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Research Article

Kinetic Model for Micro Algae Cell Concentration and Size Distribution: Application to *Nannochloropsis gaditana*

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ABSTRACT

The cell concentration and size distribution of the microalgae *Nannochloropsis gaditana* were studied over the whole growth process. Various samples were taken during the light and dark periods the algae were exposed to. The distributions obtained exhibited positive skew, and no change in the type of distribution was observed during the growth process. The size distribution shifted to lower diameters in dark periods while in light periods the opposite occurred. The overall trend during the growth process was one where the size distribution shifted to larger cell diameters, with differences between initial and final distributions of individual cycles becoming smaller. A model based on the Logistic model for cell concentration as a function of time in the dark period that also takes into account cell respiration and growth processes during dark and light periods, respectively, was proposed and successfully applied. This model provides a picture that is closer to the real growth and evolution of cultures, and reveals a clear effect of light and dark periods on the different ways in which cell concentration and diameter evolve with time.

Key words: coulter counter, growth kinetics, microalgae, modeling, size distribution.

INTRODUCTION

Microalgae grown under light-dark cycle conditions can undergo changes in important ways including size. In this regard, microalgae exhibit a naturally phased cell division which occurs only during a particular time of the day, generally at night [1]. In the dark (i.e., when the light intensity is too low to support cell growth), the cells do not grow but respire to preserve themselves. In the absence of light, of energy or of some other metabolizable organic carbon source in the medium, cells metabolize their own cell components to obtain sustenance. This leads to a decrease in cell weight. It has been reported that up to 35% of biomass produced during the daylight period may be lost through respiration at night [2].

Microalgae cell growth rates are affected by a combination of environmental parameters such as light intensity, photoperiod, temperature, and nutrient composition in the culture system [3][4]. In photoautotrophic cultures where temperature is not limiting, the light regime and photoperiod are the critical factors that determine biomass production in these cultures [5].

Growth is defined as an increase in the amount of living tissue – usually the number of cells for unicellular microorganisms [6]. Some classic growth models such as the one proposed by Monod are based on the specific growth rate. Consequently, in most models of growth kinetics and photosynthetic cell growth, the specific growth rate during the exponential growth phase is used as the growth parameter [7]. The specific growth rate is usually expressed as:

$$N(t) = N_1 \cdot e^{\mu(t-t_1)} \quad (1)$$

Where $N(t)$ and N_1 are the cell concentration at time t and t_1, respectively, and μ is the specific growth rate [5].This equation expresses the fractional increase in cell number after a specified period of time. The specific growth rate is an average over the

growth rates of all cells present in the culture, since most microbial cultures divide asynchronously [8]. Since the growth of microalgae proceeds through lag, exponential and stationary phases, the Logistic model (Equation 2) can be used to obtain a more accurate fit of cell concentration as a function of time [9]:

$$N(t) = \frac{Nf}{1+\frac{Nf-No}{No}e^{-\frac{4\mu max \cdot t}{Nf}}} \qquad (2)$$

Where N_f and $N_o$are the cell concentration during the stationary phase and at the initial time, respectively, and μ_{max} is the maximumgrowth rate, which can be obtained by nonlinear-fitting. Time scale used in these equations is total time. The effect of light cycles (night/day) on CO_2 fixation by *Aphanothece microscopica Nägeli* in photobioreactors is studied in [10]. They studied different cycles ranging from continuous lighting of 24 h every 24 h, to 0 h every 24 h (i.e. complete dark) using 2 hours interval. If plotting their results vs the time the culture was exposed to light (Figure 1) , all curves merge in a single curve, showing that the culture (taking into consideration the dispersion observed) grows depending on the time that has been exposed to light regardless the cycle. Thus, it seems that there is no reason to use light cycles in artificially lighted photobioreactors, since the highest growing rate in the total time basis is obtained for continuously lighted systems.

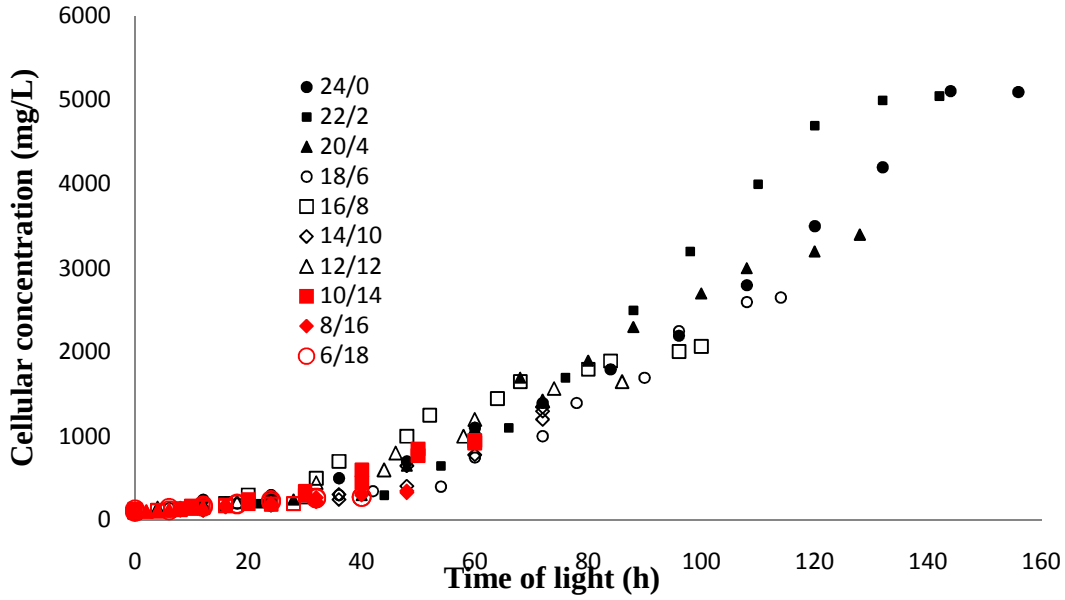


Figure 1: Cellular concentration vs lighting time for different light/dark cycles. Data read from [10].

Size-dependent growth rates have been reported in many references, indicating that cell division rates and other physiological processes are a function of cell size [7], [9], [11], [12], [13]. Therefore, as cell size varies during the whole growth process [12], [14], an analysis of size distributions is essential to obtain accurate values of the cell size and microalgae growth rate. Cell size distributions represent the frequency of cells of a given diameter (i.e. the number or concentration of cells of a given diameter divided by the total number or concentration of cells in the culture) vs the cell diameter. The Coulter Counter provides this type of data. Cell size distribution can be represented, as other similar functions, by different probability functions. The probability of a specific cell diameter falling within a particular range of values is given by the integral of the function over that range [15]. There are different types of continuous probability distributions: Gaussian or normal, log normal, uniform, chi-squared, t-functions, among others. Gaussian function (Equation 3) is used to describe the size distribution of marine phytoplankton and a median cell size can thus be calculated [9]:

$$n\left(d(\theta(k))\right) = \frac{1}{\sigma\sqrt{2\pi}}e^{-\frac{\left(d(\theta(k))-d(\theta(k))_a\right)^2}{2\sigma^2}} \qquad (3)$$

where n(d(θ(k))) is the normalized cell concentration of d(θ (k)), which is the mean diameter of every diameter interval measured by the Coulter Counter in the sample harvested after a time θ (k) in period k, σ is the standard deviation and $d(\theta\ (k))_a$ is the average cell diameter of the sample harvested after a time θ (k) in period k. According to [16], phytoplankton size affects numerous other functional traits and crucial physiological and ecological processes, including light absorption, nutrient uptake, sinking, and grazing. They develop a very interesting mechanistic model relating these variables for different ocean communities. In a photo bioreactor, the cell size distribution obviously depends on the number of cells (marked by the culture growing kinetics) and their

size that depends on the growing (cell division produces to cells of half the volume) and the respiration process (i.e: decreasing the cell size) and the CO_2 uptake (i.e.: increasing the size).

Nevertheless, to the best of our knowledge, there are no studies considering the relations among all these variables in a microalgae culture in a photo bioreactor, including the variations in size distributions during light/dark cycles and the interrelations and modeling of such variations in combination with the time evolution of cell concentration. The analysis of the time evolution of both, the cell size and the cell concentration, and their consideration in the kinetic model allows quantification and, thus, a better knowledge of the growth process. The correlation of the growth of the culture with time would be useful for designing and scaling up the cultivation system [17].

According to [18], the possibility of simultaneously know the cell number and size would allow to select the optimum time to harvest the culture. The ability to construct, analyze and predict changes in size distributions may therefore allow a more precise description of interactions within plankton communities.

The purpose of this study is to investigate the size distributions of the microalgae Nannochloropsis gaditana during light/dark cycles, in order to obtain a better understanding of the microalgal growth process and to develop a kinetic model. In order to give to the model a more predictive character, the influence of temperature and light intensity would have to be studied, what is out of our scope that mainly consists in develop and apply the equations and procedure to simultaneously correlate the cell number and size with time.

MATERIALS AND METHODS

The microalgae, *Nannochloropsis gaditana*, used in the present study was obtained from Fitoplancton Marino S.L.(Cadiz, Spain). It was cultivated in flasks of external diameter 236 mm with a working volume of 6 L, and in a clean room to create a particle-free environment. The ambient temperature was kept constant at 18 ºC and each culture was mixed by artificially aeration through a micro porous diffuser at a rate of 1.6 $L \cdot min^{-1}$. Natural sea water from the "Golfo de Mazarrón" (Spain) (latitude 37.835 and longitude -0.791586) served as growth medium for the microalgae. The sea water was filtered, sterilized with a UV lamp and enriched with modified f/2 prior to use with a concentration of 1 mL of modified f/2 medium/L of culture. The latter is a nutrient-rich solution of twice the concentration of f/2 [19], and is also supplied by Fitoplancton Marino S.L. The cultures were illuminated with a 40 W fluorescent tube (92 $\mu mol \cdot m^{-2} \cdot s^{-1}$). As previously commented on, cell cultures growth depends mainly on the light time and intensity and is almost independent on the cycle light/dark cycle. The objective of the present work is to illustrate the interrelations among the growing kinetics and the culture cell size distribution considering the cell size decrease due to respiration during the dark period and the CO_2 uptake during the light period increasing the cell size. For this purpose the light/dark period is not a relevant variable. Due to this we have selected 8:8 h cycles of light and dark, allowing collecting the samples corresponding to complete light and dark periods in a normal 8 h working day. As shown in Table I, samples were taken every 2 h from 7:30 h in the morning, when the first switch between light and dark took place, until 15:30 in the afternoon, when the second switch between light and dark took place. Therefore, the time can be expressed as: t: time; - $\tau(t)$: cumulative time that the culture has remained under dark conditions up to time t; and - $\theta(k)$: time elapsed since the beginning of the period k(which ranges from 0 to 8 hours in each period).

