultures and added to fresh medium under the same conditions to perform the flow cytometry and multisizer assessments during the growth.

The maintenance of the cultures, as well as some preliminary experimental measurements such as optical density and CFU, were done at the Microbiology Laboratory of the *Escola Superior d'Agricultura de Barcelona* (ESAB, Universitat Politècnica de Catalunya). The flow cytometry and particle size analysis were performed at the Cytometry Unit at the Scientific-Technical Services of the *Universitat de Barcelona* (SCT-UB, Serveis Científicotècnics UB).

### 5.2.5 Details of the performed experiments

Two main series of experiments have been performed. In experiments T20, the temperature of incubation is 20°C. It is a slow growth that allows the analysis of the first stages of the growth (mainly lag phase).

In experiments T35, the temperature of incubation is 35°C. This results in a fast growth that makes it possible to analyze a wider range of the growth curve (lag, exponential and, in some repetitions, stationary phases).

Each series consists of several repetitions of an experiment under similar conditions. The characteristics of each experiment are detailed below.

#### Series T20: culturing at 20°C

##### Experiment T20-061205

- Date: 2006 December 5th.
- Inoculum: 35 ml of filtered M9 medium (0.22  $\mu\text{m}$ ) with 0.5 g/l of glucose, inoculated with a colony grown at 30 °C with agitation.
- Culture under study: 70 ml of filtered M9 (0.22  $\mu\text{m}$ ) with 0.5 g/l of glucose inoculated with 300  $\mu\text{l}$  of the inoculum (0.5%), in an erlenmeyer of 500 ml. Growth at 20 °C in a bath.
- Data acquisition with Multisizer (MSZ): samples of 100  $\mu\text{l}$  of the culture with different (controlled) dilutions, depending on the cell concentration.
- Data acquisition with Flow Cytometer (FC): samples of the culture, non-diluted or diluted in Isoton, depending on the cell concentration.
- Comments: two different populations appear in the measurements.

##### Experiment T20-070220

- Date: 2007 February 20th.
- Inoculum: 35 ml of filtered M9 medium (0.22  $\mu\text{m}$ ) with 0.5 g/l of glucose, inoculated with a colony grown at 19 – 20 °C (environmental temperature controlled with central heating) for 18 h without agitation.
- Culture under study: 120 ml of filtered M9 (0.22  $\mu\text{m}$ ) with 0.5 g/l of glucose inoculated with 600  $\mu\text{l}$  of the inoculum (0.5%), in an erlenmeyer of 500 ml. Growth at 20 °C in a bath.



- MSZ: samples of 100  $\mu\text{l}$  of the culture with different (controlled) dilutions, depending on the cell concentration.
- FC: samples of the culture, non-diluted or diluted in Isoton, depending on the cell concentration.
- Comments: two different populations appeared in the initial measures, and only one population was detected after 227 *min*.

**Experiment T20-070301**

- Date: 2007 March 1st.
- Inoculum: 35 *ml* of filtered M9 medium (0.22  $\mu\text{m}$ ) with 0.5 *g/l* of glucose, inoculated with a colony grown at 19 – 20 °C (environmental temperature controlled with central heating) for 7 *days* without agitation.
- Culture under study: 100 *ml* of filtered M9 (0.22  $\mu\text{m}$ ) with 0.5 *g/l* of glucose inoculated with 500  $\mu\text{l}$  of the inoculum (0.5 %), in an erlenmeyer of 500 *ml*. Growth at 20 °C in a bath.
- MSZ: samples of 100  $\mu\text{l}$  of the culture with different (controlled) dilutions, depending on the cell concentration.
- FC: samples of the culture, non-diluted or diluted in Isoton, depending on the cell concentration.

**Experiment T20-070315**

- Date: 2007 March 15th.
- Inoculum: 35 *ml* of filtered M9 medium (0.22  $\mu\text{m}$ ) with 0.5 *g/l* of glucose, inoculated with a colony grown at 19 – 20 °C (environmental temperature controlled with central heating) for 7 *days* without agitation.
- Culture under study: 80 *ml* of filtered M9 (0.22  $\mu\text{m}$ ) with 0.5 *g/l* of glucose inoculated with 400  $\mu\text{l}$  of the inoculum (0.5 %), in an erlenmeyer of 500 *ml*. Growth at 20 °C in a bath.
- MSZ: samples of 100  $\mu\text{l}$  of the culture with different (controlled) dilutions, depending on the cell concentration.

- FC: samples of the culture, non-diluted or diluted in Isoton, depending on the cell concentration.

### Series T35: culturing at 35°C

#### Experiment T35-061121

- Date: 2006 November 21st.
- Inoculum: 30 ml of M9 with 0.5 g/l of glucose and 1.5 ml of a previous culture grown at 30°C with agitation.
- Culture under study: 4 tubes with 3 ml of M9 with 0.5 g/l of glucose, each one inoculated with 3 µl of the inoculum. Growth at  $34 \pm 1^\circ\text{C}$  in a heater.
- Data acquisition with Multisizer (MSZ): samples of 100 µl of the culture with different (controlled) dilutions, depending on the cell concentration.
- Data acquisition with Flow Cytometer (FC): samples of the culture, non-diluted or diluted in Isoton, depending on the cell concentration.
- Comments: inoculum too large, and great background noise. The medium should be filtered.

#### Experiment T35-070123

- Date: 2007 January 23rd.
- Inoculum: 35 ml of filtered M9 medium (0.22 µm) with 0.5 g/l of glucose, inoculated with a colony grown at 30°C with agitation.
- Culture under study: 70 ml of filtered M9 (0.22 µm) with 0.5 g/l of glucose inoculated with 350 µl of the inoculum (0.5%), in an erlenmeyer of 500 ml. Growth at 35°C in a bath.
- MSZ: samples of 100 µl of the culture with different (controlled) dilutions, depending on the cell concentration.
- FC: samples of the culture, non-diluted or diluted in Isoton, depending on the cell concentration.
- Comments: The filtration of the medium reduces the background noise.

