Algal Research 27 (2017) 235–243

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

# Assessment of synergistic interactions between environmental factors on *Microcystis aeruginosa* growth and microcystin production

Pedro Geada[a,*], Ricardo N. Pereira[a], Vítor Vasconcelos[b], António A. Vicente[a], Bruno D. Fernandes[a]

[a] CEB-Centre of Biological Engineering, University of Minho, Braga, Portugal
[b] CIIMAR/CIMAR - Interdisciplinary Centre of Marine and Environmental Research and Department of Biology, Faculty of Sciences, University Porto, Porto, Portugal

ARTICLE INFO

*Keywords:*
Cyanobacteria
Cyanotoxin
Combined effect
High added-value product
Photoautotrophic growth

ABSTRACT

The combined effect of four abiotic factors on *Microcystis aeruginosa* growth and toxin production was assessed by culturing the cyanobacterium under different light intensities (10–190 μmol photons·m$^{-2}$·s$^{-1}$), $CO_2$ concentrations (0–10% (v/v)), temperatures (15–40 °C), and pH values (6.5–9.5). Results indicate a significant influence caused by the synergistic effect of environmental factors over growth-related parameters and cyanobacteria toxicity. The combined use of low to medium light intensities (50–120 μmol photons·m$^{-2}$·s$^{-1}$) and $CO_2$ concentration (1–6% v/v) led to higher cell concentrations, while specific growth rate and biomass productivity were favoured by medium to high light intensities (110–190 μmol photons·m$^{-2}$·s$^{-1}$), $CO_2$ concentrations (4–9.5% v/v) and temperatures (29–39 °C). Regarding microcystin (MC) production, higher concentrations were obtained at low light intensities and low $CO_2$ concentrations while approximately 2000-fold lower MC concentrations were achieved by simultaneous use of high values of light intensity, $CO_2$ concentration and temperature.

## 1. Introduction

As a result of continuous climate changes and environmental pollution caused by anthropogenic activities over the last decades, cyanobacteria have proliferated in water bodies throughout the globe emerging as a major concern for national and international authorities [1,2]. Among harmful cyanobacterial bloom (HCB) forming organisms, *Microcystis aeruginosa* is considered to be the most widespread, presenting a serious risk for human (and animal) health due to its ability to produce cyanotoxins (MC) as well as other metabolites that affect water's taste and odour [3,4]. Due to the large dissemination of this cyanobacterium, humans might be exposed to its hepatotoxins. MC-LR is the most frequent either by drinking and recreational water or aquatic and terrestrial foodstuffs (e.g. fish, shellfish, vegetables, plants, supplements) potentially causing severe health problems such as liver tumours [5–11]. Besides the environmental and health issues, the increasing occurrence of HCBs may also represent economic losses because of the higher costs of water treatment processes and the drop observed in water recreational and fishery activities [12].

In order to avoid similar human lethality events as happened in Brazil [13], the World Health Organization (WHO) established a guideline value for MC-LR in drinking water, 1 μg·L$^{-1}$, and a tolerable daily intake of 0.04 μg·kg$^{-1}$ [14]. Numerous laboratory analytical methods, including liquid chromatography, in vitro bioassays, and immunoassays, have been extensively used in MC detection and quantification [15]. However, the limited availability of commercial standards along with their low reliability in terms of matching the required purity and quantity, threw some suspicious thoughts over the research work already published [16,17]. To overcome this issue as well as reduce the high prices charged for pure standards used in monitoring assays, increasing MC production capacity became a necessity for research groups working on this field [18].

Additionally, it is important to understand how environmental factors affect *M. aeruginosa* growth and MC production in order to avoid or control blooms of this toxic cyanobacterium. Since growing HCBs and cyanotoxin production are complex events comprising a large number of variables, much is still unknown. This is mainly due to the lack of information regarding synergistic interactions between different abiotic factors and the contradictory data previously attained [19,20]. Over the last years, many studies have been performed in order to assess the influence of light [21,22], $CO_2$ [23,24], nutrients [22,25], temperature [22,26], and pH [23,27] on *M. aeruginosa* growth and MC content. However, all these studies aimed to explore the effect of each factor individually. One of the few exceptions is the study performed by

* Corresponding author.
*E-mail address:* pedrogeada@ceb.uminho.pt (P. Geada).

http://dx.doi.org/10.1016/j.algal.2017.09.006
Received 26 May 2017; Received in revised form 31 July 2017; Accepted 6 September 2017

[20] where the combined effect of light intensity, temperature and nitrogen concentration on *M. aeruginosa* growth was evaluated, showing significant interactions affecting cyanobacterium growth. However, the impact of such abiotic factors on toxin production was not determined in this study. Thus, there is a need for testing the influence of combined environmental factors so that we can better understand the response behaviour of these blue-green algae in their natural environment and, if needed, manipulate their growth under laboratory conditions.

The objective of this study is to determine the impact of combined use of light intensity (10–190 μmol photons·$m^{-2}$·$s^{-1}$), $CO_2$ concentration (0–10% (v/v)), temperature (15–40 °C) and pH (6.5–9.5) on *M. aeruginosa* LEGE 91094 growth and toxicity. Our expectations about the insights from this study rely on two completely opposite perspectives: *i)* increase the knowledge about *M. aeruginosa* growth and MC production which will contribute to optimize culturing conditions and consequently decrease the high prices of analytical standards employed in control and monitoring methodologies as well to assist all the research groups working in different areas around the control of HCBs and the mitigation of their consequences; *ii)* further understanding of the real impact of environmental conditions on *M. aeruginosa* growth and toxicity in order to improve HCBs predicting mechanisms.

## 2. Materials and methods

### 2.1. Microorganism, culture and experimental conditions

The unicellular cyanobacterium *Microcystis aeruginosa* LEGE 91094 from the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR - Porto, Portugal) collection was maintained in Z8 medium [28] under 10 μmol·$m^{-2}$·$s^{-1}$ using a 12:12-hour light-dark cycle at 20 °C. Stock cultures were renewed on a monthly basis.

Batch culture experiments were carried out in 40 mL glass test tubes containing Z8 medium subjected to several ranges of light intensity, $CO_2$ concentration (added to the original air stream), temperature and pH summarized in Table 1. Initial biomass concentration was 0.05 g·$L^{-1}$ (dry weight – DW) in all cultivations.

#### 2.1.1. Study of combined effect of light intensity and $CO_2$ concentration

The combined influence of light intensity and $CO_2$ concentration (independent variables) on *M. aeruginosa* growth and MC content (dependent variables) was assessed through a $2^2$ full-factorial central composite design (CCD). The choice of pairing up these two abiotic factors was due to the fact of being light the source of energy and $CO_2$ the source of carbon, making more sense to vary both simultaneously. Experiments were performed at 25 °C by varying light intensity and $CO_2$ concentration conditions (levels described in Table 1) and combine them, reaching a total of 18 different arrangements. The tested combinations are presented in Table 2. Following the experimental design, three central points (CP) were executed. The pH was kept at 8 by adjusting its value with NaOH (0.1 M) or HCl (0.1 M) and no $CO_2$ was added to the aeration stream. The volume lost due to water evaporation was replaced using sterilized distilled water and samples for determination of biomass concentration were collected every 24 h under sterilized conditions (i.e. using a laminar flow box) until the stationary phase was reached.

**Table 1**
Range of tested environmental factors.

| Environmental factor tested | Tested values |
|---|---|
| Light intensity (μmol photons·$m^{-2}$·$s^{-1}$) | 10, 55, 100, 145, 190 |
| $CO_2$ concentration (% v/v) | 0, 2.5, 5, 7.5, 10 |
| Temperature (°C) | 15, 25, 30, 35, 40 |
| pH | 6.5, 8, 9.5 |

#### 2.1.2. Study of combined effect of temperature and pH

After determining and validating the optimal conditions of light intensity and $CO_2$ concentration for *M. aeruginosa* growth, the optimal values (based on biomass productivity) of these variables were fixed and the combined effect of temperature and pH was assessed doing a similar process as shown before in Section 2.1.1 (Tables 1 and 4). The sampling and evaporation compensation was performed as described in Section 2.1.1.