Table 1: Light/dark conditions of the culture depending on the time

Cycle	Period	Dark/Light	t (h)	τ (h)	θ(h)	Sample [(a)]
1	1	Dark	0	0	0	X
		Dark	2	2	2	X
		Dark	4	4	4	X
		Dark	6	6	6	X
		Dark	8	8	8	X
	2	Light	8		0	
		Light	10		2	
		Light	12		4	
		Light	14		6	
		Light	16		8	

Table 1: Light/dark conditions of the culture depending on the time (cont…

Cycle	Period	Dark/Light	t (h)	τ (h)	θ(h)	Sample (a)
2	3	Dark	16	8	0	
		Dark	18	10	2	
		Dark	20	12	4	
		Dark	22	14	6	
		Dark	24	16	8	
	4	Light	24		0	X
		Light	26		2	X
		Light	28		4	X
		Light	30		6	X
		Light	32		8	X
3	5	Dark	32	16	0	
		Dark	34	18	2	
		Dark	36	20	4	
		Dark	38	22	6	
		Dark	40	24	8	
	6	Light	40		0	
		Light	42		2	
		Light	44		4	
		Light	46		6	
		Light	48		8	
4	7	Dark	48	24	0	X
		Dark	50	26	2	X
		Dark	52	28	4	X
		Dark	54	30	6	X
		Dark	56	32	8	X
	8	Light	56		0	
		Light	58		2	
		Light	60		4	
		Light	62		6	
		Light	64		8	
5	9	Dark	64	32	0	
		Dark	66	34	2	
		Dark	68	36	4	
		Dark	70	38	6	
		Dark	72	40	8	
	10	Light	72		0	X
		Light	74		2	X
		Light	76		4	X
		Light	78		6	X
		Light	80		8	X

Table 1: Light/dark conditions of the culture depending on the time (cont…

Cycle	Period	Dark/Light	t (h)	τ (h)	θ(h)	Sample (a)
6	11	Dark	80	40	0	
		Dark	82	42	2	
		Dark	84	44	4	
		Dark	86	46	6	
		Dark	88	48	8	
	12	Light	88		0	
		Light	90		2	
		Light	92		4	
		Light	94		6	
		Light	96		8	
7	13	Dark	96	48	0	X
		Dark	98	50	2	X
		Dark	100	52	4	X
		Dark	102	54	6	X
		Dark	104	56	8	X
	14	Light	104		0	
		Light	106		2	
		Light	108		4	
		Light	110		6	
		Light	112		8	
8	15	Dark	112	56	0	
		Dark	114	58	2	
		Dark	116	60	4	
		Dark	118	62	6	
		Dark	120	64	8	
	16	Light	120		0	X
		Light	122		2	X
		Light	124		4	X
		Light	126		6	X
		Light	128		8	X
9	17	Dark	128	64	0	
		Dark	130	66	2	
		Dark	132	68	4	
		Dark	134	70	6	
		Dark	136	72	8	
	18	Light	136		0	
		Light	138		2	
		Light	140		4	
		Light	142		6	
		Light	144		8	

Table 1: Light/dark conditions of the culture depending on the time (cont…

Cycle	Period	Dark/Light	t (h)	τ (h)	θ(h)	Sample (a)
10	19	Dark	144	72	0	X
		Dark	146	74	2	X
		Dark	148	76	4	X
		Dark	150	78	6	X
		Dark	152	80	8	X
	20	Light	152		0	
		Light	154		2	
		Light	156		4	
		Light	158		6	
		Light	160		8	
11	21	Dark	160	80	0	
		Dark	162	82	2	
		Dark	164	84	4	
		Dark	166	86	6	
		Dark	168	88	8	
	22	Light	168		0	X
		Light	170		2	X
		Light	172		4	X
		Light	174		6	X
		Light	176		8	X
12	23	Dark	176	88	0	
		Dark	178	90	2	
		Dark	180	92	4	
		Dark	182	94	6	
		Dark	184	96	8	
	24	Light	184		0	
		Light	186		2	
		Light	188		4	
		Light	190		6	
		Light	192		8	
13	25	Dark	192	96	0	X
		Dark	194	98	2	X
		Dark	196	100	4	X
		Dark	198	102	6	X
		Dark	200	104	8	X
	26	Light	200		0	
		Light	202		2	
		Light	204		4	
		Light	206		6	
		Light	208		8	

Table 1: Light/dark conditions of the culture depending on the time (cont…

Cycle	Period	Dark/Light	t (h)	τ (h)	θ(h)	Sample (a)
14	27	Dark	208	104	0	
		Dark	210	106	2	
		Dark	212	108	4	
		Dark	214	110	6	
		Dark	216	112	8	
	28	Light	216		0	X
		Light	218		2	X
		Light	220		4	X
		Light	222		6	X
		Light	224		8	X
15	29	Dark	224	112	0	
		Dark	226	114	2	
		Dark	228	116	4	
		Dark	230	118	6	
		Dark	232	120	8	
	30	Light	232		0	
		Light	234		2	
		Light	236		4	
		Light	238		6	
		Light	240		8	
16	31	Dark	240	120	0	X
		Dark	242	122	2	X
		Dark	244	124	4	X
		Dark	246	126	6	X
		Dark	248	128	8	X

[(a)]The "X" shows when a sample was harvested

The time evolution of the culture was recorded by measuring the optical density at 540 nm. The dry weight of the cultures ($g \cdot L^{-1}$) was obtained by means of a predetermined correlation, i.e. dry weight ($g \cdot L^{-1}$) = $0.33 \cdot OD_{540}$ (R^2 = 0.99). This correlation was obtained using the method proposed by [20] consisting in filtration with a 0.2 µm membrane average pore and dried in an oven until constant weight. The number of cells per unit volume (cell concentration) was determined by placing the cells in a 0.1 mm deep Neubauer counting chamberand using a light microscope. Information about the cell size distribution (CSD) was obtained by means of a Coulter LS230 counter, which can measurecell sizes ranging from 0.04 µm to 2000 µm. The calculations needed for the obtaining of the kinetic parameters for each sample was carried out following the algorithms described in Appendix A and using non-linear least-squares regression with the solver add-in in Microsoft Excel.

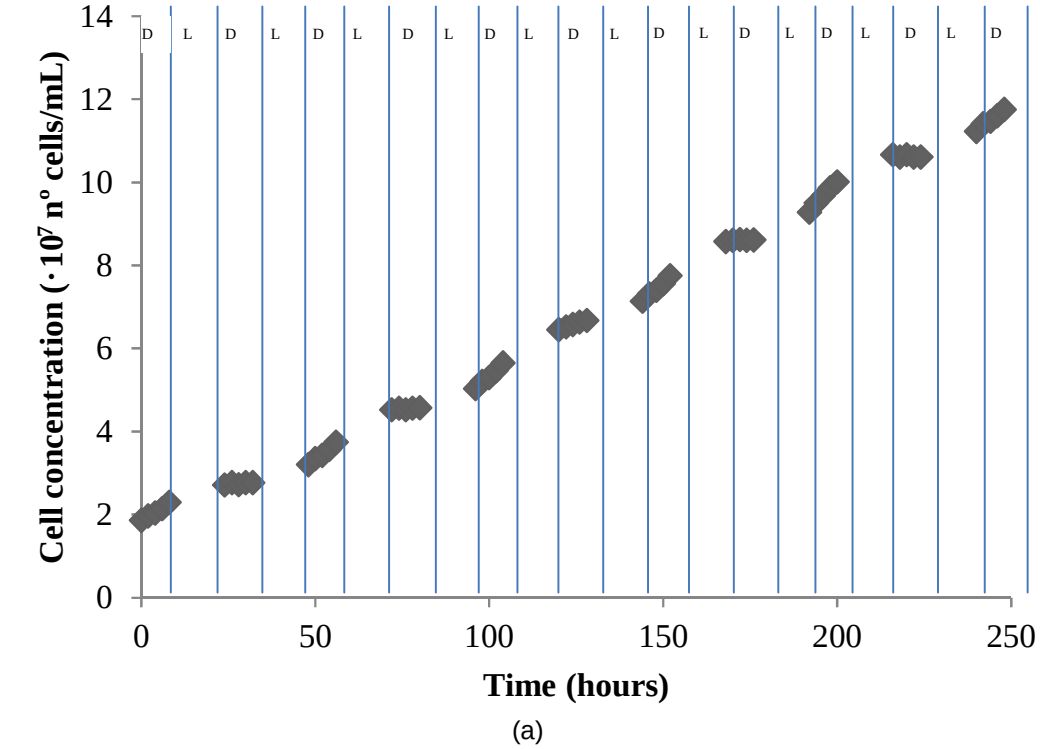
RESULTS AND DISCUSSION

Results presented correspond to the average of three determinations; the dispersion of the results was lower than 10% in all cases, including the cell size distributions.

