Aquaculture 448 (2015) 87–92

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Short communication

A simple spectrophotometric method for biomass measurement of important microalgae species in aquaculture

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ARTICLE INFO

Article history:
Received 13 October 2014
Received in revised form 27 May 2015
Accepted 29 May 2015
Available online 3 June 2015

Keywords:
Microalgae
Aquaculture
Calibration curve
Cell counting
Absorbance

ABSTRACT

The development of microalgae culture technology has been an integral part of the production of commercially important species in aquaculture. This has been the reason to make great efforts in order to understand several aspects of the microalgae production. The detailed research on monitoring cell growth, which is considered a fundamental part for the use of microalgae in aquaculture, has been, however, overlooked. In this paper, a calibration curve is proposed; correlating the specific absorbance and measured cell density by cell counting (cells mL^{-1}), from four microalgae species important for aquaculture. The final result allows the prediction of cell counting from absorbance using cells $mL^{-1} = \left\{\left[\beta_1^2 - 4\beta_2(\beta_0 - \mathrm{Abs})\right]^{1/2} - \beta_1\right\}/(2\beta_2)$ which is valid for $\mathrm{Abs} < \beta_0 - \beta_1^2/(4\beta_2)$. The adjusted determination coefficients (r^2_{adj}) were 0.998, 0.995, 0.991, and 0.998 for *Chaetoceros calcitrans*, *Isochrysis* affinis *galbana* (T-Iso), *Nannochloropsis gaditana* and *Phaeodactylum tricornutum*, respectively. The results showed that the obtained equations can be used with an error of less than 5% for microalgae densities of up to 1.01×10^7 cells mL^{-1}, 2.11×10^7 cells mL^{-1}, 8.52×10^7 cells mL^{-1}, and 1.67×10^7 cells mL^{-1} in the abovementioned species, respectively.

Statement of relevance: A rapid, simple and specific spectrophotometric methodology for biomass measurements of four microalgae species with high importance in aquaculture was developed. The specific wavelength for the maximum absorbance was established for each of the studied species. With the methodology described here, the harvest time will be easier to identify in facilities where these algae are cultured, and can be also easily tested in other monocultures. In addition, a predictive model was satisfactory and useful for a wide range of cell densities yielding a methodology with high potential for automation. A useful tool was developed for cell counting of microalgae in aquaculture.

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1. Introduction

Microalgae represent the first nutritional base on the aquatic food chain due to their ability to synthesize organic molecules using sunlight and carbon dioxide (Fuentes-Grünewald et al., 2012). Thus, the cultivation of microalgae has been an integral part for the production of commercially important species on aquaculture. Microalgae could be used as live food for various stages of growth in marine filter feeders (Ferreira et al., 2008), as food for larvae of some gastropods (during their juvenile stages), and also as food for some crustaceans and some fish species in its earliest growth stages (Brown and Robert, 2002). Microalgae are also used as indirect food when used in the zooplankton production (i.e. artemia and rotifers), essential food for several carnivorous larvae (Welladsen et al., 2014). Other application of microalgae in aquaculture is the so-called "green water", wherein the microalgal biomass is grown simultaneously in tanks with larvae (Brown et al., 1997). Table 1 shows the main microalgae species used in aquaculture and their main uses. In recent decades, a large number of microalgae species have been studied as a source of nutrients in aquaculture, but only a limited number of these have been established (Guedes and Malcata, 2012). The microalgae must have specific characteristics such as the availability of the strain, easiness of cultivation, high growth rates, physical characteristics of cell (such as the proper size for ingestion, depending on the organism), nutritional composition, easy digestibility and the absence of toxins or irritants in the different growth stages (Brown and Robert, 2002). One of the main difficulties associated with the microalgae in aquaculture is its high operating cost, which can

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http://dx.doi.org/10.1016/j.aquaculture.2015.05.044
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Table 1
Principal microalgae species used in aquaculture, either as individual diets or components of mixed diets (++ means more popular than +).
Adapted from Brown and Robert (2002) and Guedes and Malcata (2012).

