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Mathematical modeling of *Microcystis aeruginosa* growth and [D-Leu^1] microcystin-LR production in culture media at different temperatures

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ABSTRACT

The effect of temperature (26 °C, 28 °C, 30 °C and 35 °C) on the growth of native CAAT-3-2005 *Microcystis aeruginosa* and the production of Chlorophyll-a (Chl-a) and Microcystin-LR (MC-LR) were examined through laboratory studies. Kinetic parameters such as specific growth rate (μ), lag phase duration (LPD) and maximum population density (MPD) were determined by fitting the modified Gompertz equation to the *M. aeruginosa* strain cell count (cells mL^{-1}). A 4.8-fold increase in μ values and a 10.8-fold decrease in the LPD values were found for *M. aeruginosa* growth when the temperature changed from 15 °C to 35 °C. The activation energy of the specific growth rate (Eμ) and of the adaptation rate (E_1/LPD) were significantly correlated ($R^2 = 0.86$). The cardinal temperatures estimated by the modified Ratkowsky model were minimum temperature = 8.58 ± 2.34 °C, maximum temperature = 45.04 ± 1.35 °C and optimum temperature = 33.39 ± 0.55 °C.

Maximum MC-LR production decreased 9.5-fold when the temperature was increased from 26 °C to 35 °C. The maximum production values were obtained at 26° C and the maximum depletion rate of intracellular MC-LR was observed at 30–35 °C. The MC-LR cell quota was higher at 26 and 28 °C (83 and 80 fg $cell^{-1}$, respectively) and the MC-LR Chl-a quota was similar at all the different temperatures (0.5–1.5 fg ng^{-1}).

The Gompertz equation and dynamic model were found to be the most appropriate approaches to calculate *M. aeruginosa* growth and production of MC-LR, respectively. Given that toxin production decreased with increasing temperatures but growth increased, this study demonstrates that growth and toxin production processes are uncoupled in *M. aeruginosa*. These data and models may be useful to predict *M. aeruginosa* bloom formation in the environment.

1. Introduction

Cyanobacterial blooms are a global problem for freshwater ecosystems including waters for potable and recreational uses (Paerl et al., 2001). High water temperatures have been known to lead to cyanobacterial bloom development in temperate (Robarts and Zohary, 1987 Jöhnk et al., 2008; Wu et al., 2014) and semi-arid (Mitrovic et al., 2003) regions. The genus *Microcystis* is one of the most important cyanobacteria due to its great ecological importance and public health implications (Sanchis et al., 2004). Its blooms are frequently found in eutrophic waters, affecting fish (Gaete et al., 1994), zooplankton communities (Amé et al., 2010), aquatic plants and vertebrates (MacKintosh et al., 1990). They are detrimental to water quality and negatively affect the development of nautical sports and other recreational activities (Turner et al., 1990; Giannuzzi et al., 2011). Many *Microcystis* strains can produce the potent hepatotoxin microcystin (MC), of which there are more than 100 variants, causing liver damage as well as nephrotoxicity (Milutinović et al., 2003; Merel et al., 2013; Niedermeyer, 2013). Harke et al. (2016) conclude that the occurrence of Microcystis toxic blooms appears to be expanding, since 108 countries or territories around the world have documented their presence in recent years compared with fewer than 30 countries in earlier years (Zurawell et al., 2005).

As the global climate changes, the occurrence and intensity of toxic cyanobacterial blooms are expected to increase (Paerl and

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Huisman, 2009; Michalak et al., 2013 Paerl and Otten, 2013). Direct effects, such as rising temperatures, and indirect effects, such as intensified stratification, favor cyanobacterial blooms. Increased water temperature is expected to give cyanobacteria a selective advantage over competing phytoplankton because of the high optimal growth temperature of cyanobacterial species (Elliott et al., 2006; Jöhnk et al., 2008). Laboratory studies on *Microcystis aeruginosa* have demonstrated increasing growth rates with increasing temperatures from 20 to 32 °C (van der Westhuizen and Eloff, 1985; Watanabe and Oishi, 1985; Imai et al., 2009). Robarts and Zohary (1987) found that temperatures of 25–35 °C resulted in the highest growth rates of bloom-forming cyanobacteria, including *Microcystis*. The maximum growth rate usually occurs within the temperature range of 25 °C–30 °C (Reynolds, 2006).

Temperature has been found to affect toxin production in *Microcystis* species (Gehringer and Wannicke, 2014). Laboratory studies have shown that the toxicity (LD_{50}, mg dry algae kg^{-1} mice) of temperate strains of *M. aeruginosa* were highest at 18–20 °C, and decreased greatly as the temperature increased above 28 °C (van der Westhuizen and Eloff, 1985; Watanabe and Oishi, 1985). Culture-based studies have shown that MC concentrations are generally highest between 20 and 25 °C (van der Westhuizen and Eloff, 1985; van der Westhuizen et al., 1986; Codd and Poon, 1988; Amé and Wunderlin, 2005).

Temperature has also been shown to differently affect MC variants in cyanobacterial species in several laboratory studies on hepatotoxic cyanobacteria (Gehringer and Wannicke, 2014). Amé et al. (2003) found that cyanobacterial growth was not necessarily associated with MC content during a field study in the San Roque reservoir (Córdoba, Argentina). Amé and Wunderlin (2005) observed that the content of MC-RR increased 82-fold at 20 °C compared with 28 °C; however, these differences were attributed to genotypic characteristics. Evidence in the literature therefore indicates that the most favorable temperature for MC production is different from that for optimal growth conditions (Watanabe, 1996; Codd and Ponn, 1988).

Several authors have conducted laboratory experiments using *M. aeruginosa* under different temperature conditions (van der Westhvizen and Eloff, 1985; Fujimoto et al., 1997; Jiang et al., 2008; Davis et al., 2009; Yang et al., 2012), but the modelling of toxin growth and production under the influence of temperature has not been studied to date.

Some findings suggest that toxic *Microcystis* are favored over nontoxic ones at warmer temperatures (Davis et al., 2009). However, the effect of temperature on toxin production remains largely unknown (El-Shehawy et al., 2012). Higher temperatures related to climate change could therefore constitute a possible trigger of increased *M. aeruginosa* toxigenicity.

One useful way to evaluate *M. aeruginosa* growth systems in laboratory assays under different abiotic factors is to examine the three parameters that characterize the three phases of bacterial growth: the lag phase duration (LPD), the maximum growth rate for the exponential growth phase (μ) and the maximum population density (MPD) for the stationary phase. These three parameters can be calculated by the modified Gompertz equation for *Microcystis* growth. The Gompertz model is one of the most widely used and recommended models from which lag time, maximum growth rate and maximum population density (stationary phase) can be obtained directly from nonlinear regression of the cell numbers versus time data (Zwietering et al., 1990; Whiting, 1995). In previous studies *M. aeruginosa* growth and average MC concentrations throughout the entire batch culture period, including lag, exponential and stationary phases, were described using the Gompertz model (Rosso, et. al., 2014). A Gompertz model describing the growth of *M. aeruginosa* would be a better tool to analyze the effect of temperature on the parameters of kinetic growth curves in batch culture. Similarly, the effect of temperature on MC production could be analyzed by applying a dynamic model of two differential equations describing *M. aeruginosa* growth and MC production, making it possible to study the relationship between the specific growth rate and the rate of MC production in batch cultures of *M. aeruginosa* under different temperature conditions.

The aim of this work was to model the effect of temperature (26 °C, 28 °C, 30 °C and 35 °C) on the parameters of growth kinetics and MC-LR production in a native strain of *M. aeruginosa* culture under laboratory conditions.

2. Material and methods

2.1. Strain preservation

A toxic native strain of *M. aeruginosa* (CAAT2005–3) previously reported as a [D-Leu^1] MC-LR producer was used (Rosso et al., 2014). Strain preservation was performed by periodic (every 15 days) passage through modified BG11 culture medium (Rippka et al., 1979), N:P ratio of 10, cultured at 28 °C and at 30 μmol photons $m^{-2}.s^{-1}$.