### 2.2. Growth kinetics

Samples collected during cultivations were used to determine the biomass concentration as well as biomass productivity and specific growth rate attained throughout the assays performed.

#### 2.2.1. Biomass concentration

The absorbance of cultures was measured at 670 nm and 750 nm (following the recommendations given by [29]) using a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Vermont, USA). Through a calibration curve previously performed for this purpose, where the variation of cell concentration ($X$, g·$L^{-1}$ DW) was represented as function of absorbance (Eqs. (1) and (2)), it was possible to follow biomass concentration during tests.

$$X = 0.821 \times \mathrm{Abs}(670\mathrm{nm}) + 0.014 \quad (R^2 = 0.995) \tag{1}$$

$$X = 1.208 \times \mathrm{Abs}(750\mathrm{nm}) + 0.023 \quad (R^2 = 0.984) \tag{2}$$

#### 2.2.2. Determination of biomass productivity and specific growth rate

Biomass productivity ($P$, g·$L^{-1}$·$d^{-1}$) was obtained from the following equation:

$$P = \frac{X_t - X_0}{t - t_0} \tag{3}$$

where $X_t$ refers to biomass concentration (g·$L^{-1}$ DW) at a certain period of time ($t$, d) and $X_0$ is the biomass concentration (g·$L^{-1}$ DW) observed at the beginning of growth ($t_0$, d).

Specific growth rate ($\mu$, $h^{-1}$) was determined from:

$$\mu = \frac{ln(X_2) - ln(X_1)}{t_2 - t_1} \tag{4}$$

where $X_1$ and $X_2$ represent biomass concentration (g·$L^{-1}$ DW) in two consecutive moments ($t_1$ and $t_2$) of the exponential phase.

### 2.3. Microcystin quantification

The Microcystins-ADDA ELISA Kit (Abraxis, Inc., Pennsylvania, USA) was used to determine the concentration of total MC toxin (*[T]*) at the beginning of stationary phase of each growth. In order to disrupt cells, samples were frozen and thawed three times following the instructions of the kit's protocol). The amount of MC in each sample was determined following the instructions of the Microcystins-ADDA ELISA Kit. Samples were diluted according to the manufacturer's recommendations and the absorbance was measured at 450 nm using a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Vermont, USA).

Toxin productivity ($P_{toxin}$, μg toxins·$L^{-1}$·$d^{-1}$) was obtained from the following equation:

$$P_{toxin} = [T]_f \times P \tag{5}$$

where $[T]_f$ refers to toxin concentration (μg toxins·g $cells^{-1}$) at the beginning of the stationary phase and P is the biomass productivity at that point (g cells·$L^{-1}$·$d^{-1}$).

**Table 2**
Experimental design including the combination of light intensity and $CO_2$ concentration and the respective responses of $\mu_{max}$, $X_{max}$, and $P_{max}$.

| Run | $CO_2$ concentration (% v/v) | Light intensity (μmol photons·$m^{-2}$·$s^{-1}$) | $\mu_{max}$ ($h^{-1}$) | $X_{max}$ ($g·L^{-1}$) | $P_{max}$ ($g·L^{-1}$ $d^{-1}$) |
|---|---|---|---|---|---|
| 1 | 0 | 10 | 0.0118 | 1.295 | 0.067 |
| 2 | 0 | 55 | 0.0171 | 1.939 | 0.173 |
| 3 | 2.5 | 55 | 0.0190 | 1.949 | 0.149 |
| 4 | 0 | 100 | 0.0090 | 1.092 | 0.050 |
| 5 | 2.5 | 10 | 0.0125 | 0.614 | 0.040 |
| 6 | 2.5 | 100 | 0.0206 | 1.905 | 0.145 |
| 7 | 5 | 10 | 0.0108 | 1.451 | 0.049 |
| 8 | 5 | 55 | 0.0189 | 1.113 | 0.066 |
| 9 (CP)[a] | 5 | 100 | 0.0207 | 1.108 | 0.112 |
| 10 (CP)[a] | 5 | 100 | 0.0231 | 1.125 | 0.105 |
| 11 (CP)[a] | 5 | 100 | 0.0267 | 1.208 | 0.137 |
| 12 | 7.5 | 55 | 0.0250 | 1.081 | 0.090 |
| 13 | 7.5 | 100 | 0.0297 | 1.338 | 0.132 |
| 14 | 7.5 | 145 | 0.0351 | 1.266 | 0.135 |
| 15 | 10 | 55 | 0.0134 | 0.861 | 0.054 |
| 16 | 10 | 100 | 0.0233 | 0.987 | 0.059 |
| 17 | 10 | 145 | 0.0293 | 1.139 | 0.077 |
| 18 | 10 | 190 | 0.0252 | 1.168 | 0.108 |
| 19 | 5 | 145 | 0.0310 | 1.317 | 0.190 |
| 20 | 2.5 | 145 | 0.0269 | 0.936 | 0.114 |

[a] CP: Central point.

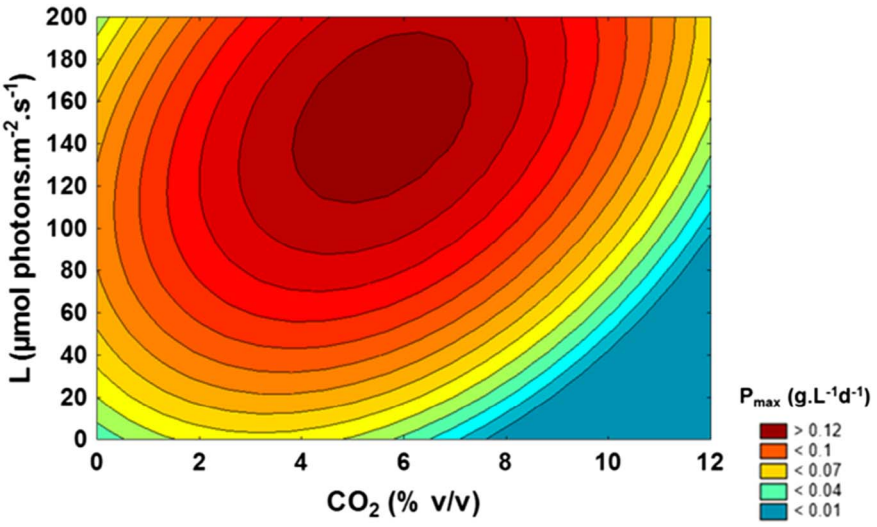


**Fig. 1.** Contour plot of maximum biomass productivity ($P_{max}$) determined for the set of light intensity (L) and $CO_2$ concentration combinations tested.

### 2.4. Statistical analysis

The experiments for quantification of microcystin were performed in triplicate. Mean values and standard errors were calculated from triplicates and used in corresponding tables and graphical representations. Statistical analyses of experimental data were performed using the Statistica 10.0.228.8 software (Statsoft Inc., USA). Analysis of variance (ANOVA) was used to estimate any statistically significant difference at a confidence level of 95%. Fitting quality of response surface models applied to the experimental data was evaluated by the coefficient of determination ($R^2$) and the root mean square error (RMSE). Desirability function analysis (DFA) derived from the predictive response surface models was applied and allowed to have a better understanding of the relationship between predictor variables (environmental factors) and response variables, as well as to verify which growth conditions can produce the most desirable/undesirable response on studied growth and toxin-related parameters.

## 3. Results and discussion

### 3.1. Effect of cultivation conditions on cell growth and toxin production

The effect of cultivation conditions (i.e. light intensity, $CO_2$ concentration, temperature and pH) on cell growth kinetics ($\mu$, $P$ and $X$) and toxin production ($[T]$ and $P_{toxin}$) was assessed through a CCD (Tables 2, 4 and 6). Overall, the study showed that the data were well fitted with a linear and/or quadratic model, where lack of fit test was not statistically significant ($p < 0.05$) and while the $R^2$ and RMSE were satisfactory.