	Bivalve molluscs	Crustacean larvae	Juvenile abalone	Zooplankton
Amphora spp.			+	
Chaetoceros calcitrans	++	++		+
Chaetoceros gracilis	++	++		+
Chlorella	+	+		++
Dunaliella spp.	+	+		+
Haematococcus pluvialis	+	+		+
Isochrysis affinis *galbana* (T-Iso)	++	+		++
Nannochloropsis atomus	++			++
Nannochloropsis gaditana	++			++
Nitzschia spp.		+	++	
Pavlova lutheri	++	+		++
Phaeodactylum tricornutum			+	++
Pyramimonas spp.	+			
Rhodomonas spp.	+			
Skeletonema spp.	+	++		
Tetraselmis chuii	+	+		++
Tetraselmis suecica	+	+		++
Thalassiosira pseudonana	+	+		

cover almost half of the economic cost of a plant of fish cultures (Guedes and Malcata, 2012). For this reason, great efforts have been made in research, development and commercialization of several aspects of microalgae production processes (Sevigné-Itoiz et al., 2012). However, even though the monitoring and control of cell growing are an essential step in the microalgae technology, its detailed investigation has been largely ignored (Havlik et al., 2013).

The growth control of the microalgae culture is very important in aquaculture. Key decisions on the process conditions such as light intensity, temperature control, and nutrient concentration, must be taken in time to prevent economic losses (Brown et al., 1997). For this reason, the development of simple and efficient systems for monitoring the cell growth is very important to achieve commercial viability, and these systems must possess characteristics such as easiness, speed, stability, selectivity, besides infrequent calibrations (Havlik et al., 2013). Within the parameters used to analyze cell growth, biomass measuring is a basic tool, used as an indirect measure for product formation, substrate consumption and process disturbances (Li et al., 2014). However, traditional direct measurements are performed by dry weight determinations or by cell counting using a microscope; being both tedious and time consuming (Ribeiro-Rodrigues et al., 2011).

The use of optical density (OD) is an indirect method widely used for non-invasive measurement of microalgae biomass, because it can be directly correlated with the number of cells in the medium and is easily adaptable to automated measurement systems, representing a useful tool in the monitoring and control of microalgal biomass growth (Ribeiro-Rodrigues et al., 2011). Although spectrophotometry is used in aquaculture, it is applied in a very general fashion. A linear dependence between absorbance and cell counting is assumed, measuring absorbance at the same wavelength (680 nm) for all species (Havlik et al., 2013).

The aim of this paper was performing growth kinetics for some key microalgae species used in aquiculture, and to develop a predictive model for estimating cell counting (cells mL^{-1}), using spectrophotometric absorbance. In addition, the wavelength of maximum absorbance for each experimental species was determined, providing a fast, efficient, selective and stable method for measurement microalgal biomass.

2. Material and methods

2.1. Cell material

The following microalgae species were used: *Chaetoceros gracilis*, *Isochrysis* affinis *galbana* (T-Iso), *Nannochloropsis gaditana*, and *Phaeodactylum tricornutum*. They were obtained from the microalgae collection of Marine Culture facilities of Facultad de Ciencias Marinas y Ambientales, Universidad de Cádiz.

2.2. Growth conditions

The microalgae were cultivated using the medium reported by Guillard and Ryther (1962), with natural seawater (average pH and salinity between 7.3–7.45 and 34–35 g L^{-1}, respectively). Cultures were grown at (19 ± 2 °C) in 10 L methacrylate containers. Microalgae cultures were subjected to a 24 h light condition. Illumination was provided by four fluorescent lamps (Sylvania Gro-Lux F30W/GRO-T8), with an intensity of 6000–6500 lx, generating an active radiation of 120–130 μmol photons m^{-2} s^{-1}. Filtered air was supplied continuously through a 1 μm cut-off cartridge and using a blower (2.5 hp) with an average flow of 50 cm^3 s^{-1}.

2.3. Maximum absorbance determination

For each analyzed microalgae species, the maximum absorbance was inspected by scanning sample cultures between 550 and 800 nm, using a UV–visible spectrophotometer (Jasco V-630, USA). The maximum absorbance value for each microalga was used to perform the growth curve by optical density (OD) (Ribeiro-Rodrigues et al., 2011).