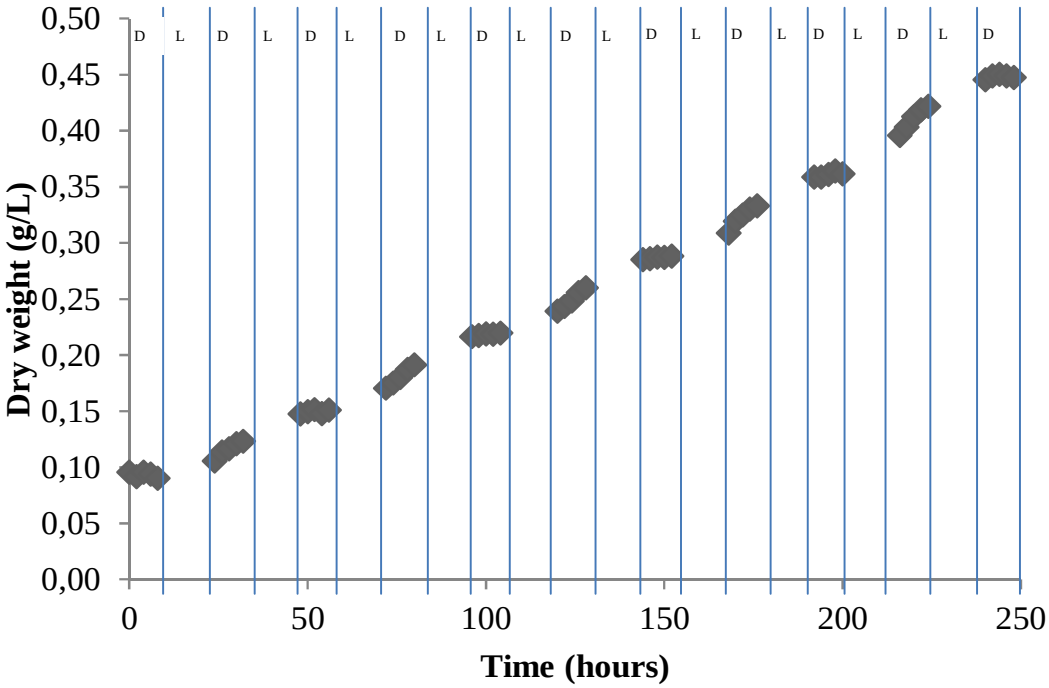
Dry weight and cell concentration

As shown in Figure 2a, the number of cells per unit volume increased during the dark periods and remainednearly constant during the light periods. However, Figure 2b shows that the dry weight followed the opposite trend: during dark periods it was

practically constant, while during light periods it increased. This is in concordance with the fact that, during dark periods, cell division takes place [1] and, during light periods, cell growth occurs instead.



(a)



(b)

Figure 2: Time evolution of the growth of *N. gaditana* in terms of a) cell concentration of the microalgae and b) dry weight.

The final cell concentration was $1.18 \cdot 10^{8}$ cells·mL^{-1}, which constitutes an increase of $9.9 \cdot 10^{7}$ cells·mL^{-1} in 248 h. The final dry weight was 0.45 g·L^{-1} and the productivity was 0.0142 g·L^{-1}·h^{-1}.

Kinetic modeling of cell concentration

Cell concentrations was fitted to Equation 1.The value of the specific growth rate obtained in this way during the exponential growth phase (μ) was $0.288 d^{-1}$(R^2 = 0.9792), which is similar to that found by [5] for the microalgae *Nannochloropsis sp*.

In Figure 3a it can be seen that the Logistic modelfits the data relatively wellwhen only the samples taken at 7:30 in the morning were taken into account. In this case, the value obtained forthe maximum growth rate was$4.82 \cdot 10^5 cells \cdot mL^{-1} \cdot h^{-1}$ (R^2= 0.9967). Nevertheless, a more detailed analysis should include all the samples that were harvested, and reveals that the model does not produce such a good fit, as Figure 3b shows. This model clearly cannot reproduce the details of the dark/light cycles because they are not accounted for explicitly in the corresponding equation. Thus, the model represents a type of average that does not consider the details of the actual process, and only takes into account the division process that occurs during the dark period.

For this reason, a modification of the Logistic model was proposed considering only the time the culture has been under dark, where the microalgae undergoes the division process. Figure 3c shows a much better fitting than that obtained by applying the Logistic model in the usual way, which permits a rather accurate prediction of the details of the observed experimental behavior. The maximum growth rate obtained was $9.50 \cdot 10^4 cells \cdot mL^{-1} h^{-1}$ in the dark, and the final cell concentration was $1.45 \cdot 10^8 cells \cdot mL^{-1}$($R^2$=0.9999).

On the other hand, since different processes take place during the light and dark periods, they were modeled independently (See section A1.1).The kinetic model proposed for fitting the cell concentration is thus a discontinuous function (Equation A.1). The results produced by this kinetic model for the evolution of the cell concentration with time are shown in Figure 3d. The obtained fit of the evolution of the cell concentration with time (R^2= 0.9991) was better than that produced by the conventional Logistic model (Figure 3b) because the newly proposed model takes into account the different behaviors that cells exhibitduring the light/dark cycles.

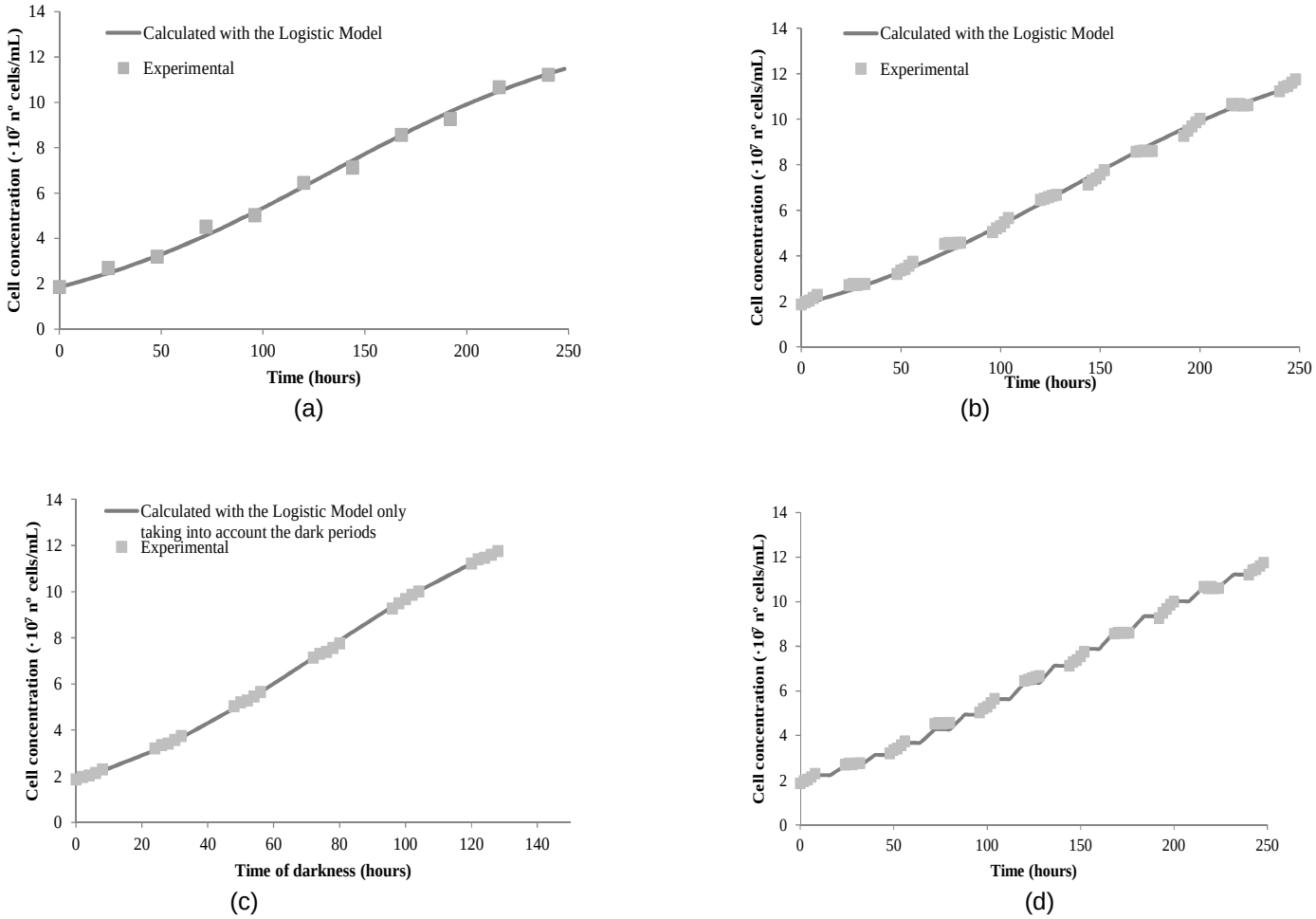


Figure 3: Kinetic analysis of the growth of *N. gaditana*. (a) Logistic model and the experimental points obtained by taking samples only every 24 hours. (b) Logistic model and all the experimental results. (c) Logistic model that takes into account only the dark periods (d) Proposed kinetic model; the solid line represents the results fitted by Equation A.1.

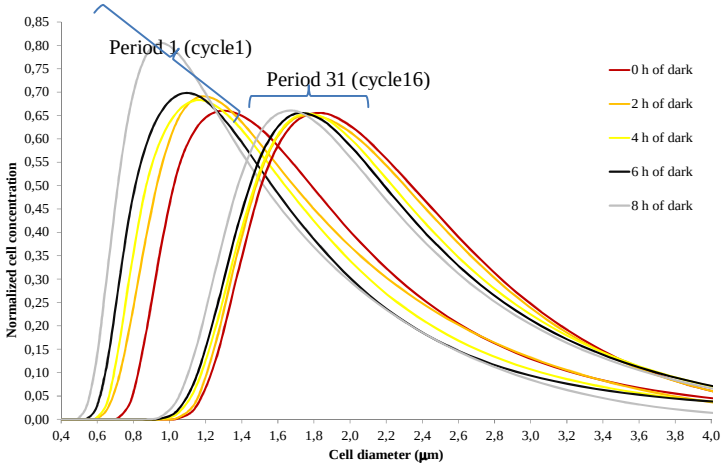
Cell size distributions

Figure 4a shows the cell size distribution as the normalized cell concentration versus the cell diameter of samples harvested in two dark periods (1 (cycle 1) and 31 (cycle 16)) and Figure4b those corresponding to two light periods: (4 (cycle 2) and 28 (cycle 14)). The remaining samples have not been included since they exhibit the same trend. The distributions were normalized in order to enable a comparison between all the curves obtained over the whole process. *N. gaditana* exhibits a distribution with positive skew, since the right tail is longer and the mass of the distribution is concentrated on the left of the figure. This distribution is said to be right-skewed, right-tailed, or skewed to the right. No variation in type of size distribution was observed during the growth process.