#### 3.1.1. Combined effect of light intensity and $CO_2$ concentration on M. aeruginosa growth

The assays performed to assess the combined effect of light intensity and $CO_2$ concentration on growth-related parameters, as well as the

**Table 3**
Effect estimates, standard errors, and *t*-test for the effect of combined use of light intensity (L) and $CO_2$ concentration ($CO_2$) on $\mu_{max}$, $X_{max}$, and $P_{max}$ according to the $2^2$ full-factorial central composite design. RMSE and $R^2$ associated with each response variable are also exhibited here.

| Variables and interactions | Estimated effects | Standard errors | *t*-Value | *p* | RMSE | $R^2$ |
|---|---|---|---|---|---|---|
| $\mu_{max}$ | | | | | | |
| [a]$CO_2$ | 0.005 | 0.002 | 2.721 | **0.020** | 0.004 | 0.810 |
| [a]$(CO_2)^2$ | − 0.005 | 0.001 | − 3.348 | **0.006** | | |
| [a]$L$ | 0.007 | 0.002 | 2.879 | **0.015** | | |
| $L^2$ | − 0.004 | 0.002 | − 1.656 | 0.126 | | |
| $CO_2 \times L$ | 0.004 | 0.002 | 1.778 | 0.103 | | |
| $X_{max}$ | | | | | | |
| $CO_2$ | − 0.062 | 0.149 | − 0.415 | 0.686 | 0.349 | 0.251 |
| $(CO_2)^2$ | − 0.137 | 0.117 | − 1.170 | 0.267 | | |
| $L$ | − 0.054 | 0.191 | − 0.282 | 0.783 | | |
| $L^2$ | − 0.228 | 0.180 | − 1.267 | 0.231 | | |
| $CO_2 \times L$ | 0.222 | 0.174 | 1.276 | 0.228 | | |
| $P_{max}$ | | | | | | |
| $CO_2$ | − 0.006 | 0.014 | − 0.425 | 0.679 | 0.032 | 0.631 |
| [a]$(CO_2)^2$ | − 0.029 | 0.011 | − 2.736 | **0.019** | | |
| $L$ | 0.038 | 0.017 | 2.190 | 0.051 | | |
| $L^2$ | − 0.018 | 0.016 | − 1.102 | 0.294 | | |
| $CO_2 \times L$ | 0.017 | 0.016 | 1.058 | 0.313 | | |

[a] Significant influence at 95% confidence level.

respective values obtained for each response variable, are shown in Table 2.

The results have shown a substantial variation of all dependent variables over the different combinations of light intensity and $CO_2$ concentration tested (Table 2). Namely, $\mu_{max}$ presents a 4-fold variation (0.0090–0.0351 $h^{-1}$), while $X_{max}$ and $P_{max}$ present a 3.2-fold (0.614–1.949 $g{\cdot}L^{-1}$) and a 4.75-fold (0.040–0.190 $g{\cdot}L^{-1}{\cdot}d^{-1}$) variation, respectively.

In order to understand in more detail the influence of light intensity and $CO_2$ concentration on each of the growth associated parameters, their relation was assessed through the response surface plots (Fig. 1 – data not shown for $\mu_{max}$ and $X_{max}$) built with the data described in Table 2.

As previously mentioned, a statistical analysis was carried out aiming at identifying which independent variables had significant influence on dependent variables. The statistical significance is given by Table 3.

Fig. 1 shows that the combined effect of light intensity and $CO_2$ concentration present in air stream influenced *M. aeruginosa* growth. However, growth-based parameters were affected in different ways. From the response surface methodology, it was found that both light and $CO_2$ linear terms and quadratic term of $CO_2$ proved to be significant to $\mu_{max}$, ($p < 0.05$), while $X_{max}$ was not affected ($p > 0.05$) by any of these experimental independent variables. Regarding the $P_{max}$, only quadratic term of $CO_2$ concentration was proven to have a significant effect ($p < 0.05$).

Results from response surface methodology also suggest that higher values of $\mu_{max}$ ($> 0.03\ h^{-1}$) are possible to obtain when high light intensities (130–190 µmol photons$\cdot m^{-2}\cdot s^{-1}$) are combined with $CO_2$-rich environments in the range of 5.5 to 9.5% (v/v) (data not shown). Although the maximum values for specific growth rate were observed by [30] within the same range of $CO_2$ concentration – i.e. 5–7% (v/v) – the values reported by these authors were approximately 2-fold higher ($\approx 1.5\ d^{-1}$).

Differently to [30], who reached the highest biomass concentrations ($\approx 1\ g.L^{-1}$) using air streams presenting 7% (v/v) of $CO_2$, in our study the highest biomass concentrations ($> 1.4\ g{\cdot}L^{-1}$) were attained for medium light intensities (50–120 µmol photons$\cdot m^{-2}\cdot s^{-1}$) and aeration stream containing low to medium $CO_2$ concentration (1–6% (v/v)) – data not shown. This difference might be related to the continuous light supply (instead L:D cycles of 12:12) or to the particular features displayed by the strains of *M. aeruginosa* used.

In contrast to what happened to $\mu_{max}$ and $X_{max}$, the results of Fig. 1 indicate that $P_{max}$ varies in a different manner since higher $P_{max}$ ($> 0.12\ g{\cdot}L^{-1}{\cdot}d^{-1}$) can be obtained combining medium to high light intensities (110–190 µmol photons$\cdot m^{-2}\cdot s^{-1}$) with medium to high concentrations of $CO_2$ (4–7% (v/v)). This response is in agreement with [30] achievements since productivity was nearly the same ($\approx 0.136$–$0.154\ g{\cdot}L^{-1}{\cdot}d^{-1}$) under similar conditions of $CO_2$ (5–7% (v/v)).

According to [20,22], increasing light intensity (up to 65 and 80 µmol photons$\cdot m^{-2}\cdot s^{-1}$, respectively) also results in higher specific growth rates and cell densities, which is confirmed by the results obtained in this work.

Through second-order equations (Eq. (6)–(8)) resulting from response-surface methods (RSM) fitting of µmax, *Xmax* and *Pmax*, respectively, it was also possible to estimate the maximum values that could be eventually attained, as well as which growth conditions should be used for that purpose.

$$\mu_{max} = 5.5 \times 10^{-3} + 3.1 \times 10^{-3} \times CO_2 - 3.7 \times 10^{-4}(CO_2)^2 + 1.7 \times 10^{-4}L - 8.7 \times 10^{-7}L^2 + 1.6 \times 10^{-5}CO_2L \quad (6)$$

$$X_{max} = 1.2 - 1.3 \times 10^{-3}CO_2 - 1.1 \times 10^{-2}(CO_2)^2 + 5.7 \times 10^{-3}L - 5.6 \times 10^{-5}L^2 + 1.0 \times 10^{-3}CO_2L \quad (7)$$

$$P_{max} = 3.3 \times 10^{-2} + 1.5 \times 10^{-2}CO_2 - 2.3 \times 10^{-3}(CO_2)^2 + 1.0 \times 10^{-3}L - 4.5 \times 10^{-6}L^2 + 7.5 \times 10^{-5}CO_2L \quad (8)$$

Accordingly to these equations the maximum estimated value for $\mu_{max}$ ($0.0312\ h^{-1}$) can be obtained at 168 µmol photons$\cdot m^{-2}\cdot s^{-1}$ and 7.8% of $CO_2$, while the maximum estimated for $X_{max}$ ($1.447\ g{\cdot}L^{-1}$) is obtained at 83 µmol photons$\cdot m^{-2}\cdot s^{-1}$ and 3.7% of $CO_2$ and $P_{max}$ ($0.146\ g{\cdot}L^{-1}{\cdot}d^{-1}$) is observed at 155 µmol photons$\cdot m^{-2}\cdot s^{-1}$ and 5.5% of $CO_2$. Since $P_{max}$ was our main goal at this stage, a series of assays (triplicate) were carried out at 155 µmol photons$\cdot m^{-2}\cdot s^{-1}$ and 5.5% of $CO_2$ in order to compare the "obtained $P_{max}$" with the "estimated $P_{max}$". The results shown that the "obtained $P_{max}$" – $0.273 \pm 0.027\ g{\cdot}L^{-1}{\cdot}d^{-1}$ – is in fact considerably higher than the "estimated $P_{max}$" value. The conditions (155 µmol photons$\cdot m^{-2}\cdot s^{-1}$ and 5.5% of $CO_2$) in which the maximum $P_{max}$ was obtained were the ones used during the study of temperature and pH effect (Section 3.1.2).