**Experiment T35-070130**

- Date: 2007 January 30th.
- Inoculum: 35 ml of filtered M9 medium (0.22  $\mu\text{m}$ ) with 0.5 g/l of glucose, inoculated with a colony grown at 30 °C with agitation.
- Culture under study: 80 ml of filtered M9 (0.22  $\mu\text{m}$ ) with 0.5 g/l of glucose inoculated with 400  $\mu\text{l}$  of the inoculum (0.5%), in an erlenmeyer of 500 ml. Growth at 35 °C in a bath.
- MSZ: does not work. Cell concentration measured with the FC.
- FC: samples of the culture, non-diluted or diluted in Isoton, depending on the cell concentration. An internal calibrator (Flow-check<sup>TM</sup> fluorospheres, Beckam Coulter, ref.: 9434559) used to measure the cell concentration.

**5.3 Results****5.3.1 Processing the experimental data**

Flow cytometry techniques give the distribution of the FS and SS among the population at the time the measurement takes place. These distributions are given in terms of channels (256), namely  $fs$  for the FS measurements and  $ss$  for the SS measurements. Each channel ( $fs$  or  $ss$ ) collects the scattered intensity within a certain interval. The intervals are set in a logarithmic scale in order to amplify the low intensity scatters and, at the same time, include a wide range of intensities. The resulting FS distribution may be represented in terms of intensity instead of channels, as shown in Figure 5.4.

Multisizer (MSZ) measurements give the size distribution among the population at a certain time. FS measurements are related with the cell size. The measures of the experiment T35-061121 are addressed to corroborate and evaluate this relationship, as well as to adjust the experimental conditions and the experimental data processing. At each time of measurement, two samples of the culture are taken and measured with the flow cytometer and the multisizer, respectively. Therefore, MSZ and FS distributions at each time can be compared.

**On the relationship between FS measures and cell size**

Although a relationship between FS and diameter exists, theory predicts, and experiments confirm, that FS amplitude is not a monotonic function of particle size, even for

uniform particles (Julià et al., 2000). However, Julià et al. (2000) proposed some second-order functions that give a relation between the FS counts and the cellular diameter  $d$  for some particular cases. Specifically, for an *Escherichia coli* culture this is (Eq. 5.1):

$$d(\mu m) = 8.54 \cdot (fs)^2 - 6.71 \cdot (fs) + 2.08 \quad (5.1)$$

where  $(fs)$  refers to the number of the channel (from 1 to 256).

The conversion from FS data to cellular diameter according to Eq. 5.1 has been verified by means of the particle analyzer for some sample cell distributions of experiment T35-061121, showing a discrepancy between multisizer and transformed FS measurements under 5% (Fig. 5.5).

The conclusion of this preliminary analysis is that the flow cytometer results can be easily transformed into equivalent sphere diameters. Therefore, from now on the result analyses will be carried out in terms of diameters by using Eq. 5.1.

### A mathematical tool to assess the evolution of the measured distributions

Among of the mathematical tools that will be used are the *distances* that were defined in Chapter 3. These distances were defined in terms of masses. In this chapter they will be used in terms of diameters. Thus, the *mean mass distance* (Eq. 3.1) is transformed into *mean diameter distance* (Eq. 5.2), and the *mass distribution distance* (Eq. 3.2) becomes *diameter distribution distance* (Eq. 5.3). Again, the *product distance* is used as the tool to follow the culture's evolution (Eq. 5.4).

$$D_{\bar{d}}(t) = \frac{|\bar{d}(t) - \bar{d}_{ref}|}{\bar{d}_{exp}} \quad (5.2)$$

$$D_{p_{k_d}}(t) = \sum_{k_d=1}^N |p_{k_d}(t) - p_{k_d,ref}| \quad (5.3)$$

$$D_d(t) = D_{\bar{d}}(t) \cdot D_{p_{k_d}}(t) = \frac{|\bar{d}(t) - \bar{d}_{ref}|}{\bar{d}_{ref}} \cdot \sum_{k_d=1}^N |p_{k_d}(t) - p_{k_d,ref}| \quad (5.4)$$

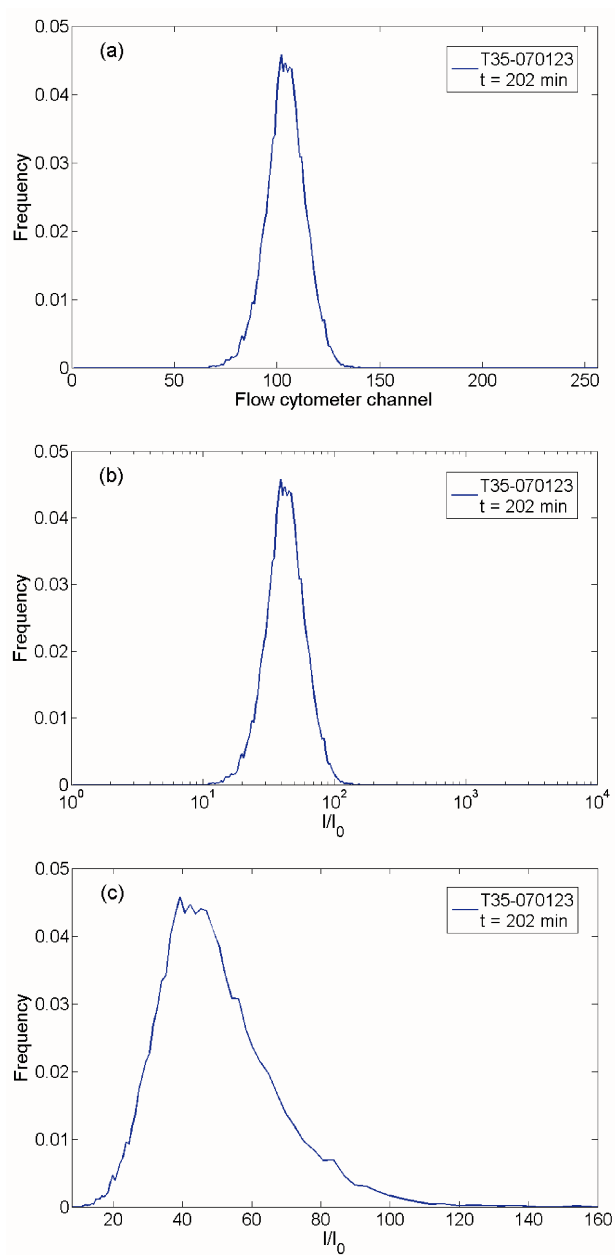


Figure 5.4: *Example of an FS measurement: T35-070123, at  $t = 202$  min (exponential phase). (a) Direct FS distribution in terms of channels, (b) FS distribution in terms of relative scattered intensity ( $I/I_0$ ) in a logarithmic scale, and (c) FS distribution in terms of relative scattered intensity ( $I/I_0$ ) in a linear scale*