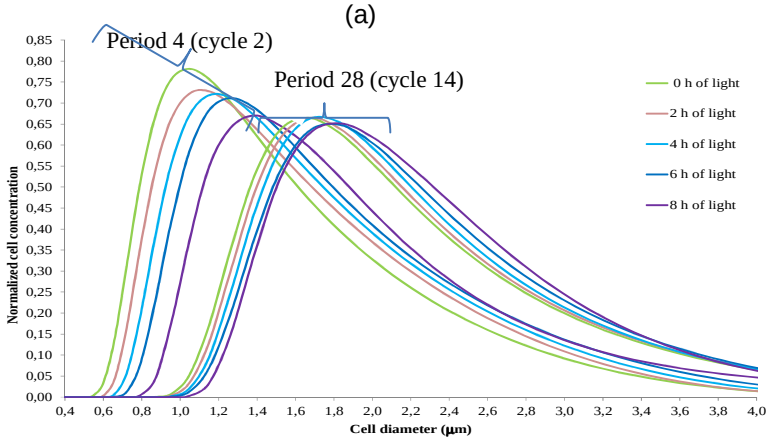
If each period is analyzed separately, the same trend becomes apparent: during the dark periods the size distribution shifts to lower diameters (Figure 4c) while during the light periods the opposite behavior occurs (Figure 4d). The fact that cell size was displaced to lower values in dark periods is consistent with the well-known behavior of microalgae, since cell division and cell respiration happens in the dark.

Figures 4c and 4d show the average cell diameter at the beginning and the end of all the dark cycles (4c) and at the beginning and the end of all the light cycles (4d). Figure 4c clearly shows this trend, the average cell diameter of the culture increases during the dark periods and this increases is lower as the number of cycle's increases. Contrarily, the cell diameter increases during the light periods, showing the effect of the CO_2 uptake on, and this increase is lower as the number of cycles increases. Reference [9] suggested that the variation of size distribution during the growth process is probably related to the evolution of the colony with time in the same way that diurnal changes in cell diameter may be attributable to the daily growth process. Nevertheless, they presented data involving only a single measurement per cycle and could not make deeper inferences. In the present work, cell size distribution was measured at different times during each period, which made it possible to track the evolution of the distribution and to correlate it with cell respiration, division and individual cell growth processes.

On the other hand, it can be clearly observed (see Figures 4c and d) that the distribution shifted to larger cell diameters during the overall process. This fact is in agreement with the results obtained in previous studies where N-limitation leads to an increase in individual cell volume [21]-[23].In this experiment, nitrates were exhausted after 192 hours.



(a)



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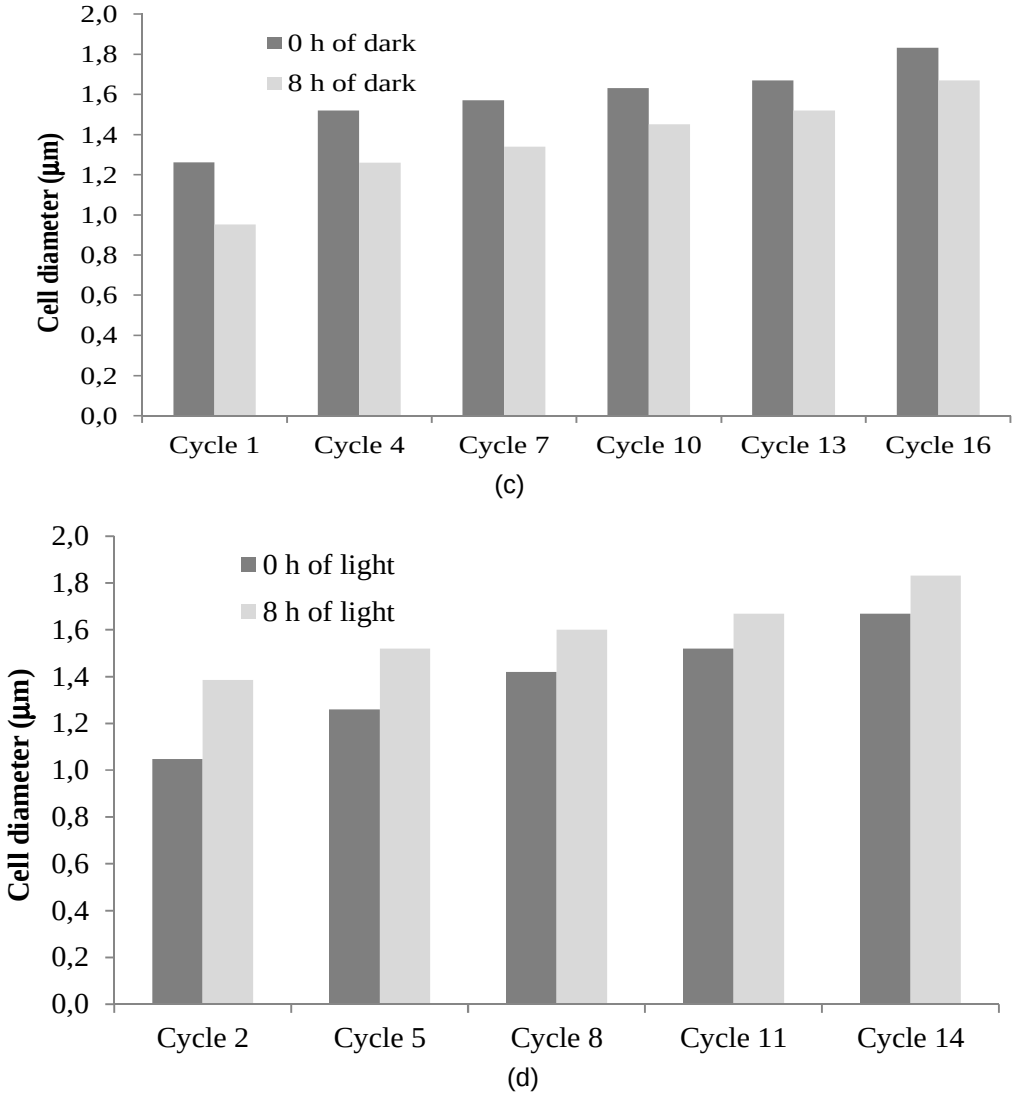

Figure 4: (a) Time evolution of the normalized cell concentration of *N. gaditana* used in the growth experiment for samples harvested in two dark periods: 1 (Cycle 1, dark) and 31 (Cycle 16, dark). (b) Time evolution of the normalized cell concentration of *N. gaditana* used in the growth experiment for samples harvested in two light periods: 4 (Cycle 2, light) and 28 (Cycle 14, light). (c) Cell diameter values of highest abundance for the dark periods of each cycle. (d) Cell diameter values of highest abundance for the light periods of each cycle.

Reference [9] also observed no variation in the type of size distribution during the growth of the phytoplankton species *Chaetoceros curvisetus, Skeletonema costatum, Phaeodactylum tricornutum, Platymonas helgolandic,* and *Heterosigma akashiwo*. However, *Gymnodinium sp.* and *Prorocentrum micans* first exhibited a normal distribution with a single-peak in the lag growth stage and then shifted to a double-peak in the exponential stage. In spite of this fact, none of the distributions obtained by [9] exhibited positive skew and the size distribution characterized by a single peak could be well described by the original. Gaussian distribution function and double-peaked curves by a combined Gaussian distribution. In the case of *N. gaditana*, we are not aware of other works concerning size distribution during the growth process. The cell size distribution curves are not symmetric and exhibit positive skew. Figure 5a shows, as an example, the CSD obtained at the initial time, as well as the fit provided by the normal Gaussian distribution (Equation 3) and a log normal distribution (Equation 4).

$$n(d(\theta(i))) = \frac{1}{d(\theta(i))\sigma\sqrt{2\pi}} e^{-\frac{(Ln\, d(\theta(i)) - d(\theta(i))_a)^2}{2\sigma^2}} \qquad (4)$$

It can be observed in Figure 5a that Gaussian distribution provides a poor fit. The log normal distribution is capable of reproducing asymmetric curves [24], [25], and provides an improved fitting with respect to the Gaussian, nevertheless, the fit provided is not suitable for our purposes of simultaneously modelling the cell division, respiration and individual cell growing processes in addition to the evolution culture cell size distribution with time. Consequently, it is very important to have a very

accurate equation to represent the cell size distribution as a function of those processes. Such a function and the procedure to handle the cell size distribution is shown in section A.1.2. Figure 5b, shows the highly satisfactory fit (R^2= 0.9988) obtained when such procedure is applied. This enables further calculations that are part of the suggested kinetic model, as can be deduced from all the obtained fits, many of which are shown in Figures 7 and 8.

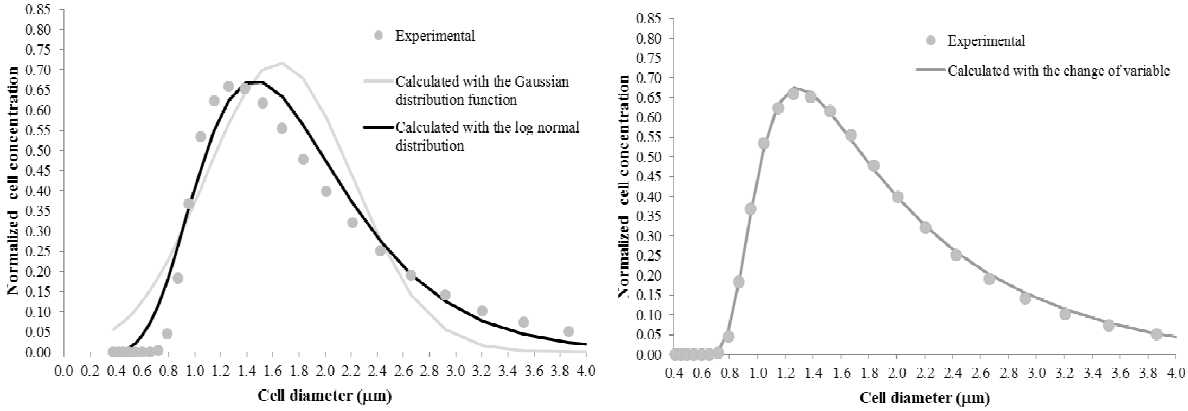

Figure 5: Kinetic analysis of the *N. gaditana* cell size distribution. (a) Using the Gaussian distribution function (Equation 3) and log normal distribution function (Equation A.10). (b) Using the change of variable proposed in Equations A.2-6.