**Table 4**
Experimental design including the combination of temperature and pH and the respective responses of $\mu_{max}$, $X_{max}$, and $P_{max}$.

| Run | pH | Temperature | $\mu_{max}$ ($h^{-1}$) | $X_{max}$ ($g.L^{-1}$) | $P_{max}$ ($g.L^{-1}d^{-1}$) |
|---|---|---|---|---|---|
| 21 | 6.5 | 15 | NG[a] | NG | NG |
| 22 | 8.0 | 15 | NG | NG | NG |
| 23 | 9.5 | 15 | NG | NG | NG |
| 24 | 6.5 | 25 | 0.0234 | 0.806 | 0.058 |
| 25 | 8.0 | 25 | 0.0261 | 1.235 | 0.124 |
| 26 | 9.5 | 25 | 0.0265 | 0.746 | 0.089 |
| 27 | 6.5 | 30 | 0.0220 | 0.571 | 0.058 |
| 28 (CP)[a] | 8.0 | 30 | 0.0326 | 1.474 | 0.179 |
| 29 (CP)[a] | 8.0 | 30 | 0.0326 | 1.437 | 0.174 |
| 30 (CP)[a] | 8.0 | 30 | 0.0326 | 1.512 | 0.184 |
| 31 | 9.5 | 30 | 0.0352 | 0.929 | 0.137 |
| 32 | 6.5 | 35 | 0.0443 | 1.681 | 0.133 |
| 33 | 8.0 | 35 | 0.0407 | 1.344 | 0.210 |
| 34 | 9.5 | 35 | 0.0429 | 1.239 | 0.219 |
| 35 | 6.5 | 40 | NG | NG | NG |
| 36 | 8.0 | 40 | 0.0343 | 0.895 | 0.124 |
| 37 | 9.5 | 40 | 0.0339 | 0.839 | 0.130 |

[a] CP: Central point; NG: No Growth.

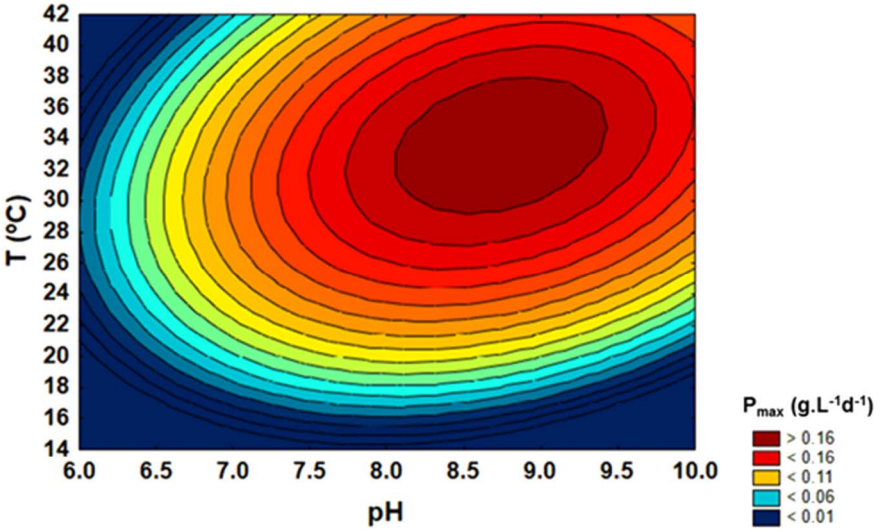


**Fig. 2.** Contour plot of maximum biomass productivity ($P_{max}$) determined for the set of temperature (T) and pH combinations tested. All growths were carried out at 155 μmol photons·m$^{-2}$·s$^{-1}$ and using a $CO_2$-rich atmosphere (5.5% (v/v)).

*3.1.2. Combined effect of temperature and pH on M. aeruginosa growth*

The combined effect of temperature and pH values on *M. aeruginosa* growth was assessed by testing 15 different combinations (Table 4). As previously mentioned, the light intensity (155 μmol photons·m$^{-2}$·s$^{-1}$) and $CO_2$ (5.5% (v/v)) conditions at which the maximum $P_{max}$ was obtained were the conditions fixed during the assays for the evaluation of temperature and pH effects.

According to these results, regardless the pH of culture medium, no growth was verified at the lowest temperature tested (15 °C). The same has happened when the highest temperature (40 °C) was applied, though it was observed just for cultures presenting a slightly acidic medium (pH 6.5). Conversely, the highest $\mu_{max}$ (0.0443 h$^{-1}$) and $X_{max}$ (1.681 g·L$^{-1}$) values were achieved in cultures grown at 35 °C and maintaining the pH of growth medium constant at 6.5. These values are contrasting with [23] where significant inhibition of *M. aeruginosa* growth is described as a consequence of acidification of the pH medium to 6.5. As shown in Table 4, our results suggest that is possible to attain great performances growing *M. aeruginosa* cells using pH values of 6.5 in culture medium; nevertheless, this behaviour can possibly occur due to the higher temperature utilized jointly with the slightly acidic medium. The highest $P_{max}$ (0.219 g·L$^{-1}$·d$^{-1}$) was obtained at the same temperature (35 °C) but using a higher pH (9.5).

Fig. 2 exhibits the contour plot of $P_{max}$, giving a clear idea about the combined effect of temperature and pH on this parameter.

Statistical analysis (summarized in Table 5) confirmed that linear term of temperature and quadratic term of pH have statistical significance on $P_{max}$ ($p < 0.05$), while $X_{max}$ and $\mu_{max}$ were only affected by the quadratic term of temperature ($p < 0.05$).

Through the response surfaces (data not shown for $\mu_{max}$ and $X_{max}$), it is possible to say that the combination of temperatures ranging between 28 and 35 °C and culture mediums presenting pH values of 7.5 to 9.0 results in favourable conditions to optimize $X_{max}$. This idea is not entirely coincident with the results obtained by [27] once the lowest cell concentration of *M. aeruginosa* was achieved using pH 7.5, while higher densities were observed when growth medium presented a pH of 9.2 and 10.5. However, those differences might exist since the assays performed by [27] were carried out solely at 24 °C while our results rely on the combined use of higher temperatures with pH variation.

On the other hand, to limit *M. aeruginosa* growth and obtain lower $X_{max}$ values, the use of temperatures below 17 °C seems to be the best strategy. Concerning $P_{max}$ behaviour, maximization is potentially obtained by coupling high temperatures (29–38 °C) with pH varying from 8.0 to 9.5. Once again, restrictions in $P_{max}$ might be found if cells are subject to temperatures lower than 18 °C. A similar reaction is also observed for $\mu_{max}$, which is negatively affected by growths carried out at temperatures under 17 °C, being the best scenarios observed when cultures are grown at 29–39 °C in mediums where pH is kept above 8.25 [31]. also studied the influence of temperature (between 27 and 36 °C) on specific growth rate but in spite of reaching greater values around 36 °C (ca. 0.65 d$^{-1}$), no significant differences were noticed among the temperatures tested. However, contrarily to what is suggested by these authors, our results showed that temperature is statistically significant for $\mu_{max}$, playing an important role in its variation, thus should not be neglected (Table 5). Furthermore, the $\mu_{max}$ achieved by [31] represents nearly half of the value obtained in our study which might be explained by the influence of the high light conditions applied here (155 μmol photons·m$^{-2}$·s$^{-1}$) when compared to their study (60 μmol photons·m$^{-2}$·s$^{-1}$).

Through the second-order equations (Eqs. (9)–(11)) resulting from RSM fitting, it was possible to obtain the maximum estimated $\mu_{max}$, $X_{max}$, $P_{max}$, respectively, and the conditions of pH and temperature under which they can be reached.