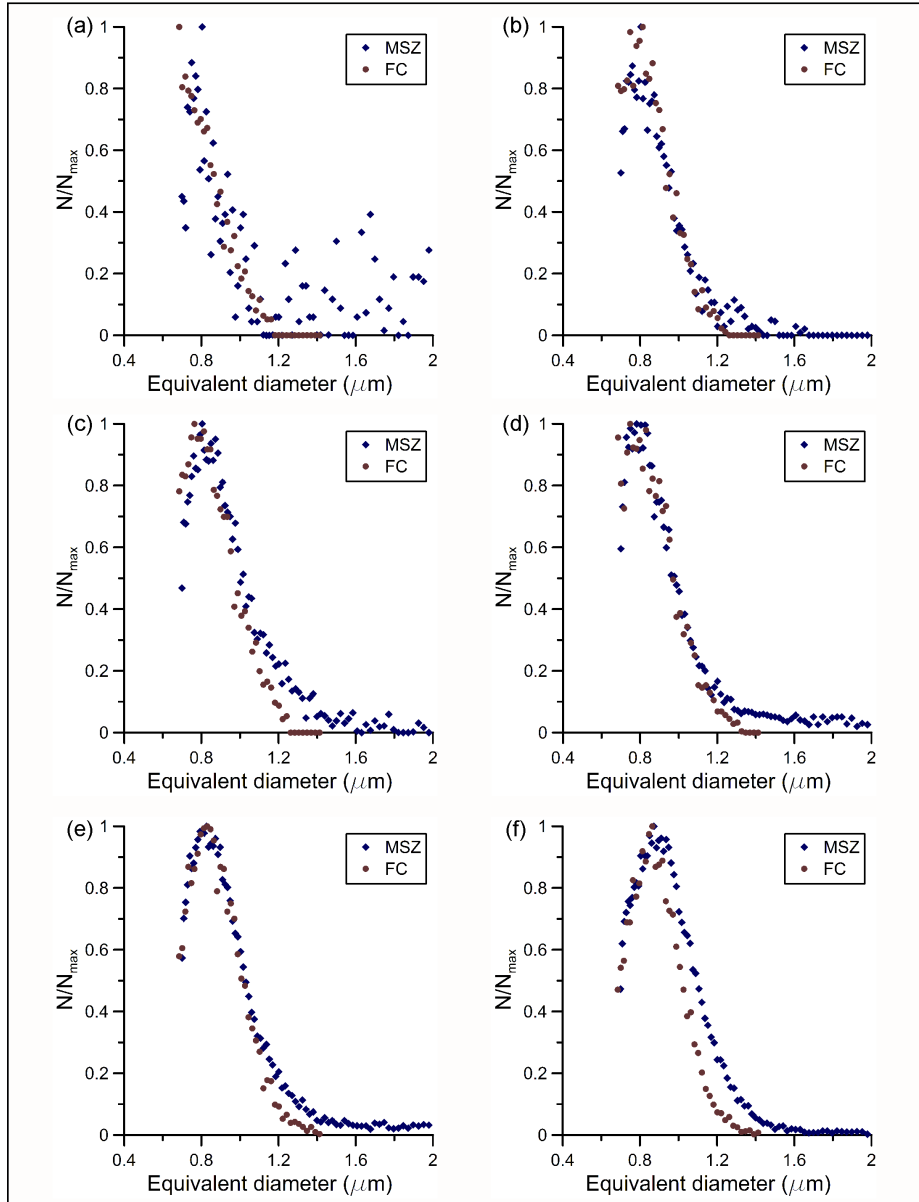


Figure 5.5: Multisizer (MSZ, blue rhombus) and transformed FS distributions (FC, red circles) for the experiment T35-061121. The expression used for transforming FS is Eq. 5.1 (Julià et al., 2000). The measurement times are (a)  $t_0 = 0 \text{ min}$ , (b)  $t_1 = 53 \text{ min}$ , (c)  $t_2 = 131 \text{ min}$ , (d)  $t_3 = 218 \text{ min}$ , (e)  $t_4 = 266 \text{ min}$ , and (f)  $t_5 = 328 \text{ min}$ .

The  $\bar{d}(t)$  is the mean diameter of the sample at the time of measurement  $t$ , and the  $p_{k_d}$  is the normalized distribution of diameters among the population of the sample. These *distances* are evaluated from a reference measured mean diameter ( $\bar{d}_{ref}$ ) and diameter distribution ( $p_{k_d,ref}$ ) that can be either the mean of the exponential phase measurements, when it is distinguishable, or one specific measurement in the cases where it is not possible to delimit a clear exponential phase. In each case it must be specified which is the reference mean diameter and distribution. The number of diameter intervals,  $N$ , is equal to 256 for both the FS and the MSZ measurements, since it is the number of channels of both instruments.

As has been seen in previous chapters, these *distances* quantify the evolution of the biomass distributions toward the characteristic stable distribution of the exponential phase. The closer the distributions, the lower the *distance* values.

### 5.3.2 Results of the series T20: culturing at 20°C

In the experiments at 20°C, the inocula were taken from old cultures in a stationary phase. Therefore, there was a lag phase when they were added into the new medium. The adaptation of the bacteria during the lag was enough slow to be observed in detail.

Four repetitions of the experiments at 20°C were performed, with the first (T20-061205) and the second (T20-070220) used to adjust the experimental protocol in order to ensure that the inoculated cells were in stationary phase. The last repetitions, T20-070301 and T20-070315, provided similar results. They showed a slow evolution of the size distributions during the culturing that consists of a wide initial forward shift followed by a small backward shift at the end (Fig. 5.6).

#### The evolution of the mean diameter and the diameter distribution as an indicator of the growth phases

The growth curves obtained from MSZ measurements do not allow the identification of the phases of the growth, due to the minimum threshold in the measurement process ( $0.7 \mu m$ ). Nevertheless, if we observe the mean diameter evolution obtained from FS measurements (Fig. 5.7), we can distinguish three phases: first it increases (lag phase of between 200 *min* and 250 *min* in both replicas), then it remains constant at some points (exponential phase), and finally it begins decreasing (transition to stationary phase). These three phases correspond to the observed evolution of the size distributions, as indicated by the arrows in Figure 5.6.