Kinetic modeling of the dark period distribution

The suggested kinetic model is described in section A.1.3. Figure 6 shows the results obtained if only the division process was taken into consideration during the dark period, in other words using a zero respiration factor (f_r (θ(i)) =0 It can be seen that the reduction in cell diameter as a result of the process of division is lower than that experimentally observed, i.e.: the calculated curve cannot attain the smaller diameters obtained experimentally. Therefore, the division process alone is not able to reproduce the evolution of cell size distribution with time.

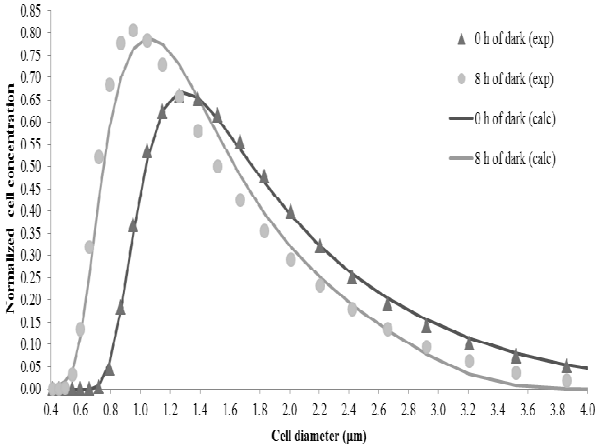

Figure 6: Example of the fit obtained when only the division process is considered in the kinetic model

Reference [26] observed in experiments carried out in a tubular photobioreactor on Spirulina Platensis that the rates of nocturnal biomass loss ranged from 5-7.6 % depending on the temperature. Reference [27], for the algae Coelastrum sphaericum and Scenedesmus falcatus, estimated that the overall loss in biomass during 12 h of darkness was between 2–10% of the biomass prior to darkening. Reference [28] reported that, under optimal operational conditions, the diurnal respiration loss by the microalga Isochrysis galbana averaged 35% of the daylight photosynthesis. Reference [29] estimated the night loss by Spirulina cultures grown in a tubular bioreactor outdoors to be from 12 to as much as 42 percent of daylight productivity. According to [30] night biomass loss by Chlorella sp. grown in their thin-layer bioreactors was 9–14%. Thus, in accordance with these observations and

given the lack of success incorrelating our experimental data by only considering the division process, the respiration process undergone by cells during dark periods must be included (See section A1.3.1).Figure 7 shows the results obtained for selected dark cycles, as examples of all obtained, indicating the very agreement obtained when considering the occurrence of both processes simultaneously and their effect on the CSD.

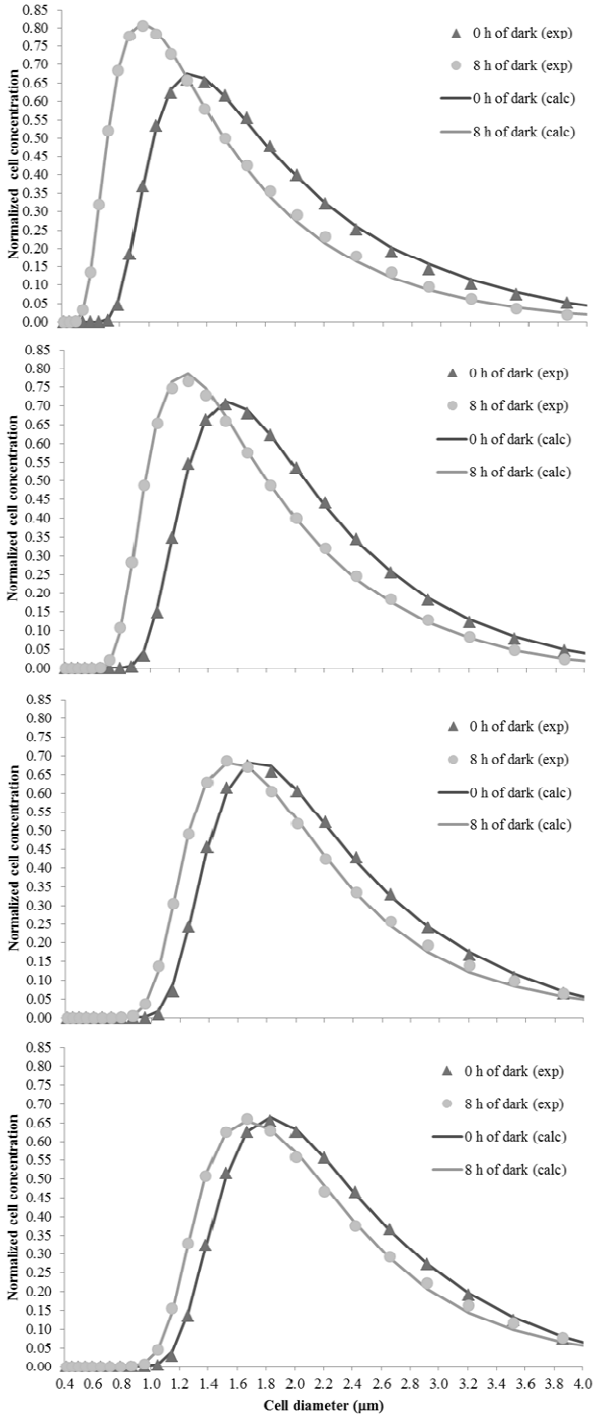

Figure 7: Results of the kinetic model for cell size distribution for the dark period; solid lines represent the results fitted by the proposed model, from top to bottom: periods 1, 7, 25 and 31.

Table 2 shows values of the division and respiration factors obtained for each sample. If the values of these factors are analyzed separately for each cycle, it can be seen that the respiration factor decreases with time during the dark period.

Table 2: Results of fitting the cell size distribution for dark periods.

t(h)	Cycle	Period	θ (h)	f_d exp·10^{-5}(a)	f_d calc·10^{-5}(b)	f_r calc·10^2 (c)	Objective function
2	1	1	2	5.02	4.74	5.59	0.007
4			4	3.44	4.87	5.56	0.008
6			6	5.58	4.99	5.53	0.010
8			8	7.44	5.11	5.49	0.010
50	4	7	2	7.36	7.29	4.81	0.003
52			4	3.52	7.37	4.78	0.006
54			6	7.36	7.45	4.74	0.006
56			8	8.64	7.53	4.71	0.011
194	13	25	2	11.10	8.76	2.46	0.004
196			4	9.27	8.72	2.43	0.004
198			6	9.27	8.67	2.39	0.005
200			8	7.42	8.62	2.36	0.006
242	16	31	2	8.98	7.20	1.67	0.001
244			4	3.37	7.11	1.64	0.003
246			6	6.73	7.02	1.61	0.004
248			8	7.85	6.93	1.58	0.005

These results are in agreement with those obtained by [31], [32]. They reported that the biomass concentration strongly influences the nocturnal loss in biomass. It was concluded that this parameter depended on the temperature and irradiance experienced by cells the preceding day, as a result of significant effects of both temperature and irradiance on cell composition. Cells that are strongly irradiated direct biomass synthesis towards carbohydrates, which can serve as a store of excess reducing power. However, at night carbohydrates are lost due to respiration, which increases the loss in biomass during the night.

Kinetic modeling during the light period

The kinetic model of the evolution with time of the cell size distribution during the light periods is described in section A.1.3.2. The results obtained for samples after 8 hours in the light period of selected cycles, as examples of all cycles, are shown in Figure 8 where the quality of the fitting obtained can be observed.

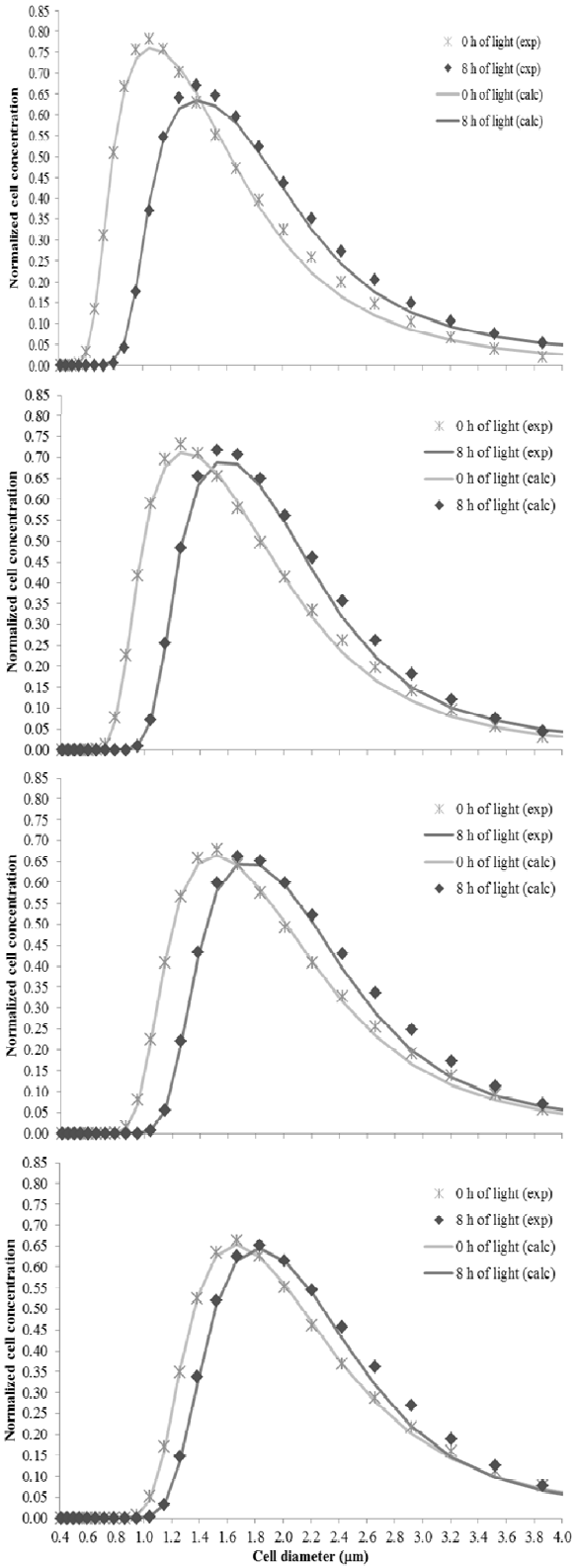

Figure 8: Results of the kinetic model for cell size distribution for the light period; solid lines represent the results fitted using the growth factor and the experimental points, from top to bottom: periods 4, 10, 22 and 28.