**Table 5**
Effect estimates, standard errors, and *t*-test for the effect of combined use of pH and temperature (T) on $\mu_{max}$, $X_{max}$, and $P_{max}$ according to the $2^2$ full-factorial central composite design. All growths were carried out at 155 μmol photons·m$^{-2}$·s$^{-1}$ and using a $CO_2$-rich atmosphere (5.5% (v/v)). RMSE and $R^2$ associated with each response variable are also exhibited here.

| Variables and interactions | Estimated effects | Standard errors | *t*-Value | *p* | RMSE | $R^2$ |
|---|---|---|---|---|---|---|
| $\mu_{max}$ | | | | | | |
| pH | 0.011 | 0.006 | 1.793 | 0.107 | 0.009 | 0.789 |
| pH$^2$ | − 0.008 | 0.010 | − 0.762 | 0.466 | | |
| T | 0.005 | 0.003 | 1.554 | 0.155 | | |
| [a]T$^2$ | − 0.006 | 0.002 | − 3.312 | **0.009** | | |
| pH × T | 0.005 | 0.003 | 1.430 | 0.187 | | |
| $X_{max}$ | | | | | | |
| pH | 0.158 | 0.240 | 0.660 | 0.526 | 0.377 | 0.718 |
| pH$^2$ | − 0.617 | 0.413 | − 1.493 | 0.170 | | |
| T | 0.065 | 0.137 | 0.476 | 0.645 | | |
| [a]T$^2$ | − 0.243 | 0.072 | − 3.386 | **0.008** | | |
| pH × T | 0.097 | 0.139 | 0.700 | 0.502 | | |
| $P_{max}$ | | | | | | |
| [a]pH | 0.070 | 0.026 | 2.719 | **0.024** | 0.041 | 0.815 |
| pH$^2$ | − 0.090 | 0.045 | − 2.018 | 0.074 | | |
| T | 0.023 | 0.015 | 1.523 | 0.162 | | |
| [a]T$^2$ | − 0.025 | 0.008 | − 3.174 | **0.011** | | |
| pH × T | 0.026 | 0.015 | 1.716 | 0.120 | | |

[a] Significant influence at 95% confidence level.

**Table 6**
Different combinations of the independent variables – light intensity, $CO_2$ concentration, pH, and temperature – and respective dependent responses of Toxin concentration (*[T]*) and toxin productivity ($P_{toxin}$) (mean of three replicates ± standard error).

| $CO_2$ concentration (% v/v) | Light intensity (μmol photons·m$^{-2}$·s$^{-1}$) | *[T]* (μg·g of cells$^{-1}$) | $P_{toxin}$ (μg·L$^{-1}$ d$^{-1}$) |
|---|---|---|---|
| 0 | 10 | 2710.432 ± 9.461 | 114.457 ± 0.400 |
| 0 | 55 | 4790.670 ± 973.488 | 308.629 ± 62.715 |
| 0 | 100 | 1326.684 ± 46.330 | 57.370 ± 2.003 |
| 2.5 | 10 | 2104.432 ± 44.088 | 76.058 ± 1.593 |
| 2.5 | 55 | 1530.736 ± 226.745 | 146.248 ± 21.663 |
| 2.5 | 145 | 8.139 ± 0.984 | 0.464 ± 0.056 |
| 5 | 10 | 2.195 ± 0.313 | 0.091 ± 0.013 |
| 5 | 55 | 3.681 ± 0.363 | 0.200 ± 0.020 |
| 5.5 | 155 | 6.608 ± 0.075 | 0.947 ± 0.011 |
| 7.5 | 55 | 4.836 ± 0.114 | 0.358 ± 0.008 |
| 7.5 | 100 | 4.707 ± 0.062 | 0.310 ± 0.004 |
| 7.5 | 145 | 8.072 ± 0.852 | 0.597 ± 0.063 |
| 10 | 55 | 4.774 ± 0.354 | 0.189 ± 0.014 |
| 10 | 100 | 5.852 ± 0.046 | 0.231 ± 0.002 |
| pH | Temperature (°C) | *[T]* (μg·g of cells$^{-1}$) | $P_{toxin}$ (μg·L$^{-1}$ d$^{-1}$) |
| 6.5 | 35 | 5.612 ± 0.529 | 0.498 ± 0.047 |
| 8.0 | 30 | 6.716 ± 0.667 | 0.820 ± 0.081 |
| 8.0 | 40 | 8.026 ± 0.924 | 0.694 ± 0.080 |
| 8.7 | 34 | 7.362 ± 0.684 | 0.930 ± 0.086 |
| 9.5 | 35 | 7.901 ± 0.530 | 0.530 ± 0.035 |

$$\mu_{max} = -1.5 \times 10^{-1} + 2.2 \times 10^{-2}\,\mathrm{pH} - 1.7 \times 10^{-3}\,\mathrm{pH}^2 + 5.0 \times 10^{-3}\,\mathrm{T} - 1.2 \times 10^{-4}\,\mathrm{T}^2 + 3.3 \times 10^{-4}\,\mathrm{pH\,T} \quad (9)$$

$$X_{max} = -10.9 + 2.1\,\mathrm{pH} - 1.4 \times 10^{-1}\,\mathrm{pH}^2 + 2.5 \times 10^{-1}\,\mathrm{T} - 4.9 \times 10^{-3}\,\mathrm{T}^2 + 6.5 \times 10^{-3}\,\mathrm{pH\,T} \quad (10)$$

$$P_{max} = -1.4 + 2.9 \times 10^{-1}\,\mathrm{pH} - 2.0 \times 10^{-2}\,\mathrm{pH}^2 + 1.8 \times 10^{-2}\,\mathrm{T} - 4.9 \times 10^{-4}\,\mathrm{T}^2 + 1.7 \times 10^{-3}\,\mathrm{pH\,T} \quad (11)$$

Accordingly to these equations the maximum estimated value for $\mu_{max}$ (0.0398 h$^{-1}$) can be obtained at 34 °C and pH of 9.4, while the maximum estimated $X_{max}$ (1.370 g·L$^{-1}$) is obtained at 31 °C and pH of 8.2 and $P_{max}$ (0.179 g·L$^{-1}$·d$^{-1}$) is obtained at 34 °C and pH 8.7. Repeating the same rationale employed for the effects of light intensity and $CO_2$ concentration (see Section 3.1.1), an assay mimicking the estimated optimal growth conditions found for $P_{max}$ was performed. The experiment was done in triplicate and the "obtained $P_{max}$" (0.186 ± 0.027 g·L$^{-1}$·d$^{-1}$) was very similar to the "expected $P_{max}$" (0.179 g·L$^{-1}$·d$^{-1}$).

### 3.1.3. Combined effect of environmental factors on microcystin production

The assays performed to assess the combined effect of light intensity, $CO_2$ concentration, pH and temperature on toxin production, as well as the respective values obtained for each response variable, are shown in Table 6.

According to the results presented in Table 6, microcystin concentration (2.2–4790.7 μg·g of cells$^{-1}$) and productivity (0.1–308.6 μg·L$^{-1}$·d$^{-1}$) experienced a strong variation (2177-fold and 3086-fold, respectively) as result of the utilization of different combinations of light intensity, $CO_2$ concentration, pH, and temperature. The results show that the highest *[T]* (4790.7 μg·g cells$^{-1}$) and $P_{toxin}$ (308.6 μg·L$^{-1}$·d$^{-1}$) were both achieved using the same growth conditions, i.e. 55 μmol photons·m$^{-2}$·s$^{-1}$ and no additional $CO_2$ input to the air stream. These conditions can be regarded as mild conditions since they are close (in average) to what cells can experience in natural conditions. It is also possible to conclude that the higher *[T]* and $P_{toxin}$ were obtained in the assays performed with $CO_2$ concentrations between 0 and 2.5%. Conversely, cultures grown under 10 μmol photons·m$^{-2}$·s$^{-1}$ and subjected to a $CO_2$-enriched environment containing 5% (v/v) presented the lowest values both for *[T]* (2.2 μg·g·cells$^{-1}$) and $P_{toxin}$ (0.1 μg·L$^{-1}$·d$^{-1}$). All the lower *[T]* and $P_{toxin}$ were obtained in the growths performed at high (≥ 5%) $CO_2$ concentrations.