2.4. Cell growth kinetics

Each of the four microalga species was grown separately; cell growth was analyzed and daily measurements were performed by triplicate, until cultures reached their death phase. Two analytical methods were used: (1) cell counting; by using a Thoma chamber and microscope (Olympus CX40, NY, USA) and determining cell density as cell number per milliliter (cells mL^{-1}) (Godoy-Hernández and Vázquez-Flota, 2006); and (2) spectrophotometric absorbance, by measuring the absorbance of cell suspensions using a UV–visible spectrophotometer (Jasco V-630, USA) (Mikschofsky et al., 2009).

2.4.1. Cell growth efficiency

Using the growth kinetics, specific growth rate and duplication time were calculated (Godoy-Hernández and Vázquez-Flota, 2006):Specific growth rate:

$$\mu = \frac{\ln X - \ln X_0}{t}. \quad (1)$$

Duplication time:

$$dt = \frac{\ln 2}{\mu} \quad (2)$$

wherein: μ, specific growth rate; dt, duplication time; X_0, initial biomass concentration; X, final biomass concentration and t, time (days).

2.5. Predictive models

The adjusted determination coefficient (r^2_{adj}) was obtained for absorbance (abs) and cell counting (cells mL^{-1}) data, using the Graphpad Prism 5.0 software. Predictive models were developed between spectrophotometric absorbance and cell counting using:

$$\text{Abs} = \beta_0 + \beta_1\left(\text{cells } mL^{-1}\right) + \beta_2\left(\text{cells } mL^{-1}\right)^2 \quad (3)$$

where the β's are the regression coefficients, estimated by a regression analysis, using the Graphpad Prism 5.0 software.

For validating the efficiency of the developed models for each microalgae species, the percentile deviation for the measured and

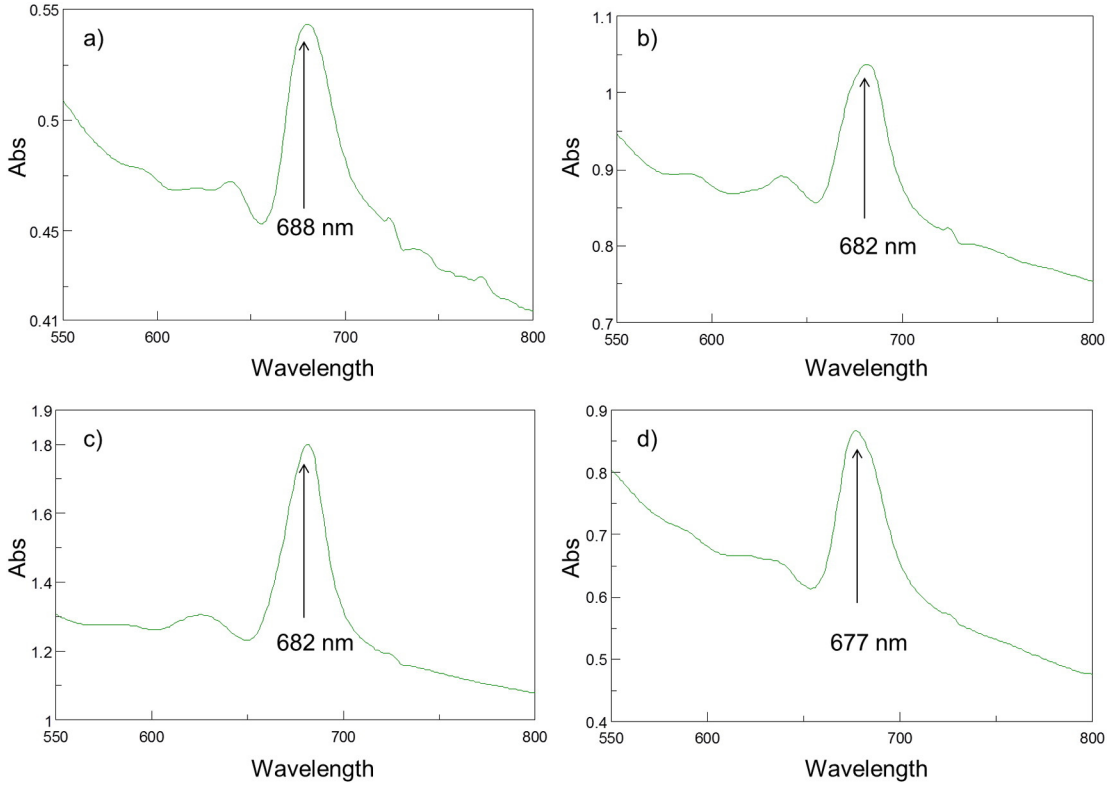

Fig. 1. Light absorbance patterns for different strains of microalgae scanned from 550 to 800 nm. a) *Chaetoceros calcitrans*; b) *Isochrysis* affinis *galbana* (T-Iso); c) *Nannochloropsis gaditana*; d) *Phaeodactylum tricornutum*. nm = nanometer.