This observed dynamic behaviour of the size distribution during the first hours of

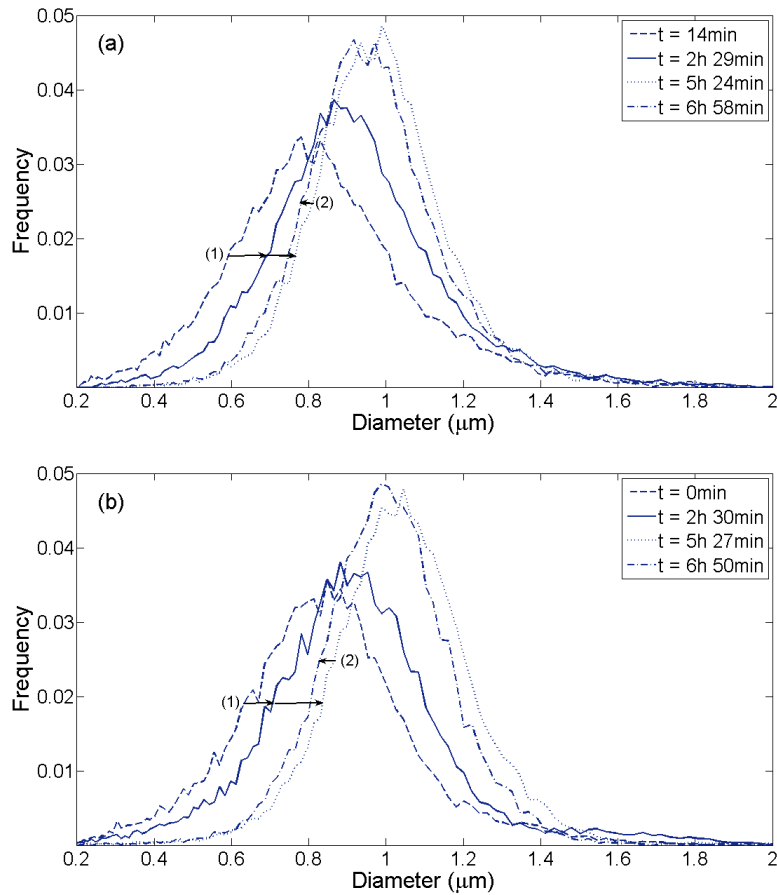


Figure 5.6: Four samples of the diameter distribution at different times for experiments T20-070301 (a) and T20-070315 (b). The arrows indicate the displacements (1) from lag to exponential and (2) from exponential to stationary phases.

growth is in accord with INDISIM predictions:

1. The pre-inoculation and the culture medium are the same. Therefore, the bacteria do not need to adapt their metabolism to a new nutrient source. As a result, no backwards shift in the size distribution is observed at the beginning and the cells start growing immediately after inoculation.
2. The lag phase is due to the small size of the inoculated bacterial cells, since they were taken from a stationary phase culture. During the lag phase, a forwards shift in



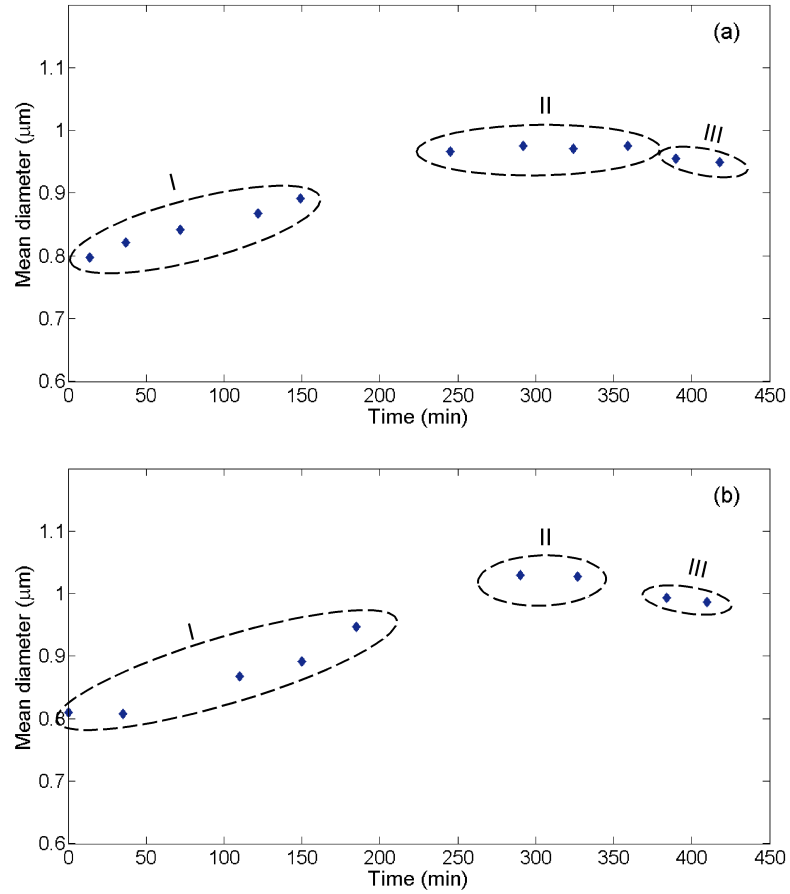


Figure 5.7: Evolution of the mean diameter in experiments T20-070301 (a) and T20-070315 (b). The three detected phases are shown: the lag phase (I), the exponential phase (II), and the beginning of the transition to stationary phase (III).

the size distribution is observed, showing the dynamic behaviour of this distribution during the lag.

Figure 5.8 shows the stability of the size distribution in three experimental points of the interval identified as exponential phase (experiment T20-070301).