2.2. Experimental design

For assays, the strain was grown in triplicate in modified BG11 (N:P 10) in 500 mL Erlenmeyer flasks under controlled temperature conditions, constant aeration with humidified sterile air, a fluorescent light intensity of 30 μmol photons $m^{-2}\,s^{-1}$ and a light-dark cycle of 10:14 h. Prior to the early growth kinetics assays, cultures were acclimated for 7 days at low inoculum, periodically adding the culture medium. The *M. aeruginosa* strain was initially maintained at 28 °C under the same light conditions as the pre-experiment culture conditions. Four treatments were applied: 26 °C, 28 °C, 30 °C and 35 °C. The cells were exposed in a controlled environment growth chamber (Ingelab I-292PF). For cool light, photosynthetically active radiance (PAR) was applied via a set of 2 fluorescent tubes (Philips TL-DI 8W/54) fixed at 20 cm above each of the triplicate cultures. The average irradiance in each incubator was 30 μmol photons $m^{-2}\,s^{-1}$. The initial cells concentration for the experiments was around 9.10^5-1.10^6 cells mL^{-1}.

Each of the cultures was examined periodically in order to record latency, exponential and stationary growth phases over 2 weeks. Subsamples of the cultures were taken periodically and the following determinations were made:

2.2.1. Cell count

The cell enumeration method is recommended for measuring the cellular growth of cyanobacteria (Orr and Jones, 1998). Direct quantification was carried out in an optical microscope with a Neubauer chamber, recommended for small culture cell counts (Villafañe and Reid, 1995). Different sample volumes depending on the density of the culture were taken and preserved in formaldehyde solution (4%). Samples were treated for 30 min at 80 °C in a water bath and shaken for 30 s to disrupt the cells (Box, 1981). The counts were performed using an Olympus microscope at 400X. Subsequently, the results were expressed in number of cells mL^{-1}.

2.2.2. Chlorophyll-a

Chlorophyll-a was determined with a spectrophotometer after extraction with 100% methanol, at 665 and 750 nm before and after acidification with 1N HCl, according to the technique of Marker et al. (1980). The concentration of Chl-a was expressed in $\mu g.L^{-1}$.

2.2.3. Total MC quantification

Aliquots of culture were submitted to ultrasonication for 30 min (Omni Ruptor 400) and then centrifuged for 15 min at 5000 rpm/min to eliminate cell debris. The supernatant was passed through conditioned (10 mL 100% methanol, 50 mL 100% distilled water) Sep-Pak C18 cartridges (Waters) and the MCs were eluted with 80% methanol (Barco et al., 2005). Quantitative chromatographic analysis of MCs was performed by HPLC/MS Shimadzu LCMS-2020 determining a principal component of [D-Leu^1] MC-LR toxins (m/z 520) using C18 column (Hyperprep HS, 5-μm pore, 250 mm 10 mm) according to Giannuzzi et al. (2016). The column was equilibrated with a mixture composed of 65% A solution [water with 0.05% (v/v) trifluoroacetic acid] and 35% B solution [acetonitrile with 0.05% (v/v) trifluoroacetic acid]. The mobile phase consisted of a discontinuous gradient of A and B solutions. The flow rate was 1.0 mL/min. Standard of MC-LR was purchased from Sigma (St Louis, MO, USA).

Cell quotas of [D-Leu^1] MC-LR (Q_{MC}, fg MC per cell^{-1}) and chlorophyll- *a* (Chl-*a*) quotas of [D-Leu^1] MC-LR ($Q_{Chl\text{-}a}$, fg.MC. ngChl-a^{-1}) were calculated over a range of incubation times in *Microcystis aeruginosa,* assuming the extracellular content of MCs to be zero.

2.3. Mathematical modelling of M. aeruginosa *growth*

The growth curve was defined as the logarithm of the relative population size [y = log (N)] as a function of time t (days) (Zwietering et al., 1991). For *M. aeruginosa*, the growth rate shows a lag phase followed by an exponential phase and finally a decreasing growth rate down to zero, resulting in a maximum value of the number of cells. The number of cells mL^{-1} obtained as a function of time was modeled using the modified Gompertz equation (Eq. (1)), a double exponential function based on 4 parameters which describes an asymmetric sigmoid curve (Zwietering et al., 1991).

$$log(N) = a + c \times exp(-exp(-b \times (t - m))) \quad (1)$$

Where **log(N)** is the decimal logarithm of the cell counts (log (cell mL^{-1}), **t** is time (days), **a** is the logarithm of the asymptotic counts when time decreases indefinitely (roughly equivalent to the logarithm of the initial levels of cyanobacteria (log (cell mL^{-1})), **c** is the logarithm of the asymptotic counts when time is increased indefinitely (the number of log cycles of growth) (log (cell mL^{-1})), **b** is the growth rate relative to time (days^{-1}); and **m** is the required time to reach the maximum growth rate (days).

The maximum or specific growth rate (μ) value is defined as the tangent in the inflection point and was calculated as μ = b.c/e with e = 2.7182 (days-^{1}). The lag phase duration (LPD) is defined as the t-axis intercept of this tangent, the asymptote, and was calculated as LPD = m−1/b, (days); and the maximum population density MPD = a + c (log (cell mL^{-1}) was derived from these parameters (Giannuzzi et al., 1998).

The equation was applied to cyanobacteria growth data by nonlinear regression using the program Systat (Systat Inc., version 5.0). The selected algorithm calculates the set of parameters with the lowest residual sum of squares and a 95% confidence interval for *M. aeruginosa* growth.

Two additional kinetic parameters were evaluated: generation time (GT) and the relative lag phase duration (RLPD). Generation time was defined as the time for the bacterial population to double in cell numbers and was calculated by dividing μ values by 0.301 (equivalent to $\log_{10}$ 2), thus, GT is a measure of the metabolic rate in a new environment. The RLPD, defined by the amount of work to be done in adjusting to a new environment and the rate at which that work is done (Mellefont et al., 2003), was calculated by dividing LPD by GT.

2.4. Effect of temperature on the specific growth rate and lag phase duration

The effect of temperature on μ, calculated from the modified Gompertz (Eq. (1)) for each growth temperature, was modeled using two common functions to describe temperature relationships.

i) Arrhenius-type equation:

$$\mu_g = A \times exp\left(-\frac{E_\mu}{RT}\right) \quad (2)$$

Where **T** is the absolute temperature (°K), $\mathbf{E_\mu}$ is the activation energy of μ (kJ mol^{-1}), also called temperature characteristic, **A** is a pre-exponential factor and **R** is the gas constant (8.31 kJ K^{-1} mol^{-1}). The activation energy (E_μ) can be considered as the sensitivity of the specific growth rate to thermal changes.

ii) Modified Ratkowsky model (Zwietering et al., 1991):

To describe the growth rate around the optimum and maximum temperatures, Zwietering et al. (1991) proposed the modified Ratkowsky et al. (1983) equation:

$$\mu = (b \times (T - T_{min}))^2 (1 - exp(c \times (T - T_{max}))) \quad (3)$$

Where **b** is a Ratkowsky parameter (°C^{-1} days^{-1}), $\mathbf{T_{min}}$ is the minimum temperature at which growth is observed (°C), **c** is a Ratkowsky parameter (°C^{-1}), and $\mathbf{T_{max}}$ is the maximum temperature at which growth is observed (°C).

The effect of temperature on LPD values reflects how the adaptation period of *M. aeruginosa* to its new environment changes with temperature; the adaptation rate is the reciprocal of LPD (Li and Torres, 1993) and was fitted to an Arrhenius–type model.

$$\frac{1}{LPD} = D \times exp\left(-\frac{E_{1/LPD}}{RT}\right) \quad (4)$$

where **1/LPD** is the adaptation rate (day^{-1}) at T (°K), **D** is the pre-exponential factor (days)$^{-1}$, $\mathbf{E_{(1/LPD)}}$ is the activation energy of 1/LPD (kJoule mol^{-1}) and **R** is the gas constant (8.31 J °K^{-1} mol^{-1}). The activation energy ($E_{1/LPD}$) can be considered as the sensitivity of the adaptation rate to thermal changes.

Table 1
Gompertz equation parameters a, c, b, m and derived kinetics parameters (μ, GT, LPD, RLPD and MPD) for *Microcystis aeruginosa* growth in culture conditions at 26 °C; 28 °C; 30 and 35 °C. Data of 15 °C from Jähnichen et al., 2011 and 25 °C from Bertoli et al., 2014.