Table 3 lists the obtained growth factor values of each sample after every light period for which samples were harvested. It can be observed that the growth factor decreases with time during the light period.

Table 3: Results of fitting the cell size distribution for light periods

t(h)	Cycle	Period	θ (h)	f_g calc·$10^{2(a)}$	Objective function
26	2	4	2	6.02	0.053
28			4	5.99	0.050
30			6	5.95	0.051
32			8	5.91	0.047
74	5	10	2	5.11	0.033
76			4	5.07	0.031
78			6	5.03	0.033
80			8	5.00	0.038
170	11	22	2	3.28	0.025
172			4	3.25	0.025
174			6	3.21	0.027
176			8	3.17	0.030
218	14	28	2	2.37	0.019
220			4	2.33	0.019
222			6	2.30	0.021
224			8	2.26	0.024

[(a)]Obtained from the fitting of the experimental values of the cell size distribution by Equations A.6 and Equation A.1.

Additionally, based on the values of the division, respiration and growth factors it is possible to predict the evolution of the average cell diameter with time. Figure 9shows the values of the time evolution of the cell diameter obtained experimentally, and calculated using the values of the division, respiration and growth factors given by the proposed model. Very good agreement has been achieved: the model reproduces the observed experimental behavior, that is, the average cell diameter decreases during dark periods and increases during light periods.

However, these variations become smaller with culture time. Moreover, the average cell diameter increases over the whole growth process due to nitrogen limitation, a fact alreadymentioned in the literature [28]-[30].

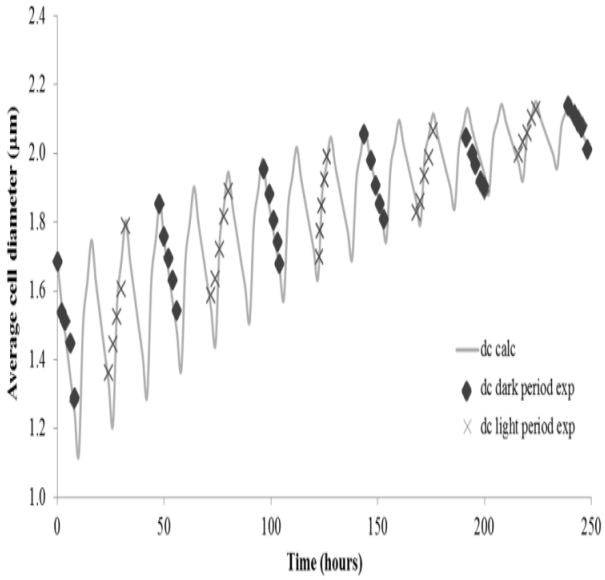

Figure 9: Time evolution of the average cell diameter over the whole experiment; the solid line represents the results obtained using the values of the division, respiration and growth factors given by the proposed model.

To sum up, in this paper it has been successfully proposed:

- A modification of the Logistic model where only the time in the dark period is considered, in order to obtain the time evolution of cell concentration since it is only in this period that cell division takes place and, as a consequence, the number of cells per unit volume is modified.
- A discontinuous model of cell concentration in which the number of cells per unit volume in light periods are taken to be constant, while increasing according to the modified Logistic model proposed for dark periods.
- A modification of the Gaussian distribution including a change of variable to adequately fit the CSD curves, in order to enable the interpolation and combination of the curves needed by the proposed kinetic model of the time evolution of CSD.
- A kinetic model of the evolution of CSD with time that takes into account the division and respiration processes in dark periods and growth process in light periods, since by the division process alone it is not possible to reproduce the experimental behavior observed. Furthermore, knowledge of the time evolution of the CSD curves allows obtaining the average cell diameter as a function of the culture time.

Despite its apparent complexity, the model is still too simple to explain the complex behavior of the microalgae under different conditions of temperature, light intensity, nutrient quality and concentration. Nevertheless, the procedure proposed and the results obtained may provide a deeper insight into the studied cell culture, yielding a more complete picture than the Logistic model or other growth models can provide. Since both cell concentration and cell size distribution are fitted simultaneously, thereby obtaining information that would not be obtained by adjusting cell concentration or considering the process of division alone. This point of view and information are very useful and provide a starting point for the analysis and optimization of the length of the light and dark cycles, the choice of which depends on the desired application of the microalgae. To our knowledge, this is the first study in which the simultaneous fitting of the time evolution of cell concentration and size distribution has been attempted.

CONCLUSIONS

A significant improvement over the results produced by the broadly used Logistic growth model has been achieved by considering only the time in the dark period instead of the total time.

A kinetic model for simultaneously correlating the time evolution of cell number and cell size distribution has been suggested and successfully applied. Consideration of the division process by itself did not reproduce the evolution of the observed cell size distributions. A satisfactory fit of the curves of the time evolution of the cell size distribution was obtained when a kinetic model was considered that included a respiration process for the cells during the dark period and a growth process during the light period. This model provides a closer approximation to reality than the Logistic model, which only takes into account the cell concentration. With the quantification that allows the kinetic model it is possible to predict the time evolution of the culture population and cell size distribution what is useful for designing and scaling up the system.

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APPENDIX

A.1. KINETIC MODEL DESCRIPTION

A.1.1. Cell concentration

A discontinuous model was proposed for calculating the growth rate and fitting the obtained experimental curves of cell concentration vs time. During light periods, the cell concentration is supposed to remain constant, while during dark periods, it can be calculated using a slightly modified version of the Logistic model that entails considering only the dark period in the functional dependence of the cell concentration on time.

$$\text{Light periods: } \frac{dN(t)}{dt}=0 \quad \ldots(A.1a)$$

$$\text{Dark periods: } N(t) = \frac{N_f}{1+\frac{N_f-N_o}{N_o}e^{-\frac{4\mu_{max}\cdot\tau(t)}{N_f}}} \quad \ldots(A.1b)$$

where $\tau(t)$ is calculated as the cumulative time that the culture has remained under dark conditions up to time t, N_o is the experimentally measured initial cell concentration, and μ_{max} and N_f are the model parameters obtained by fitting all the experimentally determined N(t) excluding N_o, which is taken to equal the experimental value. Note that the constant N(t) obtained in each light period (corresponding to Equation A.1a) is different in each period and equal to the final N(t) corresponding to the preceding dark period. A diagram of the procedure followed to obtain the kinetic parameters for fitting the evolution of cell concentration with time is shown in Figure A.1.

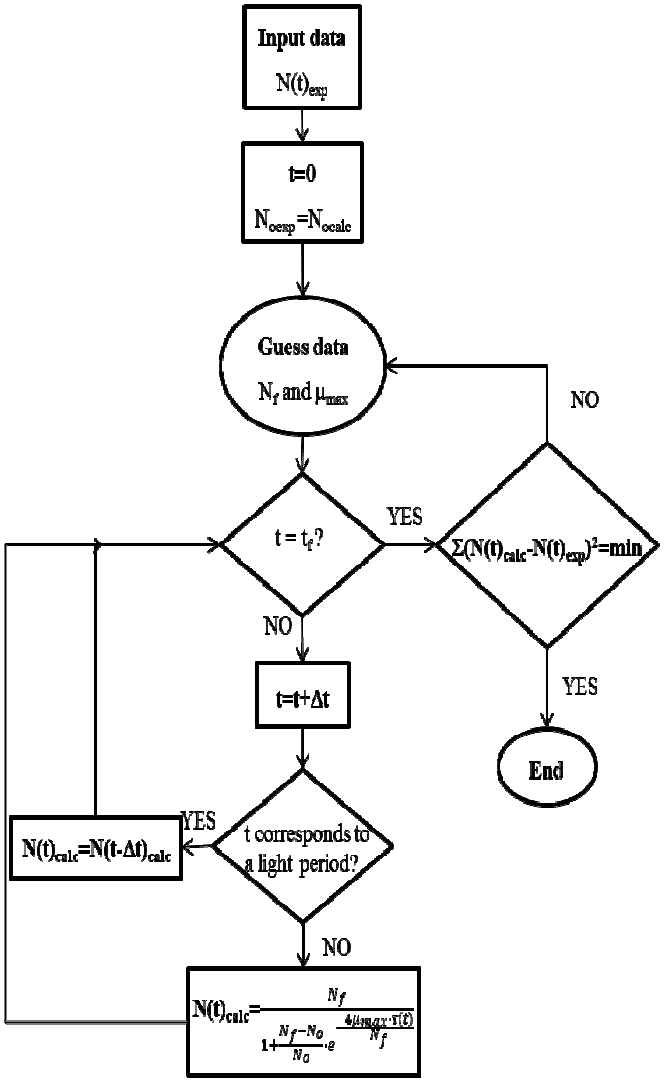


Figure A.1: Diagram of the procedure followed to obtain the kinetic parameters for fitting the evolution of the cell concentration with time.

A.1.2. Cell size distribution curves

The CSD curves at each time t were obtained after first normalizing the cell concentration by dividing the cell concentration of each cell equivalent spherical diameter d(θ(k)) by the area under the corresponding CSD curve.