predicted absorbances was calculated during the growth kinetics using the following equation (Rosas and Riveros, 1990):

$$\text{Percentile deviation} = \left(\frac{\text{Abs}-\text{Abs}_{\text{pred}}}{\text{Abs}}\right) \times 100. \quad (4)$$

3. Results and discussion

3.1. Microalgae maximum absorbance

The light absorbance patterns for different species of microalgae are shown in Fig. 1. Wavelength was scanned from 550 to 800 nm; the maximum absorbance was observed between 677 and 688 nm for the analyzed microalgae. The maximum absorbance was measured at different cellular growth stages, showing the same behavior in all experiments. The difference on the maximum absorbance for the microalgae species could be explained due to different contents of pigments, such as chlorophyll ("*a*" and "*b*") and carotenoids present in the cells (Bricaud et al., 1998). These results are similar to previous reports, where, for cell growth of some microalgae species, wavelengths were from 664 to 678 nm (Padovan, 1992), 680 nm (Geis et al., 2000), 684 nm (Ribeiro-Rodrigues et al., 2011), and 687 nm (Valer and Glock, 1998). Typically, in standard tests of microalgal growth by spectrophotometry, the 664–690 nm range is recommended, because these values are correlated with the absorbance of chlorophyll (Bricaud et al., 1998). Ebenezer and Ki (2013) analyzed the Pearson correlation coefficient, comparing the content of chlorophyll *a* and cell counting (cells mL^{-1}) for *Prorocentrum minimum* and *Tetraselmis suecica*, obtaining a very good positive correlation ($r = 0.92$ and $r = 0.99$, respectively).

3.2. Growth performance of microalgae species

Table 2 shows maximum density and absorbance obtained in the growth kinetics, and parameters for cell growth performance: specific growth rate, μ and duplication time, *dt*. The maximum absorbance was achieved at the end of the stationary phase. Previous works reported values of μ: 0.225–0.665 d^{-1} and *dt*: 3.080–1.042 d for *Chaetoceros calcitrans* (Phatarpekar et al., 2000), and values of μ: 0.33–1.97 d^{-1} and *dt*: 2.100–0.352 d for *I.* affinis *galbana* (T-Iso), obtained by optimizing growth conditions (Marchetti et al., 2012). Regarding *N. gaditana* and *P. tricornutum* values of μ: 0.2–1.44 d^{-1}, *dt*: 3.46–0.48 d, and of μ: 0.2–1.44 d^{-1}, *dt*: 3.46–0.48 d, respectively, have been also reported (Fuentes-Grünewald et al., 2012). It is clear that cell growth

Table 2
Growth performance of microalgae species.

	[max] (cells mL^{-1})	Maximum abs[a]	Days for [max] (d)	μ[b] (d^{-1})	*dt*[b] (d)
Chaetoceros calcitrans	$9.72 \times 10^6 \pm 7.82 \times 10^5$	0.9618 ± 0.0025	14	0.1464 ± 0.0183	4.7690 ± 0.5966
Isochrysis affinis *galbana* (T-Iso)	$1.74 \times 10^7 \pm 4.63 \times 10^6$	1.5453 ± 0.0034	25	0.3275 ± 0.0148	2.1186 ± 0.0962
Nannochloropsis gaditana	$8.83 \times 10^7 \pm 6.70 \times 10^6$	1.9481 ± 0.0019	23	0.2308 ± 0.0321	3.0326 ± 0.4223
Phaeodactylum tricornutum	$1.35 \times 10^7 \pm 1.27 \times 10^6$	1.1121 ± 0.0089	14	0.2930 ± 0.0043	2.3652 ± 0.0352

abs: absorbance; d: days; *dt*: duplication time; [max]: maximum density; mL: mililiter; μ: specific growth rate.
[a] Maximum absorbance at the corresponding wavelength for each microalgae species.
[b] Average from the growth kinetics developed using absorbance and cell counting.