T (°C)	a	c	b	m	μ (day^{-1})	GT (days)	LPD (days)	RLPD	MPD cell ml^{-1}	R^2	RMSE
15	5.98 ± 0.02	1.03 ± 0.04	0.14 ± 0.14	15.72 ± 0.62	0.05 ± 0.01^{a}	6.02 ± 1.21	8.17 ± 0.561^{a}	1.36 ± 0.36	7.01 ± 0.068^{a}	0.997	0.028
25	5.50 ± 0.06	1.49 ± 0.08	0.31 ± 0.02	6.14 ± 0.05	0.17 ± 0.02^{a,b}	1.77 ± 0.21	4.14 ± 0.171^{b}	2.33 ± 0.37	6.99 ± 0.140^{a}	0.987	0.010
26	6.36 ± 0.02	0.89 ± 0.03	0.55 ± 0.08	5.83 ± 0.22	0.18 ± 0.03^{a,b}	1.67 ± 0.25	4.01 ± 0.121^{b,c}	2.40 ± 0.47	7.25 ± 0.057 ^{a}	0.978	0.085
28	6.29 ± 0.02	1.07 ± 0.04	0.53 ± 0.09	4.95 ± 0.03	0.20 ± 0.03^{a,b}	1.50 ± 0.11	3.04 ± 0.113 ^{b}	2.02 ± 0.37	7.33 ± 0.063 ^{a}	0.988	0.070
30	6.22 ± 0.02	1.11 ± 0.04	0.68 ± 0.13	3.92 ± 0.26	0.28 ± 0.03^{b}	1.07 ± 0.11	2.47 ± 0.424 ^{b,c}	2.30 ± 0.64	7.33 ± 0.061 ^{a}	0.991	0.085
35	5.92 ± 0.00	1.18 ± 0.10	0.53 ± 0.11	2.64 ± 0.43	0.24 ± 0.01^{b}	1.25 ± 0.05	0.75 ± 0.041 ^{c}	0.60 ± 0.05	7.10 ± 0.114 ^{a}	0.981	0.082

Different letters in each column indicate significant differences between temperatures at the 5% level of probability ($p < 0.05$).

The Q_{10} values (which measure the change in growth rate for a temperature increase of 10 °C) were calculated (Visser et al., 2016) for the temperature range from 15 to 25 °C, and 25–35 °C.

2.5. Mathematical modelling of [D-Leu^1] MC-LR production

According to the dynamic model proposed by Jahnichen et al. (2001, 2008); Jahnichen et al., 2001, [D-Leu^1] MC-LR production is growth rate-dependent:

$$\frac{dM}{dt} = p \times \frac{dX}{dt} - d_m \times M \quad (5)$$

where **M** is the MC concentration, **p** is the MC production coefficient describing a constant amount of MC passed to every new cell during cell division and $\mathbf{d_m}$ is a first-order MC depletion rate accounting for decreases in the intracellular MC quota over time in batch culture (Jahnichen et al./, 2001). Applying the Gompertz equation, **dX/dt** corresponds to the derivative of growth of *M. aeruginosa*:

$$\frac{dX}{dt} = 10^{a+exp(c\times exp(-exp(-b\times(t-m))))\times c\times exp(-exp(-b\times(t-m)))\times b\times exp(-b\times(t-m))\times ln(10)} \quad (6)$$

where ***a***, ***c***, ***b*** and ***m*** were taken from fitting parameters presented in Table 1. The coefficients p (fg cell^{-1}) and d_m (days^{-1}) were determined with the least square method, using a non-linear fitting procedure (MATLAB, Mathworks Inc., Natinck, Massachusetts). For this aim, Eq. (5) was numerically solved using a 4th order Runge-Kutta method.

2.6. Statistical analyses

Analysis of variance (ANOVA) and comparison tests according to the Fisher significant differences table (least significant difference) were applied with significance levels of 0.05. The statistical computer program Systat (Systat Inc., version 5.0) was used. The statistical requirements for the ANOVA (normal distribution, homogeneity of variance) were performed.

Fitting was carried out for each temperature condition by nonlinear regression, minimizing the sum of the squares of the deviations between the experimental and predicted values. The goodness-of-fit was evaluated by two parameters: coefficient of determination (R^2) and the root-mean-square error (RMSE) defined as Eq. (7).

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(experimental_i - predicted_i)^2}{n-k}} \quad (7)$$

where $\mathbf{experimental_i}$ are the experimental data, $\mathbf{predicted_i}$ are the values predicted by the model, ***n*** is the number of experimental data, and ***k*** is the number of parameters of the assessed model. Lower RMSE values indicate a better fit of the model to describe the data (Snedecor and Cochran, 1969).

3. Results

3.1. Modelling of M. aeruginosa growth at different temperatures in culture media

Cell concentrations showed a characteristic sigmoid behavior for batch cultures reaching a stationary phase. During the experiment, the number of cells mL^{-1} increased exponentially in all cultures after a lag phase. Fig. 1 shows *M. aeruginosa* counts growing in BG11 modified medium at 26 °C, 28 °C, 30 °C and 35 °C, respectively. Data from Jähnichen et al. (2011) and Bertoli et al. (2014) corresponding to *M. aeruginosa* growth at 15 °C and 25 °C, respectively, under similar conditions to the present work, have been included in the same Figure. The Figure also shows the application of the mathematical model: full lines represent the Gompertz equation. A good agreement between experimental data and predicted values was obtained with a determination coefficient of R^2 ranging between 0.981 and 0.997 and *RMSE* less than 0.085 log (cells ml^{-1}) for *M. aeruginosa* growth. Both observed and predicted cells mL^{-1} levels obtained from the model are shown in Fig. 2. In addition, Table 1 presents the kinetic parameters obtained by applying the Gompertz equation: μ, LPD and MPD at 15 °C, 25 °C, 26 °C; 28 °C, 30 °C and 35 °C obtained for *M. aeruginosa* growth in culture conditions.

It can be seen that as the temperature increases, the value of μ increases and the LPD decreases. Thus, when the temperature

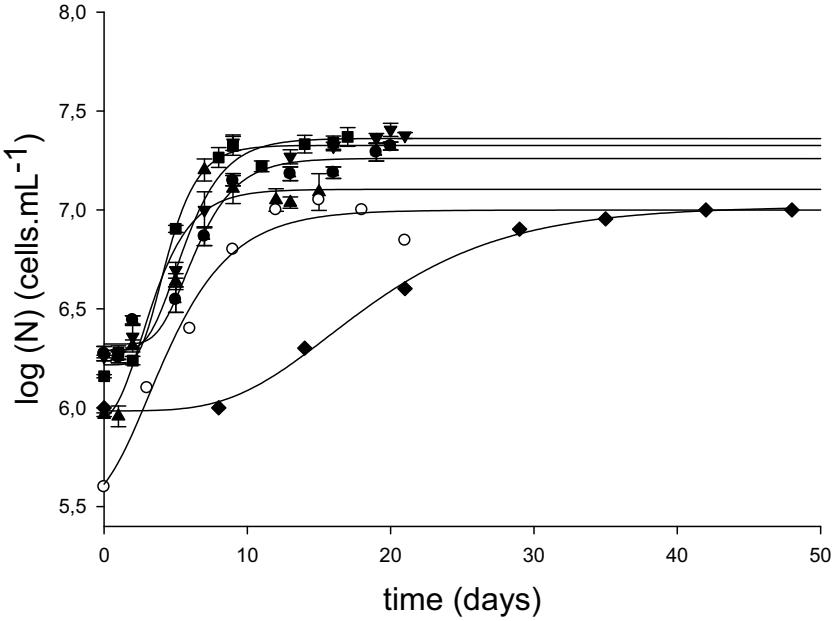

Fig. 1. Modelling of the native CAAT-03-2005 *Microcystis aeruginosa* growth in culture media, solid lines correspond to Gompertz model fitted to the experimental data at ●26 °C, ▼28 °C, ■30 °C y ▲35 °C. ◆15 °C data from Jähnichen et al. (2011). O 25 °C data from Bertoli et al. (2014). Error bars denote standard deviation (SD).