The suggested overall algorithm for determining the curves of the evolution of CSD with time involved calculating linear combinations of CSD curves. This was done in order to obtain CSD curves after a period of time in which division, respiration or growth processes may have taken place. Several typical basis functions that are frequently used in the representation of data

such as CSDs were tried. Nevertheless, the obtained results were not satisfactory enough to enable suitable calculations to be done. Consequently, because a good fit of the CSD curves is the objective, a change of variable was proposed. The Gaussian distribution function of this new variable permitted attainment of an adequate fit of the CSD curves, which are needed to execute the suggested algorithm. It also provides a mean which makes it somewhat easier to handle the curves. The Gaussian distribution was redefined as a function of the new variable "α(d(θ(k)))" as:

$$n(d(\theta(k))) = A_t \cdot e^{-B_t \cdot (\alpha(\mathrm{d}(\theta(\mathrm{k}))) - C_t)^2} \quad \text{... (A.2)}$$

Where A_t, B_t and C_t are parameters to be optimized for the sample harvested at time t. Equations A.3-6 define the variables in Equation A.2:

$$x(\mathrm{d}(\theta(\mathrm{k}))) = \frac{d(\theta(\mathrm{k})) - d_s(\theta(\mathrm{k}))}{d_l(\theta(\mathrm{k})) - d_s(\theta(\mathrm{k}))} \quad \text{...(A.3)}$$

$$z_1(\mathrm{d}(\theta(\mathrm{k}))) = \frac{x(\mathrm{d}(\theta(\mathrm{k})))}{x(\mathrm{d}(\theta(\mathrm{k}))) + D_t(1 - x(\mathrm{d}(\theta(\mathrm{k})))) + E_t x(\mathrm{d}(\theta(\mathrm{k})))(1 - x(\mathrm{d}(\theta(\mathrm{k}))))} \quad \text{...(A.4)}$$

$$z_2(\mathrm{d}(\theta(\mathrm{k}))) = \frac{1 - x(\mathrm{d}(\theta(\mathrm{k})))}{1 - x(\mathrm{d}(\theta(\mathrm{k}))) + F_j\left(1 - x\left(\mathrm{d}(\theta(\mathrm{k}))\right)\right) + G_j x(\mathrm{d}(\theta(\mathrm{k})))\left(1 - x\left(\mathrm{d}(\theta(\mathrm{k}))\right)\right)} \quad \text{...(A.5)}$$

$$\alpha(\mathrm{d}(\theta(\mathrm{k}))) = \frac{z_1(\mathrm{d}(\theta(\mathrm{k})))}{z_1(\mathrm{d}(\theta(\mathrm{k}))) + z_2(\mathrm{d}(\theta(\mathrm{k})))} \quad \text{...(A.6)}$$

where $\mathrm{d}(\theta(\mathrm{k}))$, $d_s(\theta(\mathrm{k}))$ and $d_l(\theta(\mathrm{k}))$ are the cell diameter, the smallest cell diameter and the largest cell diameter after a time $\theta(\mathrm{k})$ in period k, respectively, and D_t, E_t, F_t and G_t are parameters to be optimized, and $x(\mathrm{d}(\theta(\mathrm{k})))$, $z_1(\mathrm{d}(\theta(\mathrm{k})))$ and $z_2(\mathrm{d}(\theta(\mathrm{k})))$ are intermediate variables. This set of equations produced a very good fit of the obtained CSD curves (as can be appreciated from the R^2 value yielded by this procedure and shown in the results section). These functions serve no other purpose than to allow interpolation and combination of the size distributions obtained at different times. Other functions or interpolating procedures can be employed for this purpose if the results are as good as those obtained by the proposed modified Gaussian function used in the present work. A diagram of the procedure that was followed to obtain the parameters for fitting the CSD curves is shown in Figure A.2.

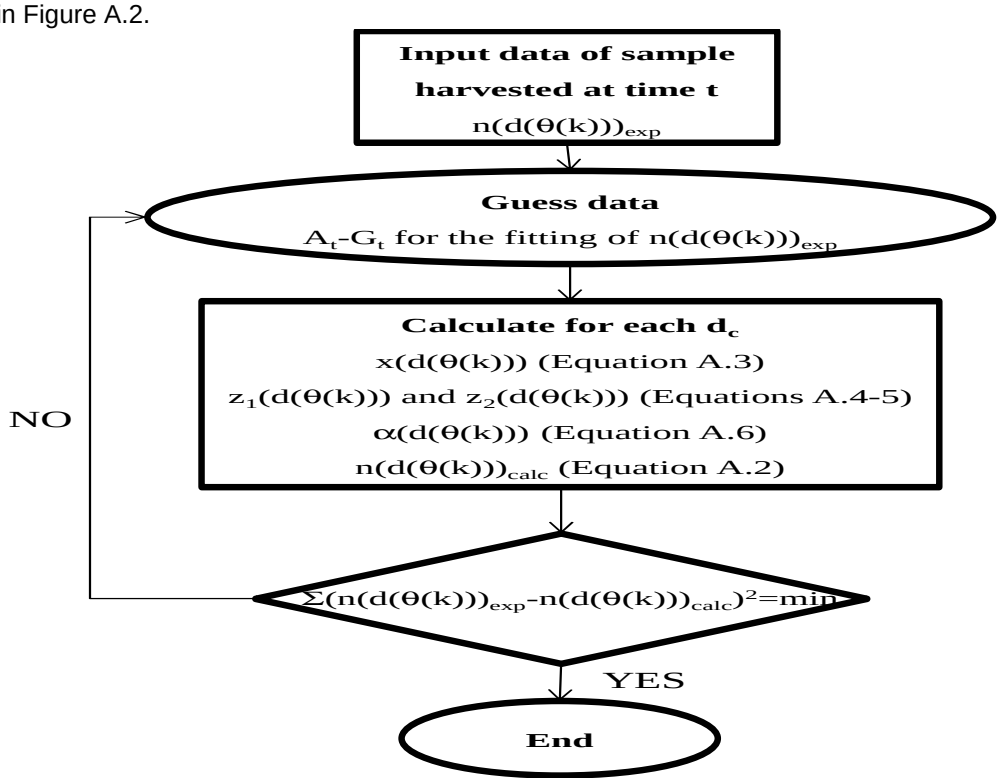


Figure A.2: Diagram of the procedure followed to obtain the kinetic parameters for fitting the cell size distribution curves.

A.1.3. Cell size distribution evolution with time

With the aim of obtaining a deeper insight into microalgal growth processes, the evolution with time of CSD curves was modeled to explain the experimental observations during both dark and light periods. Thus, a kinetic model was developed that takes into account three processes affecting cell concentration and size:

- cell division, affecting both cell size and concentration
- respiration, affecting only the size of the cells
- Growth, also affecting only the size of the cells.

In a first attempt, only cell division and growth were considered in the model, but the results obtained (only one example has been included to illustrate this point) were not satisfactory enough (Figure 5). Consequently, the process of respiration was also taken into account in the proposed model. This permitted an adequate fit of the CSDs and cell evolution, as it is shown in the results section (Figure 6).
Obviously, all cells respire; nevertheless, and in order to keep the model simple, the respiration process was included in the model only for undivided cells.

The CSD curve at time t was calculated from the curve obtained at the initial time of the period in which the sample was collected. Therefore, the factors that characterize the different processes are defined as a function of the time elapsed since the beginning of the period k ($\theta(k)$) and the average derivatives instead of the instantaneous derivatives were used in order to reduce the effect on the final fitting of the dispersion in such derivatives. To summarize, the proposed kinetic model is based on the following assumptions: The division process is characterized by the division factor $f_d(t)$ at time t, which is defined as:

$$f_d(\theta(i)) = \frac{N(t) - N_o(i)}{\theta(i)} \qquad \text{...(A.7)}$$

Where N(t) is the cell concentration at time t, $N_o(i)$ is the cell concentration at the start of the dark period i, corresponding to time t, and $\theta(i)$ is the time elapsed since the beginning of that period i. As a consequence of the respiration process, a decrease in cell sizes occurs in every dark period. As mentioned earlier, it has been assumed that only undivided cells decrease in size as a function of the associated dark period respiration factor $f_r(\theta(i))$, which was taken as constant over each interval of time between samples and defined according to the following expression:

$$f_r(\theta(i)) = \frac{d_o(i) - d_u(\theta(i))}{\theta(i)} \qquad \text{...(A.8)}$$

Where $d_o(i)$ represents the CSD diameters considered at the start of the dark period (i), $d_u(\theta(i))$ corresponds to the diameters of the undivided cells and $\theta(i)$ is the time elapsed since the beginning of that period i. In a similar way, as a consequence of the growth process, an increase in cell sizes occurs in every light period. Thus, in order to calculate the resulting CSD after a period between samples during a light period, in which a growth process has taken place, it has been assumed that all cells increase their size according to the expression that follows below. It is a function of the corresponding light period growth factor $f_g(\theta(j))$, which was considered constant over each interval of time between samples:

$$f_g(\theta(j)) = \frac{d_g(\theta(j)) - d_o(l)}{\theta(j)} \qquad \text{..... (A.9)}$$