Table 3
Regression coefficients for predictive models.

	$\beta_0 / 10^{-2}$	$\beta_1 / 10^{-7}$	$\beta_2 / 10^{-15}$	$\beta_0 - \beta_1^2 / (4\beta_2)$	rmse	r^2_{adj}
Chaetoceros calcitrans	2.991	1.446	−5.059	1.063	0.013	0.998
Isochrysis affinis *galbana* (T-Iso)	−4.053	1.148	−1.314	2.467	0.035	0.995
Nannochloropsis gaditana	12.77	0.4016	−0.2241	1.926	0.056	0.991
Phaeodactylum tricornutum	−1.582	1.063	−1.875	1.492	0.016	0.998

β_0, β_1, and β_2 are regression coefficients for Eqs. (3) and (5).
$\beta_0 - \beta_1^2 / (4\beta_2)$ is the maximum absorbance for the validity of the model.
rmse is the root of mean square error.
r^2_{adj} is the adjusted determination coefficient.

characteristics differ within the different species of microalgae. This may be explained because the microalgal cell growth can be impacted by several factors, including the reactor characteristics, culture conditions (light, nutrients, temperature, pH, aeration) and the physiological demands for the microalgae species (Guedes and Malcata, 2012). Despite this, the results obtained in this work are close to previous reports, corresponding with the proper behavior for growth kinetics and were used to obtain predictive models.

3.3. Predictive models

Table 3 shows the regression coefficients of the predictive models for abs and cell counting (cells mL^{-1}). Determination coefficients ranged from 0.991 to 0.998, indicating that measured density by cell counting (cells mL^{-1}) shows a high positive correlation with absorbance from the microalgae suspensions, explaining more than 90% of the variance (Rosas and Riveros, 1990).

Fig. 2 shows the relation between absorbance and cell counting for the studied microalgae species. In addition, the theoretical values calculated using the developed predictive models are represented by lines. Predictive models for cell counting (cells mL^{-1}) were developed as a function of absorbance, solving the obtained equations, at the correspondent wavelength for each microalgae species:

$$\text{Cells } mL^{-1} = \left\{ \left[\beta_1^2 - 4\beta_2(\beta_0 - \text{Abs}) \right]^{1/2} - \beta_1 \right\} / (2\beta_2). \quad (5)$$

This model is valid for $\text{Abs} < \beta_0 - \beta_1^2/(4\beta_2)$. At low cell densities, predictive values showed deviations from experimental ones (Fig. 2).

Fig. 3 shows the absolute value of percentile deviation (PD) for the fitted models for each microalgae species. The percentile deviation was lower than 5% for the following ranges: from 1.9×10^6 to 1.01×10^7 cells mL^{-1} for *C. calcitrans*; from 2.9×10^6 to 2.11×10^7 cells mL^{-1} for *Isochrysis* affinis *galbana* (T-Iso); from 2.01×10^7 to 8.52×10^7 cells mL^{-1} for *N. gaditana*; and from 1.37×10^6 to 1.67×10^7 cells mL^{-1} for *P. tricornutum*. Within these cell density ranges, cell counting (cells mL^{-1}) can be accurately estimated by absorbance at the specific wavelength for each microalgae species.