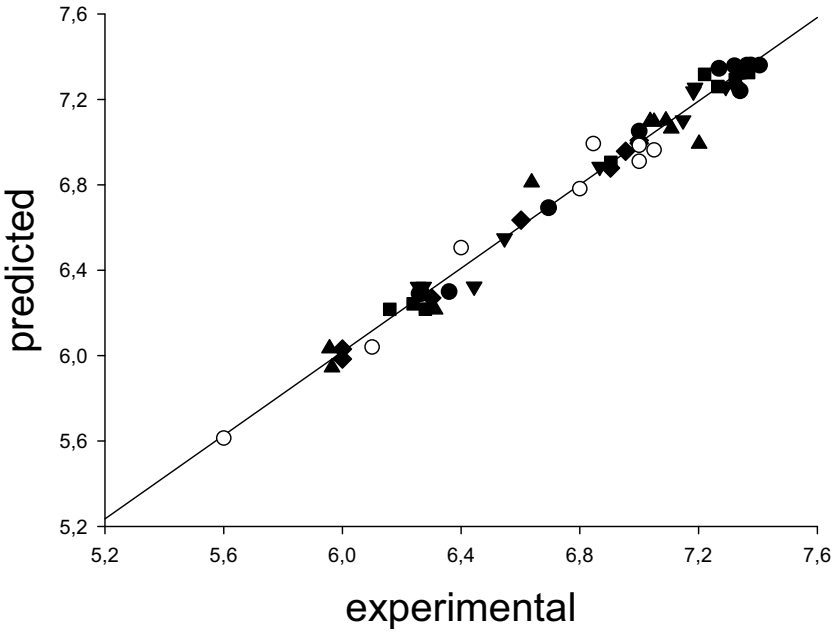

Fig. 2. Correlation between experimental data and predicted values corresponding to cell mL^{-1} of *M. aeruginosa*. Estimated data were obtained through application of Eq. (1) for *M. aeruginosa* growth at ●26 °C, ▼28 °C, ■30 °C, ▲35 °C, ◆15 °C (data from Jähnichen et al., 2011). O 25 °C (data from Bertoli et. al., 2014). The center line is the 'line of equivalence'.

changed from 15 °C to 35 °C, there was a 4.8-fold increase in μ values and a 10.8 fold decrease in LPD. Statistically significant differences ($p < 0.05$) were observed for μ values. Pairwise comparison indicated that μ values at 15 °C were significantly different from those at 28 °C ($p = 0.029$) 30 °C ($p = 0.003$) and 35 °C ($p = 0.009$). The LPD values were statistically significant different between 15 °C and the assays at other temperatures (p values ranged 0.000–0.001) and 35 °C was statistically significant different from those at 25 °C ($p = 0.002$), 26 °C ($p = 0.002$), 28 °C ($p = 0.013$) and 30 °C ($p = 0.048$). No statistically significant differences ($p > 0.05$) were found for the MPD values at the six temperatures analyzed (Table 1).

3.2. Temperature effect on specific growth rate (μ) and lag phase duration (LPD) for M. aeruginosa growth

The temperature dependence of biological rate constants is usually modeled by the Arrhenius equation. This is secondary model used to describe the temperature effect on μ and the adaptation rate (1/LPD) (Eqs. (2) and (4)). When logarithms of μ values were plotted against the reciprocal of the absolute temperature (Arrhenius plot), a low coefficient of determination ($R^2 = 0.86$) was found. The μ values in the range of temperature study (15–35 °C) was best fitted by two linear segments whose convergence determined the critical temperature at approximately 28 °C (Fig. 3a). This temperature separates two linear domains: below the optimal temperature: (from 15 °C to 28 °C) and above it (from 28 to 35 °C). Segment slopes determined two activation energies ($E_{\mu 1}$ and $E_{\mu 2}$), as shown in Eq. (8).

$$\mu = k_1 \times exp\left(-\frac{E_{\mu 1}}{RT}\right) - k_2 \times exp\left(-\frac{E_{\mu 2}}{RT}\right) \quad (8)$$

In the 15 °C to 28 °C domain, the activated energy $E_{\mu 2}$ was 83.08 ± 0.9 kJmol^{-1} with R^2 0.999; and in the 28 °C to 35 °C domain, the activation energy ($E\mu_1$) was 13.30 kJ mol^{-1} and $R^2 = 0.995$ (Table 2).

To evaluate the influence of temperature on the growth throughout the full temperature range assayed, equation 3 was applied. For this propose, the μ values found in the present work (26, 28, 30 and 35 °C), and the μ values calculated at 15, 25 and 40 °C taken from Bertoli et al. (2014), Jähnichen et al. (2011) and Krüger and Eloff (1978), respectively (Table 1) were used.

The equation fitted consistently with data ($R^2 = 0.87$), making it possible to calculate the 'cardinal temperatures' (Tminimum=8.58 ± 2.34 °C, Tmaximum=45.04 ± 1.35 °C and Toptimum = 33.39 ± 0.55 °C) characterizing *M. aeruginosa* growth in culture media (Fig. 4a). The constants of equation 3 were: $b = 0.080 \pm 0.002$ (°C^{-1} days^{-1}) and $c = 0.005 \pm 0.001$ (°C^{-1}).

Temperature variation in *M. aeruginosa* cultures also had an impact on the LPD parameter. Similarly, when logarithms of the 1/LPD values were plotted against the reciprocal of the absolute temperature, two linear domains were found (Eq. (9)) (Fig. 3b).

$$ln\left(\frac{1}{LPD}\right) = k_3 \times exp\left(-\frac{E_{1/LPD1}}{RT}\right) - k_4 \times exp\left(-\frac{E_{1/LPD2}}{RT}\right) \quad (9)$$

In these case, from 15 to 28 °C, the activated energy ($E_{1/LPD1}$) was 50.32 kJ mol^{-1} with $R^2 = 0.989$ and in the temperature range 28 °C to 35 °C the activation energy ($E_{1/LPD2}$) was 149.91 kJ mol^{-1} with $R^2 = 0.998$.

To fit the lag time, a logarithmic transformation was used throughout the full temperature range because the data showed a larger measuring error at high numerical values (the standard deviation was proportional to the mean value). After the transformation, the distribution of measuring errors at different temperatures was almost the same. A hyperbolic behavior between the lag time and the temperature was found and a hyperbolic equation was also therefore used (Eq. (10)).

$$ln(LPD) = \frac{p}{T + q} \quad (10)$$

The parameter q is the temperature at which the lag time is infinite (no growth). The parameter p is a measure for the decrease in lag time when the temperature is increased. Thus, the effect of temperature on the LDP parameter over the full temperature range (Fig. 4b) was studied. Lag phase duration values obtained in the present work (26, 28, 30 and 35 °C) and the LPD calculated at 15 °C