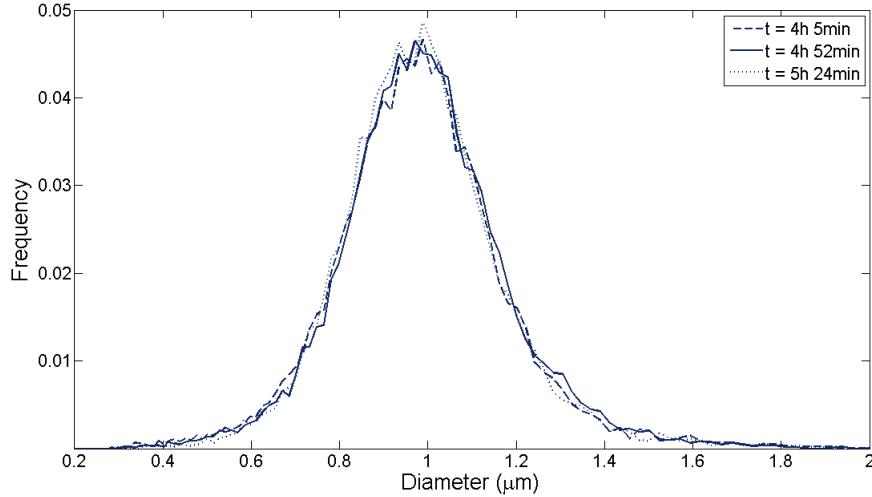


Figure 5.8: Three FS measurements of the diameter distribution among populations taken from the exponential phase (exp. T20-070301).

### Analyzing the results with the *distances*

These slow adaptations can be analyzed with the distances (Eq. 5.4). We take as the reference mean diameter and size distribution ( $\bar{d}_{ref}$  and  $p_{k_d,ref}$ ) the mean of their values at the exponential points identified in Figure 5.7.

The INDISIM simulation results showed the following behaviour for the *product distance*  $D(t)$  during the growth cycle: an approximately linear decrease during the lag phase, small oscillations around zero during the exponential phase, and an increase at the beginning of the stationary phase (see Section 3.2.2, Fig. 3.3 and Fig. 4.10).

If we plot the *product distance*  $D_d(t)$  for these experimental results (Fig. 5.9), we observe the same behaviour: a slow decrease in this variable during the lag phase which is approximately linear, followed by small oscillations around zero during the exponential phase. The last two points start increasing slightly. The slope of the initial decrease reflects the adaptation rate of the bacteria to the new medium: the greater the slope, the faster the adaptation and the shorter the lag phase.

This concord between simulations and experiments is neither trivial nor accidental; it is the result of the individual behaviours and interaction between cells. Thus, it strengthens the suitability of INDISIM to tackle the lag phase and highlights its potential for further study in this field. It is also a demonstration that INDISIM may be used to

optimize experimental designs when using FC or MSZ to perform quantitative studies of the evolution of microbial populations.

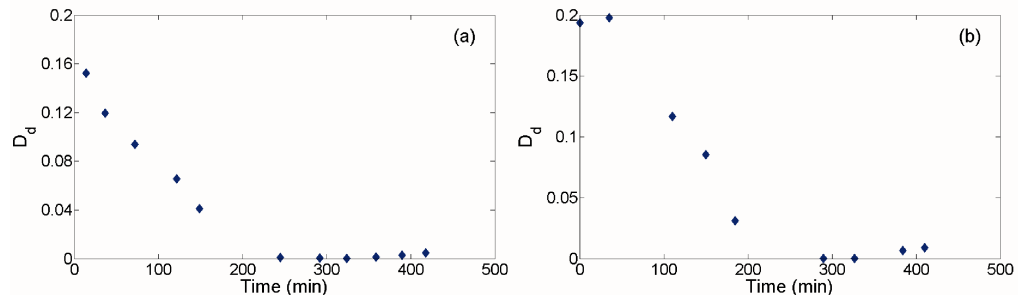


Figure 5.9: Evolution of the product distance in experiments T20-070301 (a) and T20-070315 (b).

### 5.3.3 Results of the series T35: culturing at 35°C

The 20°C experiments allowed the observation of a long lag phase, a short exponential phase, and the very beginning of the stationary phase. Now, the study of a faster growth should provide the opportunity of studying a longer exponential phase and stationary phase. Experiments at 35°C are designed to pursue this aim.

The three independent experiments were performed under the same growth conditions, and they provided similar results for lag and exponential phases. Experiment T35-061121 showed a high level of background noise that was reduced in successive experiments by filtering the medium. Experiments T35-070123 and T35-070130 reproduced the same behaviour detected in the first experiment, but without such a high background.

In experiment T35-070123, a clear transition from lag to exponential can be observed, followed by a long exponential phase. The evolution of the measured size distribution is in accord with the growth curve obtained with multisizer measurements (forward shift during lag and stability during exponential phase). This experiment ends before the culture reaches the stationary phase.

In experiment T35-070130, the three phases of the growth are clearly observed in the growth curve (Fig. 5.10), where a sigmoidal curve has been fitted ( $r^2 = 0.993$ ). The lag parameter of this culture, calculated by means of the geometrical definition, is  $\lambda = 43 \text{ min}$ . This lag is substantially (20 – 25%) shorter than the one observed in 20°C culturing, as was expected. In the following sections, the results of these experiments are studied with more detail.

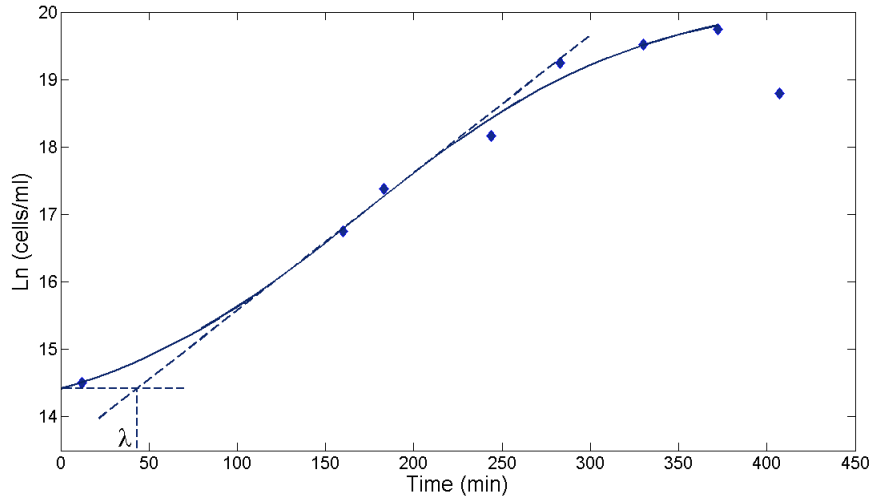


Figure 5.10: Cell concentration, measured with the flow cytometer by using an internal calibrator (exp. T35-070130). A sigmoid curve has been fitted ( $r^2 = 0.993$ ), and the lag phase calculated by means of the geometrical definition is  $\lambda = 43$  min.