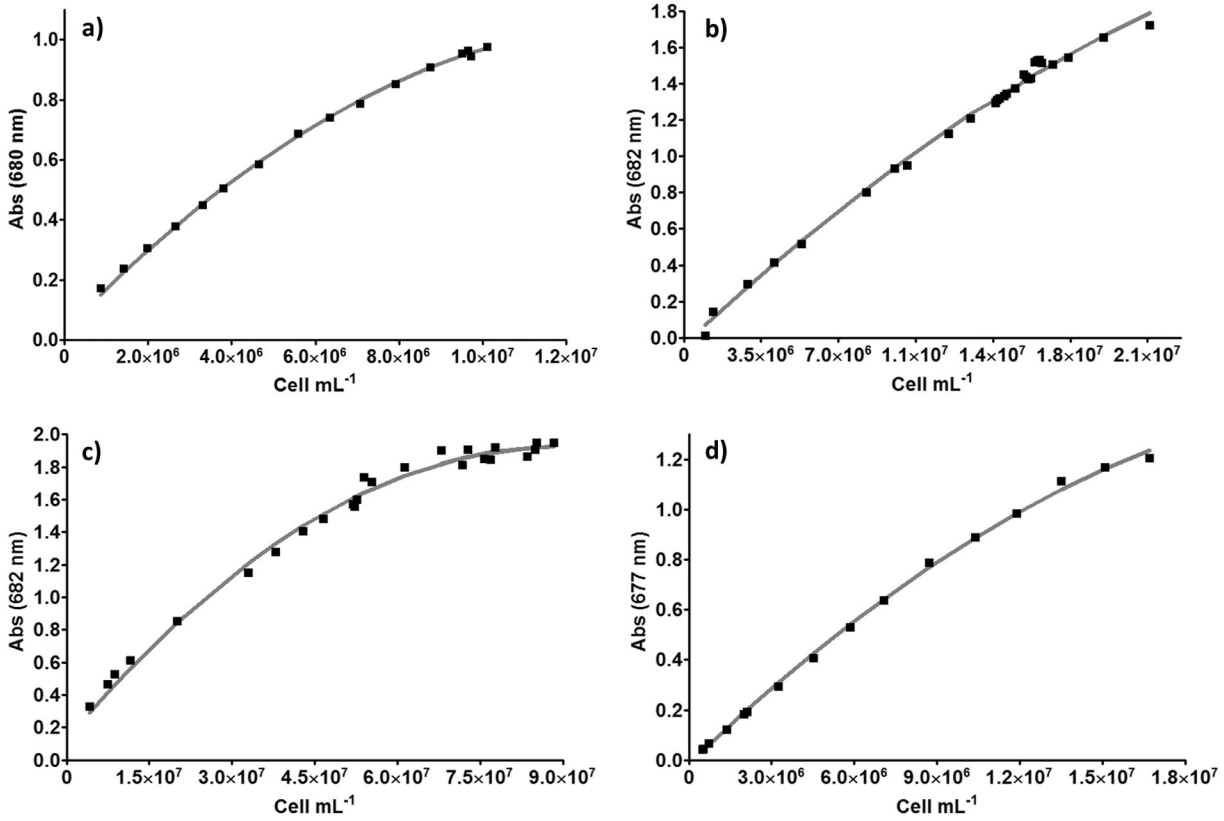

Fig. 2. Relation between absorbance and cell counting for different microalgae species. Black dots represent the experimentally observed values and dotted curve represents the theoretical values calculated using the developed predictive models. a) *Chaetoceros calcitrans*; b) *Isochrysis* affinis *galbana* (T-Iso); c) *Nannochloropsis gaditana*; d) *Phaeodactylum tricornutum*. nm = nanometer; mL = mililiter.