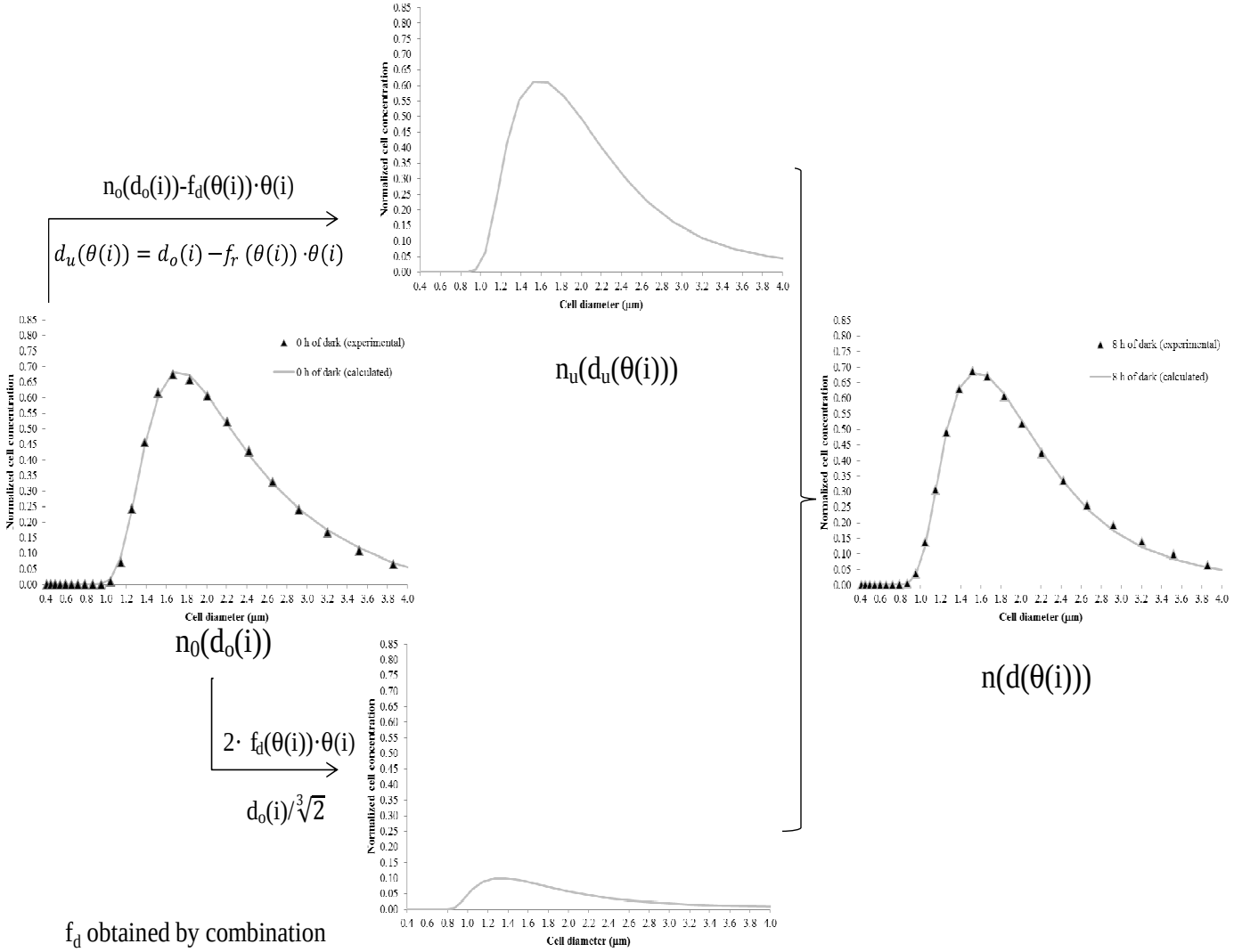
where $d_o(j)$ represents the CSD diameters considered at the start of the light period j, $d_g(\theta(j))$ is the diameter of the cells after the corresponding growth process that takes place during that light period, and $\theta(j)$ is the time elapsed since the beginning of that period j.

- Cell death is not accounted for since dead cells remain in the culture and are also counted by the Neubauer chamber and the Coulter counter and experimental data would not allow validating a model of cell death. However, a more realistic model should include it since dead cells do not undergo growth, division or respiration and this fact has a significant importance for very long cultures.

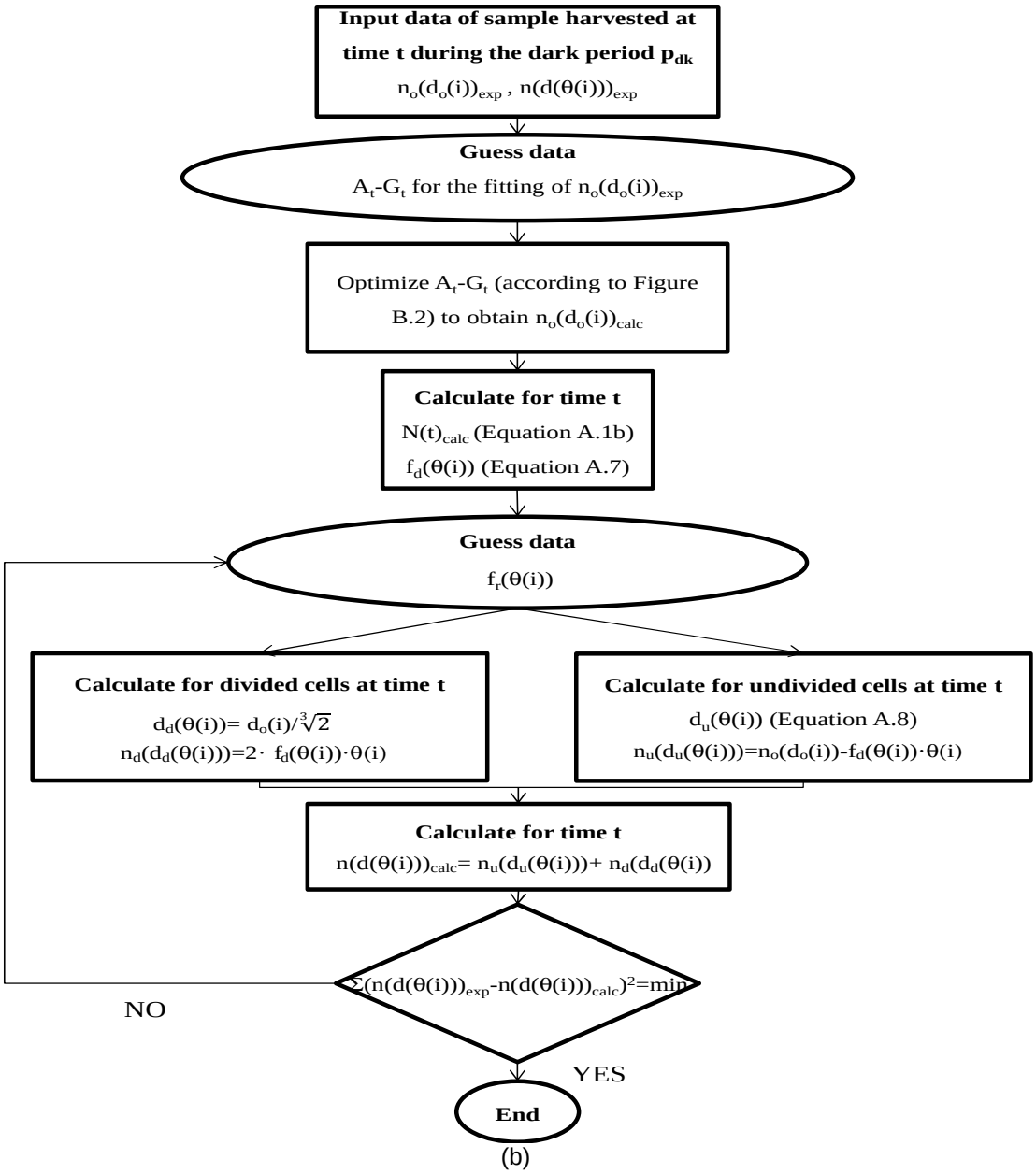
A.1.3.1. Cell size distribution evolution during dark periods

Figure A.3a shows an example of the procedure followed in the case of a dark period. The CSD of the sample harvested at the beginning of the period ($n_0(d_o(i))$ vs $d_o(i)$)is used to calculate the CSD curve obtained after a time $\theta(i)$ ($n(d(\theta(i)))$ vs $d(\theta(i))$) after taking into account that a fraction of the original number of cells has divided, so that their final volume is half their initial volume, and also that the undivided cells shrink as a result of the respiration process. The sum of these two fractions yields the CSD curve obtained after a time $\theta(i)$.

Figure A.3b details step by step the calculations outlined in Figure A.3a for the dark periods.



(a)



(b)

Figure A.3: Outline of the procedure followed to obtain the kinetic parameters for fitting the evolution of cell size distribution with time during dark periods. (a) Example of the results obtained by this procedure for a sample. (b) Diagram of the proposed calculation based on the model

NOMENCLATURE

$N(t)$	Cell concentration at time t (cells/mL)
N_0	Cell concentration at initial time(cells/mL)
N_f	Cell concentration at final time (cells/mL)
μ	Specific growth rate (h^{-1})
μ_{max}	Maximum growth rate (cell/mL h)
I	Dark period
J	Light period
K	i or j (light or dark period)
t	Time (h)
$\tau(t)$	Time that the culture has remained in the dark up to time t (h)
$\theta(k)$	Time elapsed since the beginning of the period k (h)
$d(\theta(k))$	Mean diameter inevery diameter interval measured by the Coulter Counter in the sample harvested after a time$\theta(k)$ in period k (μm)
$d(\theta(k))_a$	Average cell diameter of the sample harvested after a time$\theta(k)$ in period k (μm)
Σ	Standard deviation
OD	Optical density
$n(d(\theta(k)))$	Normalized cell concentration of $d(\theta(k))$
CSD	Cell size distribution ($n(d(\theta(k)))$ vs $d(\theta(k))$)
$f_d(\theta(i))$	Division factor for the sample harvested after a time$\theta(i)$ in period i
$f_r(\theta(i))$	Respiration factor for the sample harvested after a time$\theta(i)$ in period i
$f_g(\theta(j))$	Size increasing factor (due to CO_2 uptake) for the sample harvested after a time$\theta(j)$ in period j
$d_o(k)$	Diameter of the original cells at the start of the period k (μm)
$d_u(\theta(i))$	Diameter of the undivided cells after a time $\theta(i)$in period i (μm)
$d_g(\theta(j))$	Diameter of the cells after a time $\theta(j)$in period j (μm)
$d_d(\theta(i))$	Diameter of the divided cells after a time $\theta(i)$in period i (μm)
$V_d(\theta(i))$	Volume of the divided cells after a time $\theta(i)$ in period i (μm^3)
$V_o(i)$	Volume of the cells at the beginning of the dark period i (μm^3)
A_t-G_t	Parameters to be optimized to fit the CSD of the sample harvested after a time $\theta(k)$ in period k
$\alpha(d(\theta(k)))$	Variable defined for the Gaussian function that fits the CSD curves after a time $\theta(k)$ in period k
$x(d(\theta(k)))$	Intermediate variables defined to fit the CSD curves after a time $\theta(k)$ in period k
$z_1(d(\theta(k)))$	
$z_2(d(\theta(k)))$	