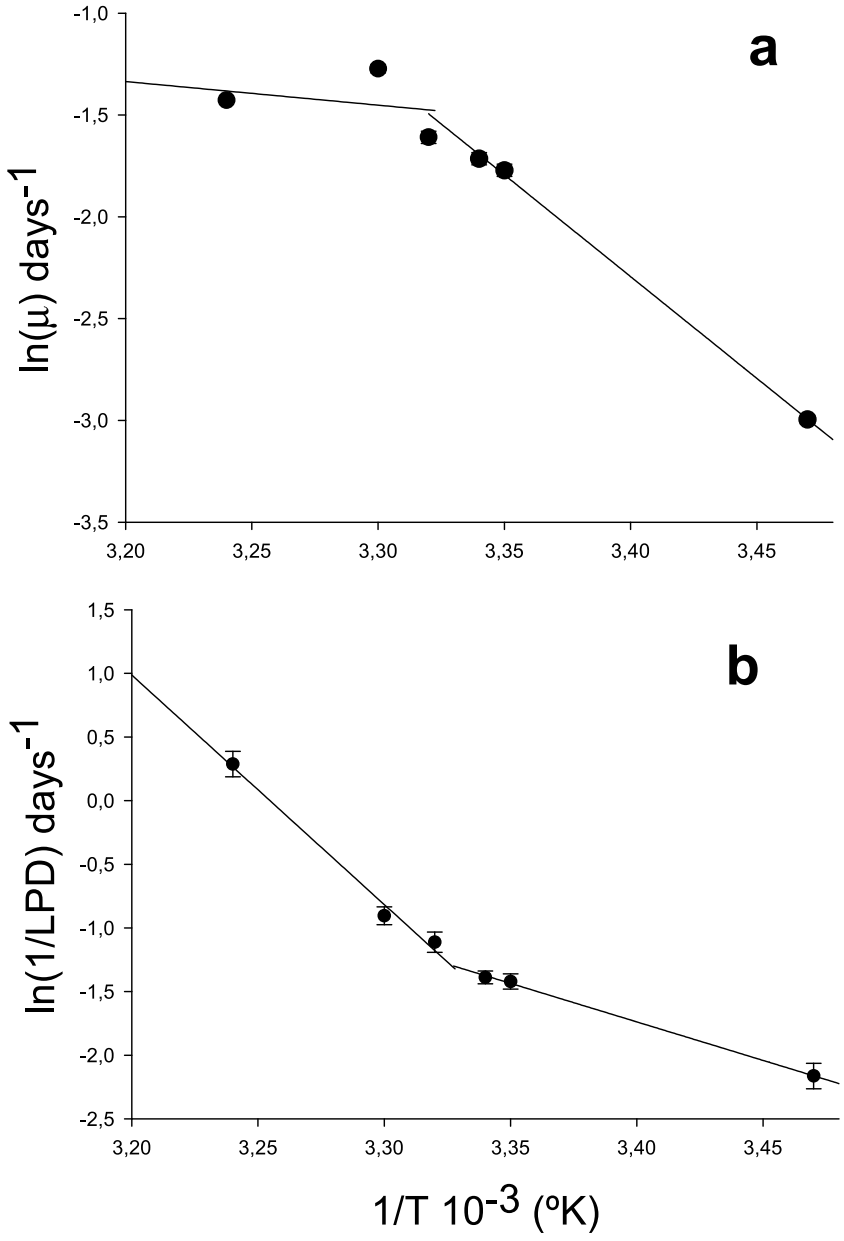

Fig. 3. Arrhenius plot of: (a) specific growth rate (μ) and (b) adaptation rate (1/LPD) for *Microcystis aeruginosa* growth in culture media.

and 25 °C taken from Bertoli et al. (2014) and Jähnichen et al. (2011), respectively, are included (Table 1). A good agreement was observed between the models and LPD values; the obtained parameters of equation 10 were p = 33.43 ± 4.56 and q = 0.73 ± 0.27 with $R^2 = 0.93$.

The Q_{10} values were 0.29 and 0.71 for temperatures ranging from 15 to 25 °C and 25 °C–35 °C, respectively, for *M. aeruginosa*. Generation time (GT) and the relative lag phase duration (RLPD) parameters were used to evaluate the temperature effect on this kinetic parameter (Table 1). In the temperature range studied (15–35 °C), GT values at 15 °C differed ($p > 0.05$) from those at other temperatures and RLPD values were very similar ($p > 0.05$).

3.3. Correlation between lag phase duration (LPD) and the reciprocal of specific growth rate (1/μ)

An early report by Cooper (1963) and Giannuzzi et al. (1998) noted that in some examples the ratio of growth rate to adaptation rate was nearly constant. This suggested a linear relationship between lag phase duration (LPD) and the reciprocal of specific

Table 2
Application of the Arrhenius type equation for evaluating the effect of temperature on the specific growth rate (μ) and adaptation rate (1/LPD) of *Microcystis aeruginosa*.

$\mu = k_1 * \exp(-\frac{E\mu_1}{RT}) - k_2 * \exp(-\frac{E\mu_2}{RT})$	k1 = 2.37 ± 0.05 (day)$^{-1}$ $E_{\mu 1}$ = 13.30 ± 0.81 (kJ.mol^{-1}) R^2 = 0.995	k_1, k_2, k_3 and k_4.2 = 31.127 ± 0.56 (day)$^{-1}$ $E_{\mu 2}$ = 83.08 ± 0.9 (kJ.mol^{-1}) R^2 = 0.999
$\frac{1}{LPD} = k_3 * \exp(-\frac{E_{(1/LPD)1}}{RT}) - k_4 * \exp(-\frac{E_{(1/LPD)2}}{RT})$	k3 = 58.72 ± 6.22 (day)$^{-1}$ $E_{(1/LPD)1}$ = 149.91 ± 1.9 (kJ mol^{-1}) R^2 = 0.998	k4 = −18.0 ± 0.06 (day)$^{-1}$ $E_{(1/LPD)2}$ = 50.32 ± 0.19(kJ mol^{-1}) R^2 = 0.989

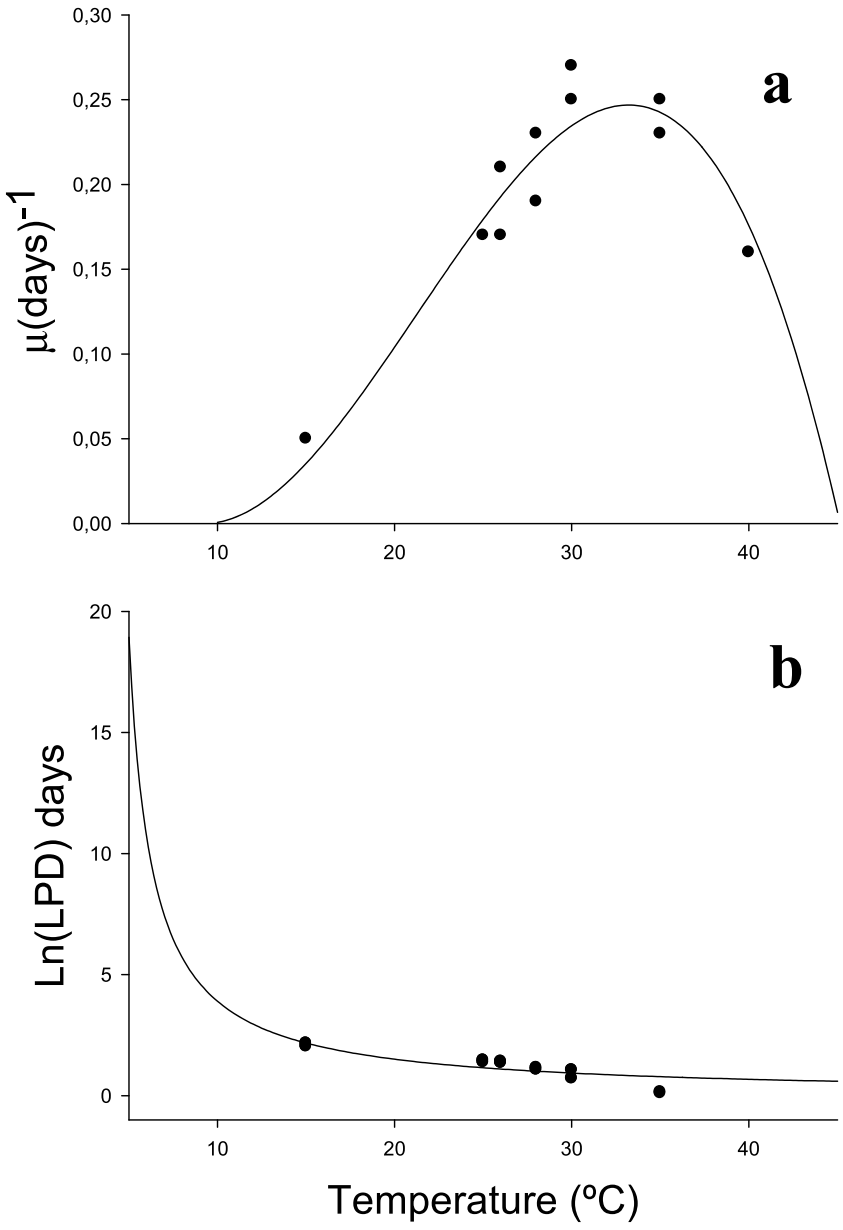

Fig. 4. Effect of incubation temperature on (a) the specific growth rate and (b) lag phase duration of *M. aeruginosa* growth in culture media. Data of μ and LPD values 15 °C and 25 °C are taken from Jähnichen et al. (2011), and Bertoli et al. (2014), respectively, and showed in Table 1. Data of μ at 40 °C are taken from Krüger and Eloff (1978).

growth rate (1/μ), a hypothesis that is confirmed in the present work for *M. aeruginosa* growth in culture media at different temperatures. In Fig. 5, a linear relationship between 1/μ and LPD was found and the correlation coefficient obtained was 0.86.