A more clear perception of how different combinations of light intensity and $CO_2$ concentration might affect *[T]* and $P_{toxin}$ can be visualized through the contour plot (Fig. 3) built with data from Table 6.

According to Fig. 3A, and confirming the conclusions withdrawn from Table 6, higher concentrations of toxin are attained by coupling very low to medium light quantities (below 60 μmol photons·m$^{-2}$·s$^{-1}$) with low levels of $CO_2$ available (0–1% (v/v)). These results are corroborated by [22] where a sharp decrease was observed for toxin production of *M. aeruginosa* cells grown under 8 μmol photons·m$^{-2}$·s$^{-1}$ when compared to another ones performed at 65 μmol photons·m$^{-2}$·s$^{-1}$.

According to Fig. 3B, $P_{toxin}$ also follows the behaviour found for *[T]* reaching values > 160 μg·L$^{-1}$·d$^{-1}$ under similar growth conditions (light intensities below 80 μmol photons·m$^{-2}$·s$^{-1}$ combined with $CO_2$ concentrations never exceeding 1% (v/v)). Lower *[T]* and $P_{toxin}$ are obtained under high light intensities (> 140 μmol photons·m$^{-2}$·s$^{-1}$) or combining light intensities below 100 μmol photons·m$^{-2}$·s$^{-1}$ with high $CO_2$ concentrations (> 6% (v/v)). Despite the marked differences observed, the results do not show a statistically significant effect ($p > 0.05$) of neither of the two environmental factors on *[T]* and $P_{toxin}$ (Table 7).

To sum-up, extreme conditions (i.e., very different from natural environment) like high light intensities but mainly $CO_2$-rich environments seems to reduce *M. aeruginosa* toxicity, while conditions that are more close to the natural environments might be more prone to improve toxin productivity rates.

Regarding *M. aeruginosa* toxicity changes observed as a result of variation on temperatures and pH (Table 6), one can see these results are in the same range as those obtained when high $CO_2$ concentrations were applied, maintaining values below 10 μg·g·cells$^{-1}$ and 1 μg·L$^{-1}$·d$^{-1}$ for *[T]* and $P_{toxin}$, respectively. The low values obtained for *[T]* and $P_{toxin}$ in this set of experiments confirm that despite the combined use of 155 μmol photons·m$^{-2}$·s$^{-1}$ of light and 5.5% (v/v) of $CO_2$ being suitable to reach maximum $P_{max}$ (Section 3.1.1), these conditions do not favour the production and productivity of MC.

Although these variations verified for *[T]* and $P_{toxin}$ are not in the same magnitude as the ones observed when the influence of light intensity and $CO_2$ concentration was tested, the fact is that linear term and quadratic term of pH were found to have a statistically significant effect on *[T]* and $P_{toxin}$ (Table 8), respectively.

Fig. 4 shows contour surfaces *[T]* and $P_{toxin}$ under the effects of temperature and pH.

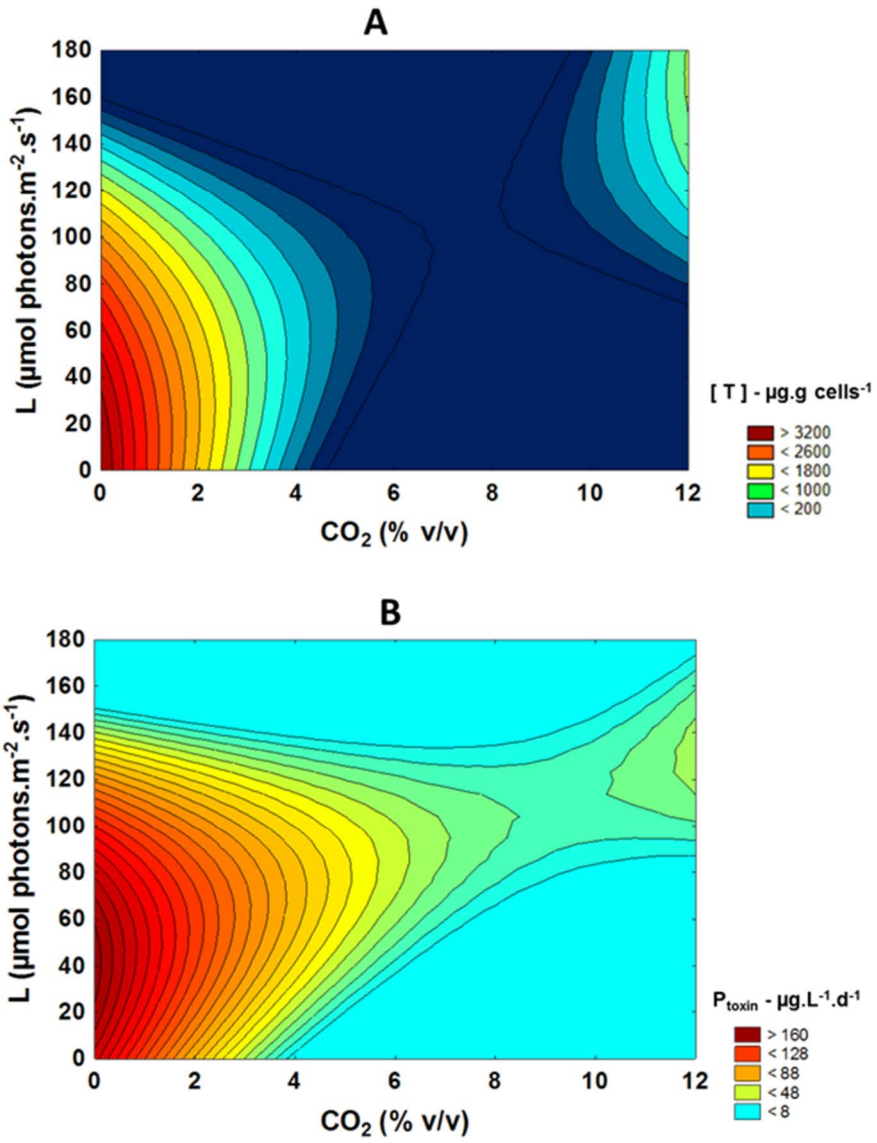


**Fig. 3.** Contour plot of A) toxin concentration (*[T]*) and, B) toxin productivity ($P_{toxin}$) determined for the set of light intensity (L) and $CO_2$ concentration combinations tested ($n = 3$).

**Table 7**
Effect estimates, standard errors, and *t*-test for the effect of combined use of light intensity (L) and $CO_2$ concentration ($CO_2$) on *[T]* and $P_{toxin}$ according to the $2^2$ full-factorial central composite design. RMSE and $R^2$ associated with each response variable are also exhibited here.

| Variables and interactions | Estimated effects | Standard errors | *t*-Value | *p* | RMSE | $R^2$ |
|---|---|---|---|---|---|---|
| *[T]* | | | | | | |
| $CO_2$ | − 1849.810 | 814.216 | − 2.272 | 0.053 | 807.307 | 0.811 |
| $(CO_2)^2$ | 1735.780 | 1237.350 | 1.403 | 0.198 | | |
| $L$ | − 728.980 | 573.789 | − 1.270 | 0.240 | | |
| $L^2$ | − 505.450 | 518.396 | − 0.975 | 0.358 | | |
| $CO_2 \times L$ | 1653.090 | 892.231 | 1.853 | 0.101 | | |
| $P_{toxin}$ | | | | | | |
| $CO_2$ | − 115.908 | 63.756 | − 1.818 | 0.107 | 63.215 | 0.690 |
| $(CO_2)^2$ | 53.628 | 96.888 | 0.554 | 0.595 | | |
| $L$ | − 59.118 | 44.929 | − 1.316 | 0.225 | | |
| $L^2$ | − 60.666 | 40.592 | − 1.495 | 0.173 | | |
| $CO_2 \times L$ | 100.675 | 69.864 | 1.441 | 0.188 | | |