### Stability of the diameter distribution during exponential phase

Again, one of the results of INDISIM simulations that agrees with the theoretical predictions is experimentally found: the stability of the mass distribution during the exponential growth, which is a characteristic of the bacterial strain and the medium conditions (see Chapter 4). The flow cytometry measurements corroborate this behaviour, which is characteristic of balanced growth according to this variable. Figure 5.11 shows the stability of the distribution at four points of the exponential phase in experiment T35-070130.

### Evolution of the diameter distribution and the mean diameter

The experimentally measured evolution of the diameter distribution perfectly correspond with the predictions of INDISIM simulations. The inoculum is taken from a previous culture which is in stationary phase. Therefore, the bacteria have experienced a decrease in their biomass that has been caused by the starvation conditions. When the cells of the inoculum are added to the new medium, they must again increase their biomass before starting the reproduction cycle. This is detected in flow cytometry measurements by a forward shift of the diameter distribution (Fig. 5.12). During the exponential phase,

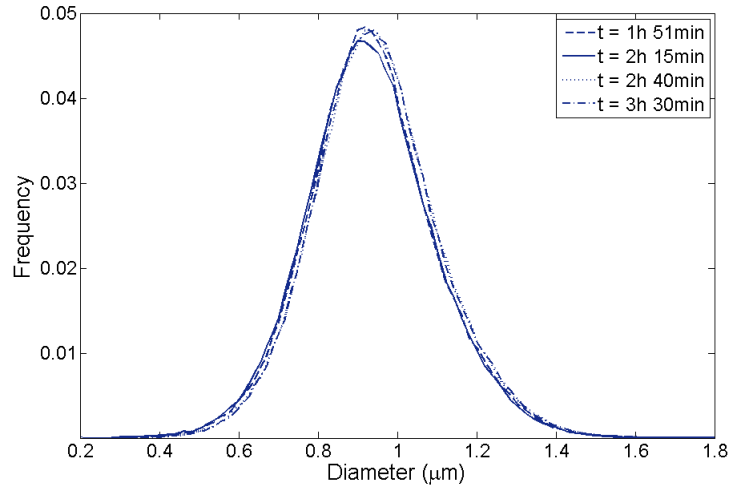


Figure 5.11: *Four FS measurements of the diameter distribution among populations taken from the exponential phase (exp. T35-070130).*

the size distribution remains stable, as shown in Figure 5.11. It is another proof that the culture is in balanced growth. Then, when the nutrient runs out and the bacteria are in starvation conditions, a backward shift of the distribution is detected (Fig. 5.12).

The reported simulations showed that when the pre-inoculation medium is the same as the culturing one, there is no need to carry out a metabolic adaptation to a new nutrient source. This hypothesis is corroborated by the experiment: the small inoculated cells start increasing their masses from the beginning, since no backward shift in diameter distribution is detected during the lag phase.

The plot of the mean diameter evolution also shows the predicted behaviour, which is related to the evolution of the diameter distribution. The three phases can be clearly distinguished: the mean diameter increases during the lag phase, it remains approximately constant during the exponential phase, and begins decreasing when the culture enters the stationary phase (Fig. 5.13). One can imagine the prolongation of the experiment, which would continue with the decrease of the mean diameter down to the value of the initial inoculum, since it had been taken from the end of an analogous stationary phase. If a sample of this culture were taken at that point and inoculated into fresh medium, the same process would be observed: an increase of the mean diameter during the lag phase up to the characteristic value of the exponential growth, at which point it would remain stable.

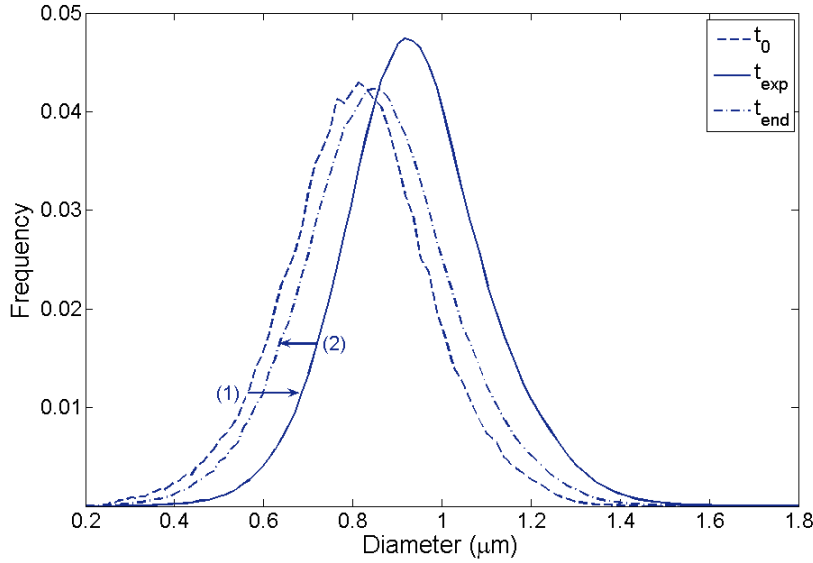


Figure 5.12: *FS measured distribution at inoculation time ( $t_0$ ), mean of the measured distributions during exponential phase ( $t_{exp}$ ) and last FS measured distribution at the end of growth ( $t_{end}$ ) (exp. T35-070130). The arrows indicate the displacements (1) from lag to exponential and (2) from exponential to stationary phases.*

An unexpected result is the evolution of the mean diameter. As explained in Chapter 4, the Helmstetter experimental equation (Helmstetter et al., 1968) states a relationship between the duplication rate  $R$  (related to the growth rate) and the mean mass of a culture in exponential phase (Eq. 5.5):

$$\bar{m} = \frac{2^R m_0}{\ln 2} \quad (5.5)$$

The cell cycle model of INDISIM, which sets a fix period for reproduction cycle during which the bacteria can keep growing, is in accord with this equation: the simulations reproduce this relationship (López, 1992). The faster the growth, the higher the mean diameter at exponential phase.