3.4. Modeling of [D-Leu^1] MC-LR production

For [D-Leu^1] MC-LR production, temperatures 26, 28, 30 and 36 °C were only tested on the CAAT-03-2005 strain since each strain has its own characteristic toxin production. The initial values for [D-Leu^1] MC-LR production ranged between 100 and 150 μg L^{-1}. These concentrations may be attributed to the toxin content of the inoculums. The [D-Leu^1] MC-LR concentrations changed during the growth of *M. aeruginosa*. At the end of the experiment (13-20 days), [D-Leu^1] MC-LR values were 950, 500, 365 and 100 μg L^{-1} at 26 °C, 28 °C, 30 °C and 35 °C, respectively. The [D-Leu^1] MC-LR production of *M. aeruginosa* grown in culture media at 26 °C, 28 °C, 30 °C and 35° C expressed as μg L^{-1} is shown in Fig. 6. It can be observed that the maximum [D-Leu^1] MC-LR values were obtained at 26° C, decreasing 9.5-fold as the temperature was increased from 26 °C to 35 °C. Fig. 6 also shows the application of the equation 6 for [D-Leu^1] MC-LR production (full lines). A good agreement between experimental data and predicted values were obtained with a correlation coefficient of R^2 ranging between 0.917–0.987. Maximum [D-Leu^1] MC-LR concentrations were observed at the end of the exponential growth phase. The production coefficient (p) and [D-Leu^1] MC-LR depletion rate (d_m) at 26 °C, 28 °C, 30 °C and 35 °C are shown in Table 3. It can be seen that as the temperature increases, the p value decreases, with a statistically significant difference ($p < 0.05$) between 26 °C and the other temperatures assayed. The maximum d_m occurred in the range of 30–35 °C (Table 3).

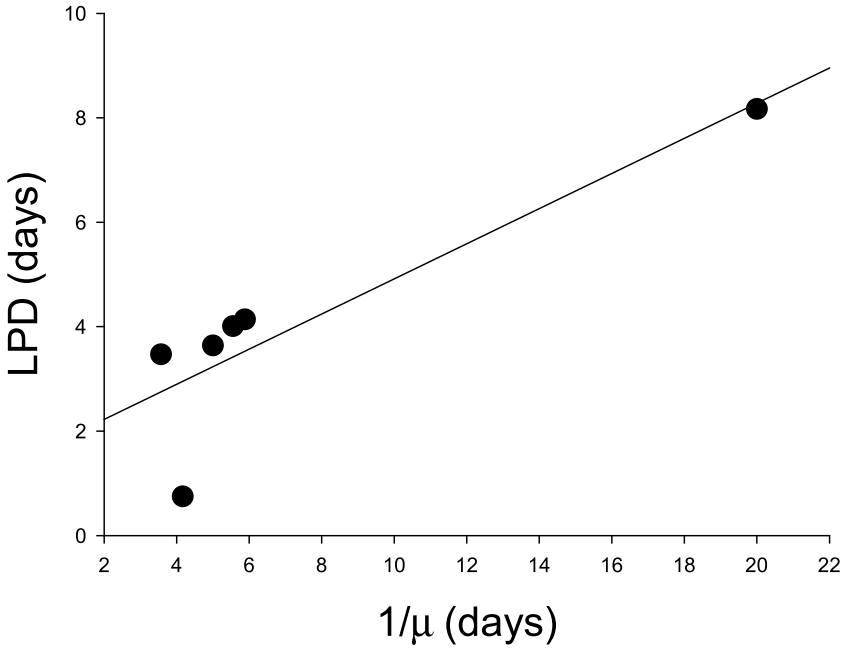

Fig. 5. Correlation of reciprocal of specify growth rate (1/μ) and lag phase duration LPD for *Microcystis aeruginosa* growth in culture media in the range 15–35 °C.

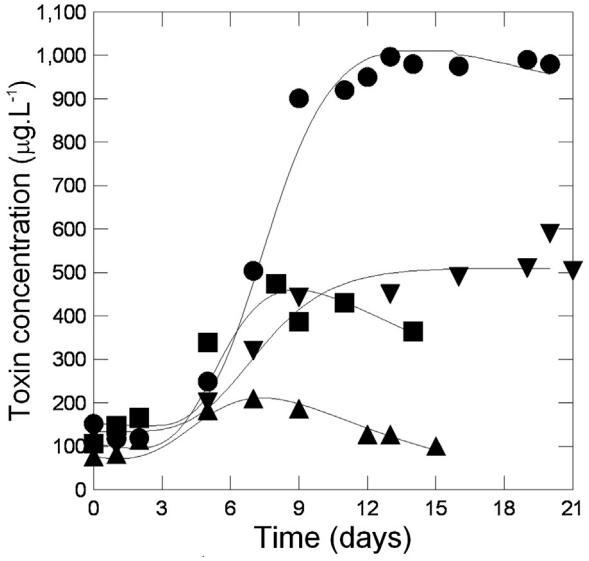

Fig. 6. Modelling of [D-Leu^1] MC-LR production in culture media, solid lines correspond to model fitted (eq. 6) to the experimental data at ● 26 °C, ▼28 °C, ■30 °C,▲35 °C.

The changes occurring in [D-Leu^1] MC-LR cell quotas (Q_{MC}) were also calculated during these experiments. The initial Q_{MC} of the inoculum ranged from 73 to 84 fg cell^{-1} (Fig. 7a). In the present work, the inoculum cultures were acclimated for 7 days at low cell level with the periodic addition of culture medium, thus allowing the cell to commence from the exponential phase. A decrease in the intracellular [D-Leu^1] MC-LR quotas was observed during the late exponential growth phase. At 30 and 35 °C, the quota decreased during the incubation time. At 26 °C, the by the end the experiment the Q_{MC} values had decreased to almost half of the initial values. The [D-Leu^1] MCLR cell quota decreased as a function of the incubation time and at the end of the study (15–20 days), Q_{MC} values were similar for the 28, 30 and 35 °C assays: 20 ± 10 fg cell^{-1}. It was observed that while there were no significant differences among temperatures in MPD, [D-Leu^1] MC-LR production increased with decreasing temperature, indicating a decrease in the [D-Leu^1] MCLR cell quota with increasing temperature.

The initial Chl-a production ranged from 948.2–1706.7 μg L^{-1}. At the end of the experiment, Chl-a values were 9850.7, 9165.9, 9323.9 and 10,851.5 μg L^{-1} at 26 °C, 28 °C, 30 °C and 35 °C, respectively. The Chl-a quota (Fig. 7b) ranged between 0.5 and 1.5 fg ng^{-1} and no significant differences ($p > 0.05$) were observed at different temperatures.

Table 3
Estimated model parameters to fitting experimental data to eq. 6: MC production coefficient (p) and MC depletion rate (d_m).

T (°C)	p (fg/cell)	d_m (day^{-1})	R^2
26	63.89 ± 3.68^a	$1.370\times10^{-2} \pm 6.185\times10^{-3}$	0.987
28	18.24 ± 2.67^b	$7.960\times10^{-4} \pm 9.302\times10^{-3}$	0.965
30	27.91 ± 8.05^b	$6.890\times10^{-2} \pm 4.8817\times10^{-2}$	0.917
35	31.35 ± 4.80 ^{b}	$1.629\times10^{-1} \pm 2.9454\times10^{-2}$	0.926

Different letters in each column indicate significant differences between temperatures at the 5% level of probability ($p < 0.05$).

4. Discussion

The general consensus is that the optimum growth temperatures for cyanobacteria are higher than those of most algae. Paerl (2014) reported that the optimum temperatures for cyanobacteria growth are greater than 25 °C, overlapping with those of green algae (27–32°8C) but clearly differing from those of dinoflagellates (17–27 °C) and diatoms (17–22 °C). In the present work, the optimum growth temperature for native *M. aeruginosa* was found to be 33.39 ± 0.55 °C.

In addition to assessing optimum temperatures, it is of interest to study how fast the growth rate increases with temperature. The temperature-dependence of growth rates in different species has gained much attention in the context of the metabolic theory of ecology (Gillooly et al., 2001; Brown et al., 2004).