**Table 8**
Effect estimates, standard errors, and *t*-test for the effect of combined use of pH and temperature (T) on *[T]* and $P_{toxin}$ according to the $2^2$ full-factorial central composite design. All growths were carried out at 155 µmol photons·$m^{-2}$·$s^{-1}$ and using a $CO_2$-rich atmosphere (5.5% (v/v)). RMSE and $R^2$ associated with each response variable are also exhibited here.

| Variables and interactions | Estimated effects | Standard errors | *t*-Value | *p* | RMSE | $R^2$ |
|---|---|---|---|---|---|---|
| *[T]* | | | | | | |
| [a]pH | 2.289 | 0.681 | 3.362 | **0.020** | 0.681 | 0.772 |
| $pH^2$ | − 0.481 | 1.636 | − 0.294 | 0.780 | | |
| T | 1.311 | 0.681 | 1.925 | 0.112 | | |
| $T^2$ | 0.748 | 1.567 | 0.478 | 0.653 | | |
| $P_{toxin}$ | | | | | | |
| pH | 0.031 | 0.104 | 0.302 | 0.775 | 0.104 | 0.834 |
| [a]$pH^2$ | − 1.041 | 0.251 | − 4.149 | **0.009** | | |
| T | − 0.126 | 0.104 | − 1.202 | 0.283 | | |
| $T^2$ | − 0.556 | 0.240 | − 2.312 | 0.069 | | |

[a] Significant influence at 95% confidence level.

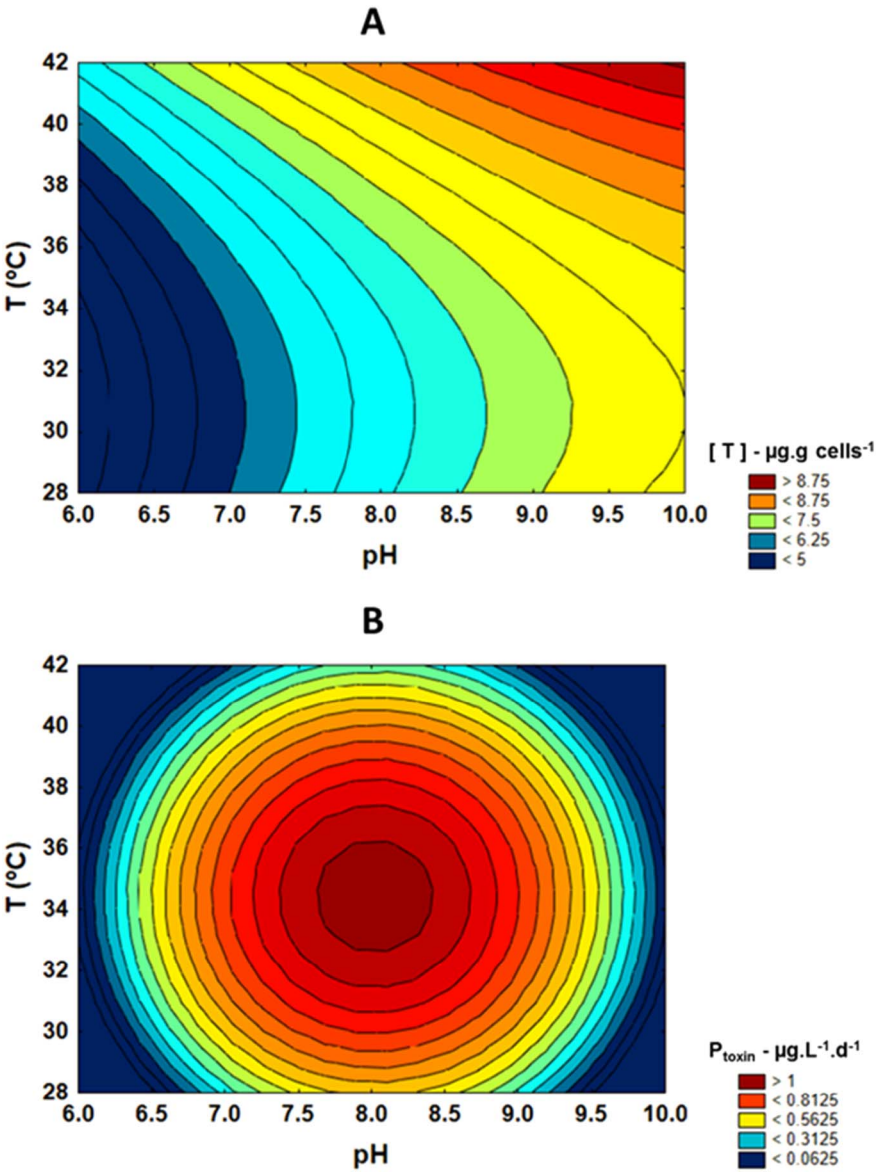


**Fig. 4.** Contour plot of A) toxin concentration (*[T]*) and, B) toxin productivity ($P_{toxin}$) determined for the set of temperature and medium pH combinations tested for cultures grown at 155 µmol photons·m$^{-2}$·s$^{-1}$ using a $CO_2$-rich atmosphere (5.5% (v/v)) (n = 3).

Among the conditions tested, it is possible to infer through Fig. 4A graph that the combination of high temperatures with high medium pH represent allow reaching greater toxin contents (> 8.75 µg·g cells$^{-1}$). These results are not in agreement with other studies reporting that increases in temperature (15–36 °C) lead to a decrease of toxin production [22,31]. On the other hand, [27] evaluated the effect of pH medium on toxin concentration by *M. aeruginosa* concluding that higher pH values (9.2–10.5) have a positive impact in opposition to lower ones (7.5), which is in agreement with results from Fig. 4A.

Combined use of temperatures below 40 °C and pH values lower than 7.5 tend to have a negative impact over the amount of toxin produced (< 5 µg·g cells$^{-1}$). Regarding $P_{toxin}$, there is a distinct region of conditions where high values can be attained (> 1 µg·L$^{-1}$d$^{-1}$). Those conditions were achieved at temperatures between 33 and 36 °C along with medium pH ranging from 7.5 to 8.5. From the tested combinations it was also possible to conclude that the joint effect of 30 °C or 40 °C with extreme pH values (6.5 and 9.5) was found to be responsible for lower productivities (< 0.0625 µg·L$^{-1}$·d$^{-1}$).

## 4. Conclusions

A great impact of the combined effect of light intensity, $CO_2$ concentration, temperature, and pH, was observed on both *Microcystis aeruginosa* growth parameters and MC production. Results suggest that maintaining cultures under light intensities below 80 µmol photons·m$^{-2}$·s$^{-1}$ and low $CO_2$ concentrations (< 1% v/v) provides suitable conditions to reach high toxin production. On the other hand, the combination of high light intensities and $CO_2$ concentrations (155 µmol photons·m$^{-2}$·s$^{-1}$ and 7.5% (v/v), respectively) with slightly acidic environments (pH 6.5) at 30 °C has been pointed as the best condition to apply when toxin production restriction is needed, indicating a significant negative effect over MC synthesis process.

These results constitute significant insights on *M. aeruginosa* growth dynamics and MC production, triggering events which can be applied to predict the response of such cyanobacteria to variations occurring in their surrounding environment. Additionally, taking into account the rising expectations on the potential uses of *M. aeruginosa*, these results represent useful information as starting point to develop a suitable strategy in order to optimize growth conditions and, eventually, the production of its high added-value products.