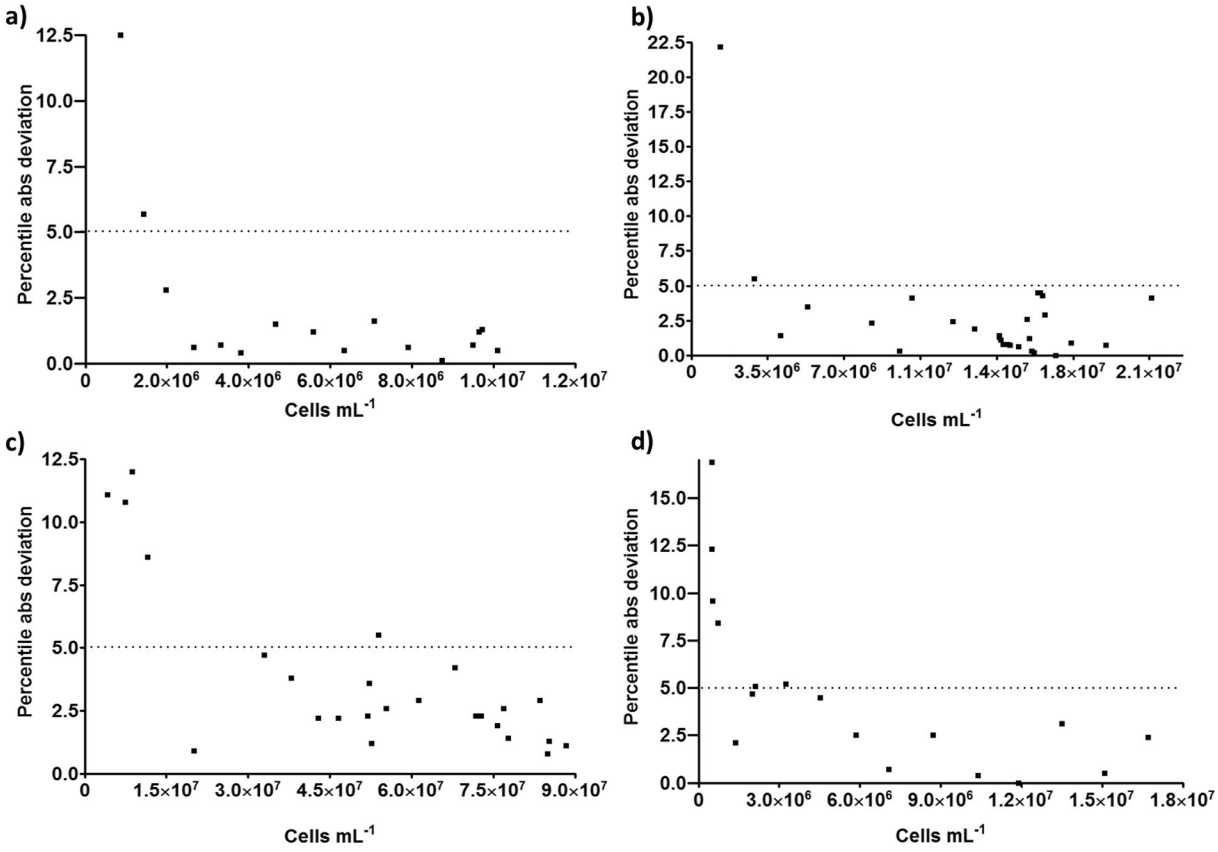

Fig. 3. Percentile deviation [(observed − predicted) × 100 / observed] for the absorbance of proposed models as of cell density (cells mL^{-1}) registered by cell counting, on the microalgae cultures. a) *Chaetoceros calcitrans*; b) *Isochrysis* affinis *galbana* (T-Iso); c) *Nannochloropsis gaditana*; d) *Phaeodactylum tricornutum*. mL = milliliter.

In previous reports, correlations between cell counting (cells mL^{-1}) and spectrophotometric absorbance of some compounds present in microalgae have been analyzed, such as chlorophyll *a*, *b* and c, as well as some carotenoids. Very high positive Pearson coefficients have been found, from 0.9338 to 0.9998 (Ebenezer and Ki, 2013; Lee et al., 2013). Even under uncontrolled conditions (at seashore or open sea), at high microalgae concentrations, strong correlations have been found between cell counting (cells mL^{-1}) and some microalgae characteristics; like chlorophyll content, carbon particles or even total lipids (Rossi and Fiorillo, 2010).

On the other hand, some effective equations to estimate cell counting from spectrophotometric absorbance have been reported for some species of microalgae, where cell counting ranged from 1×10^4 to 1×10^5 cells mL^{-1} (Valer and Glock, 1998) and for a maximum concentration of 5×10^6 cells mL^{-1} (Ribeiro-Rodrigues et al., 2011). In the present research, efficient equations were achieved for predicting most of the cell densities reported in the growth kinetics, including the highest values.

4. Conclusions

A rapid, simple and specific spectrophotometric methodology for biomass measurements of four microalgae species with high importance in aquaculture was developed. The specific wavelength for the maximum absorbance was established for each of the studied species. With this methodology, the harvest time will be easier to identify in facilities where these algae are cultured, and can be also easily tested in other monocultures. In addition, a predictive model was satisfactory and useful for a wide range of cell densities yielding a methodology with high potential for automation. A useful tool was developed for cell counting of microalgae in aquaculture.

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