Warming can selectively promote cyanobacterial growth because as prokaryotes, their growth rates are optimized at relatively high temperatures (Robarts and Zohary, 1987;

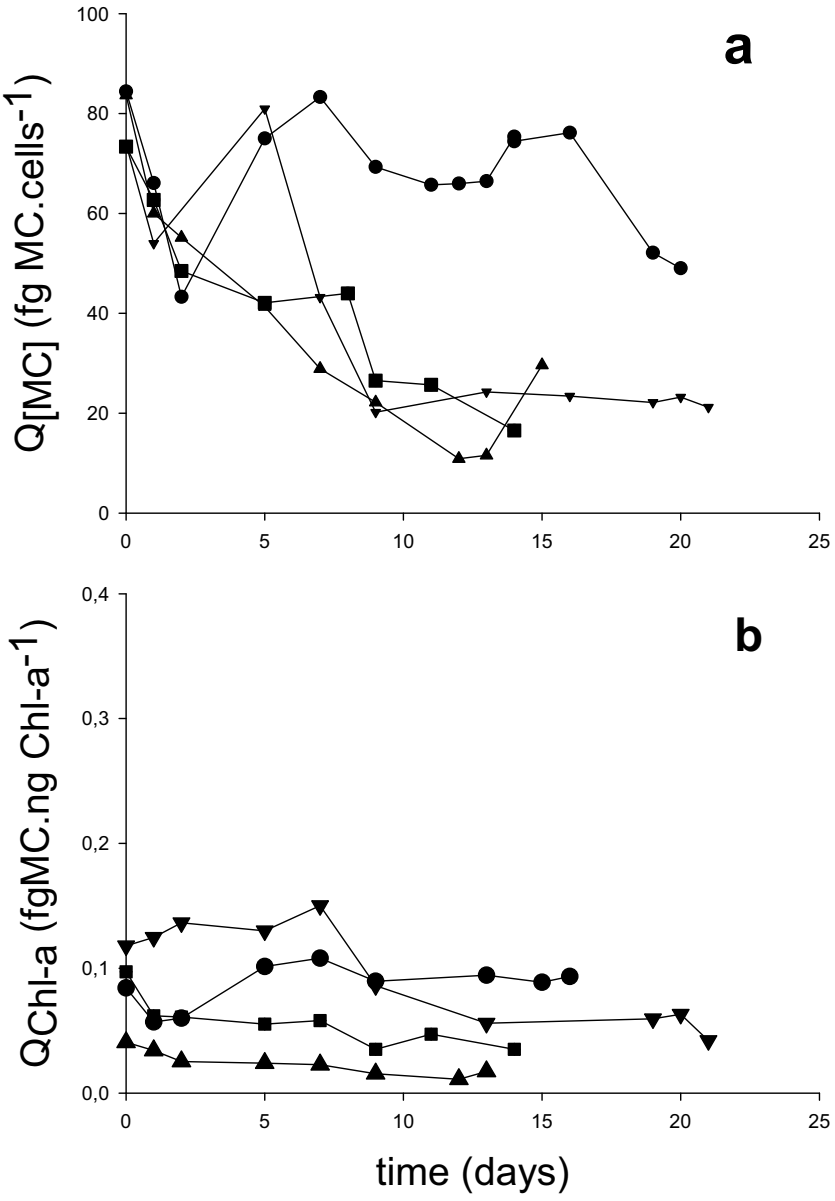

Fig. 7. Evolution of (a) $Q_{[D\text{-}Leu^1]\text{-}MC\text{-}LR}$ for cell and (b) $Q_{[D\text{-}Leu1\text{-}MC\text{-}LR]}$ for ng Chl-a during experimental assay at ●26 °C, ▼28 °C, ■30 °C and ▲35 °C.

Butterwick et al., 2005; Watkinson et al., 2005). This provides a distinct advantage for harmful cyanobacteria blooms under nutrient-enriched conditions, when competition with eukaryotic primary producers, including diatoms, chlorophytes, cryptophytes and dinoflagellates, can be intense (Paerl et al., 2011). Furthermore, intense light absorption by surface cyanobacterial blooms leads to higher temperatures within the bloom, resulting in a positive feedback loop (Ibelings et al., 2003).

In the present work μ and LPD vales of the native strains of *M. aeruginosa* tested were affected by temperature changes: as the temperature rose from 25 a 35 °C, μ values increased from 0.17 to 0.24 $days^{-1}$ and LPD values decreased from 4.14 to 0.75 days.

The μ and LPD values were calculated by applying the mathematical model of Gompertz. This, the logistic model and other predictive models are commonly used to determine important kinetic parameters relating to bacterial growth (Zwietering et al., 1990; Skinner et al., 1994). However, this is the first report to provide a simultaneous quantitative evaluation of kinetic parameters (μ, LPD and MPD) for native *M. aeruginosa* in culture media at different temperatures by modelling all points along the curve (minimum 8 points) using the Gompertz function. The Gompertz model successfully calculated the kinetic parameters of *M. aeruginosa* growth, as demonstrated by the high coefficients of determination (R^2) and low RMSE obtained. The μ values obtained in the present work are in agreement with those reported by Gorham (1964) for the optimum growth temperature (30 °C –35 °C). Imai et al. (2009) found significant differences in the growth rate of *M. aeruginosa* ($p < 0.05$) between 20 °C and 30 °C, but not between 25 °C and 30 °C, or between 30 °C and 35 °C in modified MA medium. Lyck (2004) reported a specific growth rate in the range of 0.52 to 0.54 day^{-1} for *M. aeruginosa* NIVA – CYA 228/1 in modified O^2 culture media at 20° C; however, these authors

reported the specific growth rate (day^{-1}) between successive sampling times, calculated according to a simple first-order rate function using cell concentration ($cells\ ml^{-1}$).

The present paper enhances current knowledge in the field by evaluating the effect of temperature on μ and LPD values, for which propose the activation energy was calculated. This parameter is a measure of the increase in the growth rate with temperature and can be calculated from an Arrhenius plot.

In the range from 15 °C to 28 °C, the activation energy ($E_{\mu 2}$: $83.08\ kJ\ mol^{-1}$) was found to be approximately 6.3 times higher than in the 28 °C to 35° temperature domain ($E_{\mu 1}$: $13.30\ kJ\ mol^{-1}$). This indicates that changes in temperature in the range 15–28 °C have a marked influence on μ values of *M. aeruginosa* growth. The specific growth rate (μ) values were well represented by an expanded Ratkowsky model permitting the calculation of 'cardinal temperatures'. Lürling et al. (2013) found similar optimal temperatures for two strains of *M. aeruginosa* (30.0–32.5 °C).

In an early report, Eloff (1974) reported the activation energy values calculated for the different isolates of *M. aeruginosa* from the respective Arrhenius plots. Under similar temperature (24–34.5 °C) and light intensity ($33\ \mu E\ m^{-2}\ s^{-1}$) growth conditions, the activation energy reported by these authors was 12.5 times lower than found in the present work. The reason for this divergence may lie in differences in the strains of *M. aeruginosa* and in the different methods used to calculate the μ values. The cited authors used turbidity as a measure of *M. aeruginosa* growth. In order to calculate the growth rate in a particular experiment, only the exponential portion of the growth curve was used and the authors only reported the activation energy for the specific growth rate. No information is available in the literature on the activation energy of the adaptation rate (1/LPD). In the present work, in the range from 28 °C to 35 °C was approximately 3 times higher than in the range from 15 °C to 28 °C.

Temperature not only has a direct effect on the growth rates and LPD of organisms, but also has considerable indirect effects that can tip the competitive balance between species with ecological implications.

It is important to analyze the differences in temperature sensitivity between cyanobacteria and eukaryotic algae. On average, the reported Eμ value for cyanobacterial species is $48.63\ kJ\ mol^{-1}$ and that of green algae is $41.48\ kJ\ mol^{-1}$. This indicates that the growth rate of cyanobacteria increases faster with temperature than that of green algae, although the variation among species is considerable, and the difference between cyanobacteria and green algae was therefore at best marginally significant. Another parameter to be analyzed is Q_{10}, which measures the change in growth rate for a temperature increase of 10 °C. Over the temperature range from 20.0 to 27.5 °C Q_{10} values were 2.63- 0.94 for cyanobacteria and 2.03–1.02 for green algae. Other studies also reported high Q_{10} values for cyanobacteria –for example, a study by Mehnert et al. (2010) on seven cyanobacterial species showed an average Q_{10} of 2.33–0.87.