## References

[1] H.K. Hudnell, Q. Dortch, H. Zenick, Chapter 1: an overview of the Interagency,

International Symposium on Cyanobacterial Harmful Algal Blooms (ISOC-HAB): advancing the scientific understanding of freshwater harmful algal blooms, in: H.K. Hudnell (Ed.), Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs, New York, NY, Springer New York, 2008, pp. 1–16.
[2] J.M. O'Neil, T.W. Davis, M.A. Burford, C.J. Gobler, The rise of harmful cyanobacteria blooms: the potential roles of eutrophication and climate change, Harmful Algae 14 (2012) 313–334.
[3] L. Bláhová, O. Adamovský, L. Kubala, L. Švihálková Šindlerová, R. Zounková, L. Bláha, The isolation and characterization of lipopolysaccharides from *Microcystis aeruginosa*, a prominent toxic water bloom forming cyanobacteria, Toxicon 76 (2013) 187–196.
[4] S. Zhou, Y. Shao, N. Gao, Y. Deng, J. Qiao, H. Ou, J. Deng, Effects of different algaecides on the photosynthetic capacity, cell integrity and microcystin-LR release of *Microcystis aeruginosa*, Sci. Total Environ. 463–464 (2013) 111–119.
[5] D. Gutiérrez-Praena, A. Campos, J. Azevedo, J. Neves, M. Freitas, R. Guzmán-Guillén, A. Cameán, J. Renaut, V. Vasconcelos, Exposure of lycopersicon esculentum to microcystin-LR: effects in the leaf proteome and toxin translocation from water to leaves and fruits, Toxins 6 (6) (2014) 1837.
[6] J. Martins, V. Vasconcelos, Microcystin dynamics in aquatic organisms, J. Toxicol. Environ. Health B Crit. Rev. 12 (2009) 65–82.
[7] M.L. Saker, M. Welker, V.M. Vasconcelos, Multiplex PCR for the detection of toxigenic cyanobacteria in dietary supplements produced for human consumption, Appl. Microbiol. Biotechnol. 73 (5) (2007) 1136–1142.
[8] S. Saqrane, I.E. Ghazali, B. Oudra, L. Bouarab, V. Vasconcelos, Effects of cyanobacteria producing microcystins on seed germination and seedling growth of several agricultural plants, J. Environ. Sci. Health, Part B 43 (2008) 443–451.
[9] Z.A. Mohamed, M.A. Deyab, M.I. Abou-Dobara, A.K. El-Sayed, W.M. El-Raghi, Occurrence of cyanobacteria and microcystin toxins in raw and treated waters of the Nile River, Egypt: implication for water treatment and human health, Environ. Sci. Pollut. Res. 22 (2015) 11716–11727.
[10] Z.A. Mohamed, M.A. Deyab, M.I. Abou-Dobara, W.M. El-Raghi, Occurrence of toxic cyanobacteria and microcystin toxin in domestic water storage reservoirs, Egypt, J. Water Supply Res. Technol. AQUA 65 (2016) 431–440.
[11] J. Jia, W. Luo, Y. Lu, J.P. Giesy, Bioaccumulation of microcystins (MCs) in four fish species from Lake Taihu, China: assessment of risks to humans, Sci. Total Environ. 487 (2014) 224–232.
[12] H.K. Hudnell, Q. Dortch, Chapter 2: a synopsis of research needs identified at the Interagency, International Symposium on Cyanobacterial Harmful Algal Blooms (ISOC-HAB), in: H.K. Hudnell (Ed.), Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs, New York, NY, Springer New York, 2008, pp. 17–43.
[13] E.M. Jochimsen, W.W. Carmichael, J. An, D.M. Cardo, S.T. Cookson, C.E.M. Holmes, M.B. Antunes, D.A. de Melo Filho, T.M. Lyra, V.S.T. Barreto, S.M.F.O. Azevedo, W.R. Jarvis, Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil, N. Engl. J. Med. 338 (13) (1998) 873–878.
[14] World Health Organization, Cyanobacterial Toxins: Microcystin-LR in Drinking Water Background Document for Preparation of WHO Guidelines for Drinking-water Quality, (2003).
[15] L.A. Lawton, C. Edwards, Chapter 23: conventional laboratory methods for cyanotoxins, in: H.K. Hudnell (Ed.), Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs, New York, NY, Springer New York, 2008, pp. 513–537.
[16] J.A. Meriluoto, L.E. Spoof, Chapter 21: cyanotoxins: sampling, sample processing and toxin uptake, in: H.K. Hudnell (Ed.), Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs, New York, NY, Springer New York, 2008, pp. 483–499.
[17] R.A. Pegram, A.R. Humpage, B.A. Neilan, M.T. Runnegar, T. Nichols, R.W. Thacker, S. Pflugmacher, S.M. Etheridge, A.H. Love, Chapter 15: cyanotoxins workgroup report, in: H.K. Hudnell (Ed.), Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs, New York, NY, Springer New York, 2008, pp. 513–537.
[18] P. Geada, S. Gkelis, J. Teixeira, V. Vasconcelos, A. Vicente, B. Fernandes, Chapter 17: cyanobacterial toxins as a high added-value product, in: R. Muñoz, C. Gonzalez (Eds.), Microalgae-based Biofuels and Bioproducts, Woodhead Publishing, Cambridge, UK, 2017, pp. 405–432.
[19] C.P. Deblois, P. Juneau, Relationship between photosynthetic processes and microcystin in *Microcystis aeruginosa* grown under different photon irradiances, Harmful Algae 9 (1) (2010) 18–24.
[20] Z. Yang, L. Geng, W. Wang, J. Zhang, Combined effects of temperature, light intensity, and nitrogen concentration on the growth and polysaccharide content of *Microcystis aeruginosa* in batch culture, Biochem. Syst. Ecol. 41 (2012) 130–135.
[21] C.P. Deblois, P. Juneau, Comparison of resistance to light stress in toxic and non-toxic strains of *Microcystis aeruginosa* (Cyanophyta), J. Phycol. 48 (4) (2012) 1002–1011.
[22] S. Jähnichen, B.M. Long, T. Petzoldt, Microcystin production by *Microcystis aeruginosa*: direct regulation by multiple environmental factors, Harmful Algae 12 (2011) 95–104.
[23] X. Wang, C. Hao, F. Zhang, C. Feng, Y. Yang, Inhibition of the growth of two blue-green algae species (*Microsystis aruginosa* and *Anabaena spiroides*) by acidification treatments using carbon dioxide, Bioresour. Technol. 102 (10) (2011) 5742–5748.
[24] Y. Yamamoto, H. Nakahara, Competitive dominance of the cyanobacterium *Microcystis aeruginosa* in nutrient-rich culture conditions with special reference to dissolved inorganic carbon uptake, Phycol. Res. 53 (3) (2005) 201–208.
[25] Y. Liu, L. Li, R. Jia, The optimum resource ratio (N:P) for the growth of *Microcystis aeruginosa* with abundant nutrients, Prog. Environ. Sci. 10 (Part C) (2011) 2134–2140.
[26] J.N. Bouchard, D.A. Purdie, Effect of elevated temperature, darkness, and hydrogen peroxide treatment on oxidative stress and cell death in the bloom-forming toxic cyanobacterium *Microcystis aeruginosa*, J. Phycol. 47 (6) (2011) 1316–1325.
[27] T. Krüger, N. Hölzel, B. Luckas, Influence of cultivation parameters on growth and microcystin production of *Microcystis aeruginosa* (Cyanophyceae) isolated from Lake Chao (China), Microb. Ecol. 63 (1) (2012) 199–209.
[28] J. Kotai, Instructions for Preparation of Modified Nutrient Solution Z8 for Algae, Norwegian Institute for Water Research, Blindern, Oslo, 1972, p. 5.
[29] M.J. Griffiths, C. Garcin, R.P. van Hille, S.T.L. Harrison, Interference by pigment in the estimation of microalgal biomass concentration by optical density, J. Microbiol. Methods 85 (2) (2011) 119–123.
[30] A.L. Gonçalves, C.M. Rodrigues, J.C.M. Pires, M. Simões, The effect of increasing $CO_2$ concentrations on its capture, biomass production and wastewater bioremediation by microalgae and cyanobacteria, Algal Res. 14 (2016) 127–136.
[31] M.A.D. Mowe, C. Porojan, F. Abbas, S.M. Mitrovic, R.P. Lim, A. Furey, D.C.J. Yeo, Rising temperatures may increase growth rates and microcystin production in tropical Microcystis species, Harmful Algae 50 (2015) 88–98.