In contrast, experimental results at 20°C give a mean diameter slightly greater than the series T35. If the mean diameter at exponential phase in 35°C series is around 0.95  $\mu m$ , with experiments at 20°C it increases up to almost 1  $\mu m$  (Fig. 5.14). This is not a huge quantitative difference, but it may be conceptually significant.

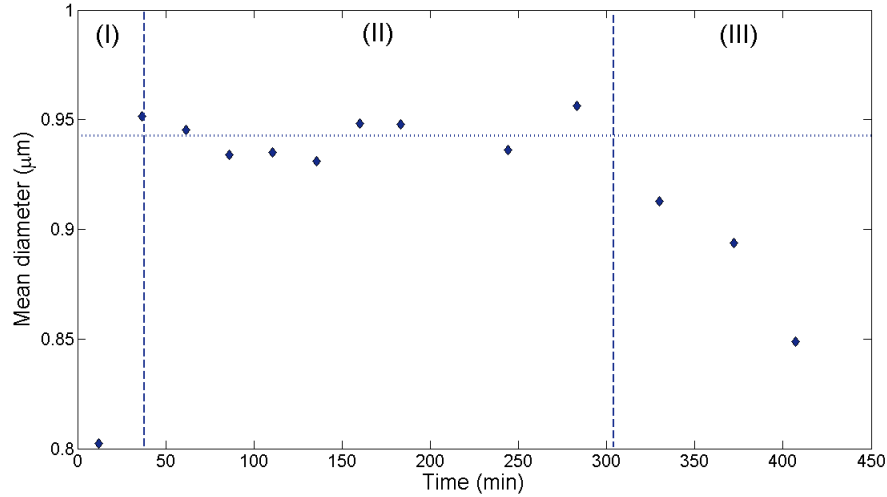


Figure 5.13: Mean diameter evolution of the culture (exp. T35-070130), from FS measurements. Dashed lines indicate the lag (I), exponential (II) and stationary (III) phases. Dotted line indicates the mean value during exponential phase.

It is necessary to design a set of experiments at intermediate temperatures to follow the evolution of the mean diameter. Then, these data should be analyzed to suggest an explanation for this unexpected phenomenon.

### Analyzing the results with the *distances*

The evolution of the diameter distribution and the mean diameter can be quantified by means of the *distances*. The mean diameter distance  $D_{\bar{d}}(t)$  (Eq. 5.2) and the diameter distribution distance  $D_{p_{k_d}}(t)$  (Eq. 5.3) for the experimental FS measurements are plotted in Figure 5.15. The product distance  $D_d(t)$  (Eq. 5.4), as shown in Chapter 3, is the best one to follow the evolution of the system. Figure 5.16 shows the evolution of  $D_d(t)$  in experimental FS measurements (exp. T35-070130), and the evolution of the *product distance* predicted by INDISIM simulations for a similar case,  $D(t)$  (Eq. 3.3). In both cases, the reference distribution to calculate the distances is taken from the mean of several points of the exponential phase. The observed behaviour is the same: an initial decrease during the lag phase, then it remains close to zero during the exponential phase, and finally increases when the culture enters the stationary phase. In this case, the scarcity of measured points during the lag phase does not allow us to see the linear decrease of

the product distance. Again, simulations and experiments are in agreement.

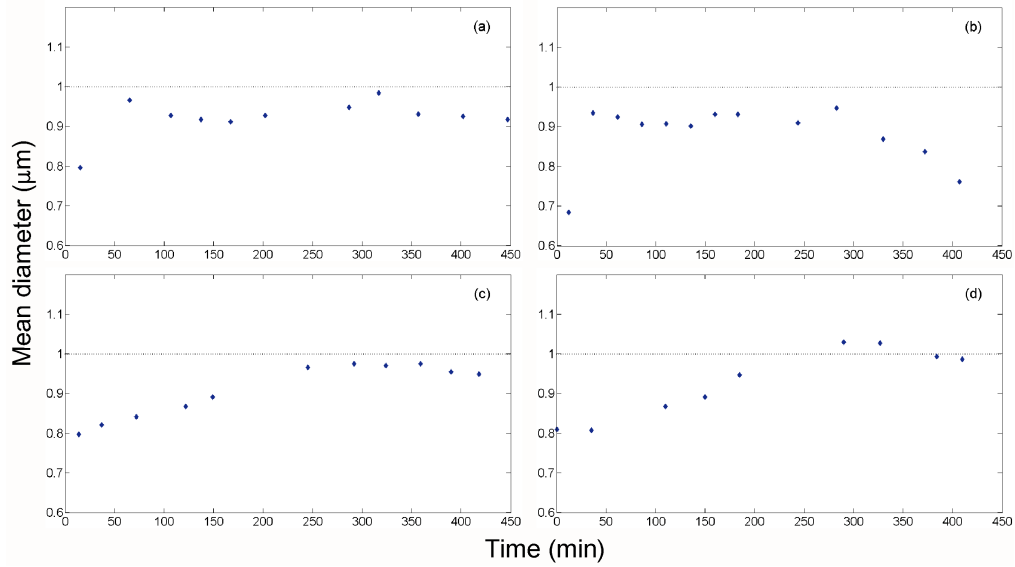


Figure 5.14: Mean diameter evolution for 35°C experiments  $T35-070123$  (a) and  $T35-070130$  (b), and 20°C experiments  $T20-070301$  (c) and  $T20-070315$  (d). The dotted lines indicate the 1 µm level in each plot.

