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

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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

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[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·$L^{-1}$·$d^{-1}$).