In the present work, the Q_{10} values were 0.29 and 0.71 in the range of 15–25 °C and 25–35 °C, respectively. These values are lower than the Q_{10} values reported by Visser et al. (2016) for *M. aeruginosa* PCC7941 ($Q_{10} = 2.21$), and CYA140 ($Q_{10} = 4.63$) calculated from the data of Lürling et al. (2013). Nevertheless, Lürling et al. (2013) calculated growth rates using Chl-a as a biomass indicator.

The above data suggest that the native strain of *M. aeruginosa* is able to compete favorably with other phytoplankton species, producing more frequent blooming events in scenarios of climate change. Further study of the temperature dependencies of the different physiological processes affecting growth (e.g., carbon fixation, photorespiration and respiration) are required in order to better understand the differences in temperature sensitivity between cyanobacteria and eukaryotic algae.

Is well known that the lag phase duration is affected by many variables, and cyanobacterial responses to changes in the environment are complex and difficult to characterize. When a cyanobacterial culture is introduced into a new environment, the ratio of the lag time divided by the generation time in that environment will be a measure of the amount of work to be done by the cell before growth is initiated (Ross, 1999) and can be considered as the relative lag phase duration (RLPD) (Mellefont et al., 2003). This parameter is defined as the amount of work that a population has to perform in order to adjust to a new environment, regardless of the rate at which that work is done. Robinson et al. (1998) defined work as the various biosynthetic and homeostatic responses needed to prepare for growth in the new environment. In the present paper, the RLPD values for *M. aeruginosa* were plotted against a scale reflecting the magnitude of the change in the environment; a horizontal straight-line relationship was observed in the range 25–30 °C. This constancy means no extra work was required to respond to changes in this portion of temperatures. For 15 and 35 °C, the RLT values decreased with temperature, meaning *M. aeruginosa* needs less work to adapt to the environment and thus is well adapted to this range of temperature.

The effect of temperature on [D-Leu^1] MC-LR production was revealed by applying a dynamic model of two differential equations describing growth and MC production (Jahnichen et al., 2001, 2008).

The production of [D-Leu^1] MC-LR decreased with increasing temperature, coinciding with the findings of Gorham, 1964; Runnegar et al., 1983; van der Westhuizen and Eloff, 1985; Cood and Poon, 1988; Sivonen, 1990; Rapala et al., 1997; Lehman et al., 2008; Wang et al., 2010 and Giannuzzi et al. (2016). van der Westhuizen and Eloff (1985) reported that the optimal growth conditions do not coincide with the production of toxins. Similarly, Gorham (1964) affirmed that the optimum temperature for growth (30 °C –35 °C) differed from that for optimal toxicity (25 °C). In the light of all these findings, the negative correlation between toxin content and cell growth can be interpreted in terms of the costs and benefits of producing MCs. MC biosynthesis occurs via a multi-enzyme complex and consumes a great deal of energy, implying a high cost for the cell. Our results suggest that under cell growth conditions, the benefits of producing MC outweigh the costs, which could explain the low level of MC production at high temperatures (30 and 35 °C). Briand at al. (2008) reported that under environmental conditions favoring cyanobacterial growth, the cost of MC production prevails over its benefits, and consequently, toxin production is lowered. On the other hand, the MC production coefficient (p values), representing a constant amount of MC passed to every new cell during cell division, was significantly higher at 26 °C compared to that at 28, 30 and 35 °C; and d_m was more than 30–35 °C. These results are in agreement with the p values informed by Jahnichen et al. (2001) for *M. aeruginosa* growth in a nonaxenic culture.

In addition, [D-Leu^1] MC-LR data were expressed as a cell quota in order to emphasize the importance of this parameter in determining the cellular physiology of MC production. Jähnichen et al. (2008) reported that the starter culture has a remarkable influence on the MC cell quota of the following batch culture. The [D-Leu^1] MC-LR cell quota of *M. aeruginosa* displayed a decreasing trend as temperature increased, being significantly higher at 26 °C compared to other temperatures, thus supporting the hypothesis that rising temperatures in the tropics and subtropics would reduce toxin production (Mowe et al., 2015). Substantial modifications in the [D-Leu^1] MC-LR cell quota can occur as a result of MC depletion, becoming apparent at the transition from exponential to stationary growth. Briand et al. (2008) observed a decrease in MC cell quota in the late exponential growth phase.

The ecophysiological role of microcystins has been a topic of intense scientific research in recent decades. It is well known that the production of microcystins is regulated by a number of factors commonly associated with the formation and senescence of blooms such as temperature, nutrients, and light. Another hypothesis is that microcystins act as feeding deterrents for predators such as zooplankton and fish. However, phylogenetic analysis suggests that the genes responsible for microcystin synthesis pre-date the eukaryotic lineage. More recently, factors such as chelation of metals, intraspecies communication, colony formation and protein-modulation have been implicated as potential functions for microcystin (Harke et al., 2016).

Dziallas and Grossart (2011) showed that warmer environments are often characterized by more radicals with a higher potential for cell damage (e.g., due to higher diffusibility), and proposed that cyanobacterial toxins act as radical scavengers, thus benefiting cyanobacterial growth at elevated temperatures. In this respect, it could be that the decrease in [D-Leu^1] MC-LR production observed at 35 °C in our experiments is related to oxidative stress caused by the temperature rise. Moreover, Alexova et al. (2011) mention that MC can bind to proteins in conditions of oxidative stress as a possible mechanism to increase the ability of toxic strains. By using high temperature conditions and inducing oxidative stress, the researchers observed a specific and covalent interaction of MC with several proteins, resulting in their accumulation, suggesting another possible function of this metabolite. Recently, Zilliges et al. (2011) proposed that MCs can bind to some phycobilins and thus protect them from degradation by reactive oxygen species. The apparent loss of MC is likely the consequence of a specific and covalent binding of the toxin to various proteins (Meissner et al., 2013, 2014). Further studies may help to confirm this putative role of MC in the cellular metabolism and to partially elucidate the toxin dynamic observed in the field. On the other hand, recent studies have also shown that a large fraction of MCs are covalently bonded to proteins within *Microcystis* cells and cannot be extracted using methanol (Zilliges et al., 2011; Meissner et al., 2013, 2014). Thus, the lower total MC could indicate a higher fraction of bound MC at higher temperatures. Since bound MCs were not measured in the present study, further research is required to confirm this possibility.

Non-significant differences were observed in [D-Leu^1] MC-LR Chl-a quota values. Some authors have reported that MCs are associated with the thylakoid membrane of *M. aeruginosa,* suggesting a close physical association between MCs and the photosynthetic machinery of the cell. The constant [D-Leu^1] MC-LR Chl-a quota ratio found in this study supports this hypothesis and suggests that MC synthesis and/or function may be linked to the photosynthetic process. Long et al. (2001) found similar results to those reported here, with values of the MC Chl-a quota at 0.59 ± 0.03 (fg ng^{-1}); however, these authors also suggest that MCs may not be essential in photosynthesis. To date there has been insufficient understanding of the temperature effects on the physiology of MCs production to draw firm conclusions.

In this context, the current paper is the first to report clear quantitative evidence of a direct relationship between MC production and cell growth as affected by temperature. Further studies are required to gain deeper insight into the factors that influence MC growth and production in order to better predict aspects related to *M. aeruginosa* blooms.

5. Conclusions

The predictive Gompertz model together with the secondary Arrhenius-type and the modified Ratkowsky models constitute a simple method of calculating biokinetic growth parameters and evaluating the influence of incubation temperature on the native *M. aeruginosa* strain in culture conditions. Furthermore, [D-Leu^1] MC-LR production and growth kinetics values can be obtained using the dynamic model proposed by Jahnichen et al. (2001, 2008); Jahnichen et al., 2001. The current study provides quantitative evidence of the effects of temperature on *M. aeruginosa* growth and MC production. It also proves that a statistical approach using mathematical modelling, previously ignored in studies on cyanobacterial toxicity, is an efficient tool for deciphering the complex variation of MCs in the cells of cyanobacteria. These data and models may be useful to predict *M. aeruginosa* bloom formation in the environment.

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