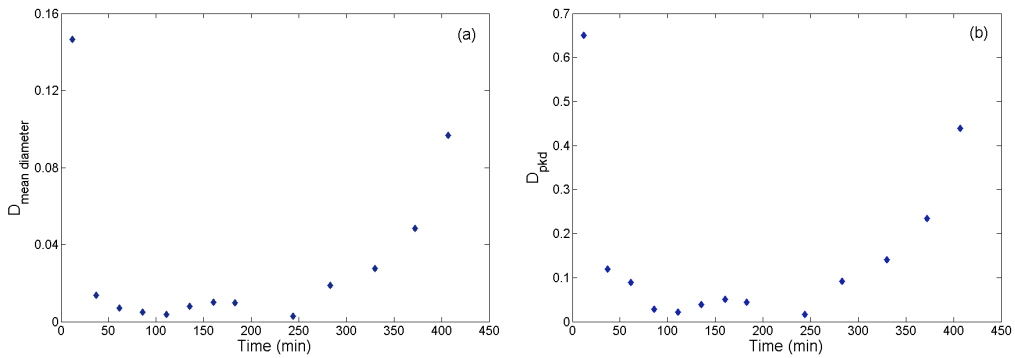


Figure 5.15: (a) Mean diameter distance ( $D_{\bar{d}}(t)$ ) and (b) diameter distribution distance ( $D_{pk_d}(t)$ ) evolution for FS measurements (exp.  $T35-070130$ ).

We can compare the product distance evolution during the lag phase for 20°C and 35°C experiments, assuming the linear decrease for all of them during the lag phase. The



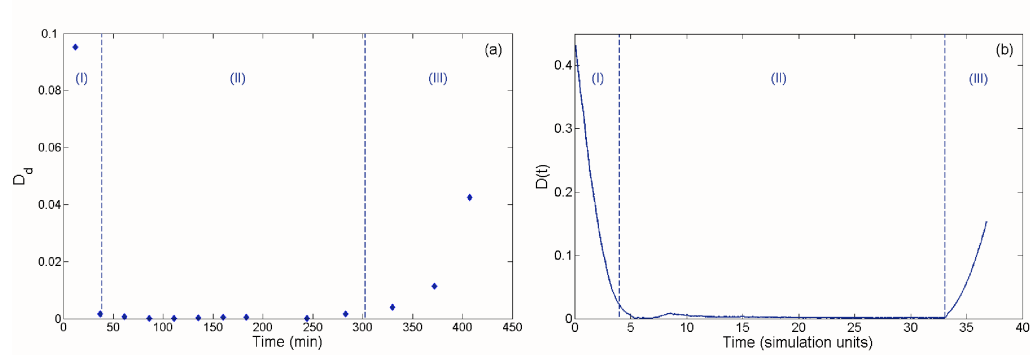


Figure 5.16: (a) Product distance ( $D_d(t)$ ) evolution for FS measurements (exp. T35-070131), and (b) INDISIM simulation prediction of the  $D(t)$  evolution for a similar culture. Dashed lines indicate the lag (I), exponential (II) and stationary (III) phases.

differences in their slopes will be a consequence of the differences in the inocula adaptation rates during the lag phase. As is shown in Table 5.1, the slopes of the  $D_d(t)$  of the 20°C experiments range between 20% and 50% of the slopes of the 35°C experiments. This is in accord with the expected results: the higher the temperature, the faster the adaptation. This should be explored in growths at higher temperature; when the upper limit is near, the adaptation should show a different behaviour.

Table 5.1: Slope of the product distance decrease during the lag phase.

Experiment	Slope ( $\text{min}^{-1}$ )
T35-070123	$-2 \cdot 10^{-3}$
T35-070130	$-4 \cdot 10^{-3}$
T20-070301	$-0.8 \cdot 10^{-3}$
T20-070315	$-1.1 \cdot 10^{-3}$

## 5.4 Discussion

The combination of flow cytometry, particle analysis and Individual-based Modelling has proved useful for the study of lag, exponential and stationary phases, providing new insights into these complex and still poorly understood phenomena. The study of a dynamic property of a population such as the biomass distribution provides additional information about the temporal evolution of the system as well as all associated processes. Thus, this methodology is appropriate to tackle the study of bacterial cultures in transient

processes, where the size distribution is not stable. Moreover, the evolution of this distribution is a reflection of the underlying processes that provides more information than the pure observation of the macroscopic growth curve with *classic* experimental methods (optical density or microscopic counts). Thus, these cell size analysis techniques are able to get measurements at an individual, mesoscopic and population level of description. As we have seen throughout this chapter, INDISIM simulations provide appropriate tools to analyze and explain these results, and to predict new ones. Cell size analysis techniques and IBM modelling and simulation work at the same scale.

With the instruments used, the flow cytometry measurements have provided better and more accurate results than the multisizer, mainly because the small size of the bacteria used is within the limits of the multisizer observations ( $0.7\ \mu\text{m}$ ). The use of the flow cytometer to assess the population counts requires the use of an internal calibrator and a larger culture sample, so it slows down and complicates the experimental measurements.

Mathematical comparisons between distributions, such as the product distance, are a new approach to the assessment of biomass variability in microbial systems. The product distance has proved to be a good tool for studying the culture evolution: the different phases of the growth can be distinguished, and the adaptation rate can be evaluated with the slope of the initial decrease.

The experimental measurements have corroborated the simulation results presented in previous chapters. The size distribution is very stable during exponential growth, and any transient process (either lag or stationary phases) can be detected through a shift in this distribution. The adaptation of the size distribution during lag phase is slower in low temperature experiments than in high temperature experiments. The experimental measurements have shown that the initial decrease in product distance is linear, as was predicted by INDISIM simulations in previous chapters.

Next FC experiments should cover two areas. On the one hand, a deeper analysis of the lag phase at high temperatures ( $35^\circ\text{C}$ ) should be done with more measurement points during the first hour, in order to check the shape of the initial decrease in product distance. On the other hand, experiments at intermediate temperatures should be carried out to corroborate the detected tendencies (such as the adaptation rate) and to solve the open question regarding the relationship between the mean size and the growth rate.

Further experiments should explore other techniques, in order to solve the technical problems such as the low threshold of the multisizer or the limitations in assessing the total counts with the flow cytometer. A combination of scattering techniques with a more efficient way for assessing the total counts would be the perfect experimental technique to study microbial population in liquid medium. Laser Diffraction Particle Size

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Analyzer (LDPSA) seems to be the appropriate experimental way for this purpose; it directly provides a size distribution of the studied sample, so data transformations are not needed, and the cell counting is also precise and efficient. Furthermore, the use of microcalorimetric measurements in parallel with scatter measurements would provide interesting additional information about the metabolic activity of the bacteria during the different phases of growth. Study of the relationship between metabolic activity and the evolution of size distribution would be an interesting vantage point from which to tackle the transient processes at the microscopic, mesoscopic and macroscopic levels.

