Universidade do Minho
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Development and optimization of cultivation systems and techniques in order to improve cyanotoxin productivity and cost effectiveness

February, 2018

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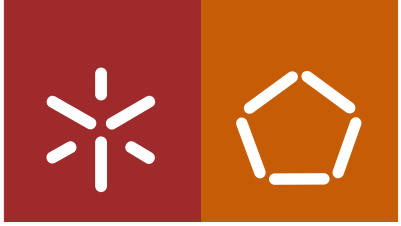

Universidade do Minho
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Development and optimization of cultivation systems and techniques in order to improve cyanotoxin productivity and cost effectiveness

PhD in Bioengineering

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February, 2018

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

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University of Minho, 09/02/2018

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"Science is different to all the other systems of thoughts...because you don't need faith in it, you can check that it works."

Brian Cox

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ABSTRACT

Development and optimization of cultivation systems and techniques in order to improve cyanotoxin productivity and cost effectiveness

Microcystis aeruginosa and its toxins, microcystins (MCs), constitute a threat for humans and animals. This cyanobacterium is spreading across the globe and with that, the risk of exposure to MCs arises, mainly through water consumption and aquatic recreational activities. Despite of the negative impact of these toxins, which was already responsible for several human and animal deaths, there is a huge biotechnological potential since MCs can be used in scientific research and environmental risk assessment studies. Additionally, MCs are also pointed as a promising agent to treat/reduce symptoms associated with cancer and Parkinson's disease. Therefore, this study focused on increasing the cost-effectiveness of the production process of MCs through the optimization of: i) environmental factors that affect cell growth and toxin production; ii) different growth strategies to enhance toxin productivity; and iii) downstream processing.

The synergistic interactions between light intensity, CO_2 concentration, temperature, and pH unveiled significant impacts on *M. aeruginosa*'s growth-related parameters and toxicity. The combined use of low to medium light intensities (50–120 $\mu mol_{photons}.m^{-2}.s^{-1}$) and CO_2 concentrations (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 $\mu mol_{photons}.m^{-2}.s^{-1}$), CO_2 concentrations (4–9.5 % v/v) and temperatures (29–39 °C). Regarding MCs production, higher concentrations were obtained at low light intensities and low CO_2 concentrations. Conversely, approximately 2000-fold lower MCs concentrations were achieved by simultaneous use of high values of light intensity (> 140 $\mu mol_{photons}.m^{-2}.s^{-1}$) and CO_2 concentration (> 6 % v/v).

The cyanobacteria growth was greatly affected by the type of photobioreactor (PBR) utilized, providing different light exposure and distribution of cells inside the vessel due to specifities of the flow patterns of the culture. These factors are influenced by the hydrodynamics of the system that, in turn, has impact on the system's mass transfer parameters. Bubble column (BC) and flat panel (FP) PBRs reached higher biomass concentrations than split cylinder airlift (SCA) – 76 and 98 %, respectively. This is an interesting finding since both PBRs present a continuously illuminated surface which is

approximately 20 % lower when compared to that of SCA. With respect to maximum biomass productivity (P_{max}) and specific growth rate (μ_{max}), FP also showed the best results – up to 40 and 57 % higher, respectively.

A two-stage growth approach was also tested as strategy to optimize MCs productivity. In a first phase, the conditions were set to maximize the cyanobacteria growth, followed by a second phase where growth conditions were changed in order to induce MCs accumulation. As result, the initial stage has presented the highest P_{max} (≈ 0.088 $g.L^{-1}.d^{-1}$) and μ_{max} (≈ 0.025 d^{-1}), whilst a 67 % increment of MCs concentration was reached in the second stage when compared to control.

The *M. aeruginosa* growth and MCs accumulation has demonstrated to be considerably affected by the presence of filtrates and extracts of other photosynthetic microorganisms. The highest growth enhancement (17 %) was achieved with 20 % of extract of non-toxic *M. aeruginosa* (NTMASF20). The production of toxin in this culture was however similar to the control. Applying 20 % of filtrate of *S. obliquus* (SOF20) and extract of *C. vulgaris* (CVSF20), there was an inhibition of toxic *M. aeruginosa* growth of approximately 13 and 12 %, respectively. The impact on MCs accumulation was inexistent for CVSF20, while SOF20 resulted in an increment of 37 %. However, the conditions that resulted in a higher production of MCs were 20 % of filtrate (CEF20) and 10 % of extract of *C. emersonii* (CESF10), with concentrations roughly 49 and 58 % greater than the control, respectively.

Several harvesting methods were tested, optimized and compared. Among the pH-induced flocculation tests, the highest harvesting efficiencies (*HE*s) were achieved at pH 3 and 4 – *HE*s > 90 % after 8 h. The adjustment of zeta potential (ZP) to values comprised between -6.7 and -20.7 mV enhanced significantly the settling rates using flocculation agents. The best results were obtained with $FeCl_3$ where the *HE* was incremented in 88 % by ZP adjustment, achieving 92 % in 4 h. The use of iron oxide magnetic microparticles (IOMMs) has shown to be a very promising alternative to conventional harvesting methods once it allowed reaching *HE*s of 93.6 % with just 4 min of treatment. The optimal conditions verified for this methodology were a IOMMs:cells (g/g) ratio of 0.8:1 and pH 12.

Different cell disruption methods were also tested, optimized and compared. The optimal conditions found for each disruption method tested were: i) 20 % of beads and

treatment time of 7 min (bead milling); ii) 800 W for 1.5 min (microwave); iii) three 12-h freezing cycles at -20 ˚C (freeze-thaw cycles - FTC); iv) 15000 rpm for 7 min (high-speed homogenization - HSH); and v) 40 kHz for 10 min (sonication). Sonication and FTC followed by sonication revealed to be the most efficient methodologies to apply on MCs release and *IOMR*. The impact on cells' viability was though more evident in FTC, FTC followed by sonication, and microwave where only 0.3, 0.05 and 0.9 % of the initial cells, respectively, maintained their viability after being treated. On the other hand, sonication and bead milling reduced the viability of the original culture to 5 and 15.5 %, respectively, while the HSH did not show any significant differences compared to control.

The results obtained in this thesis, as well as the optimization of several steps of the production process of MCs by *M. aeruginosa*, allowed concluding that the improvement of the cost-effectiveness of the process is feasible and that a widespread use of such toxins in various biotechnological fields might become a reality in the future.

Desenvolvimento e otimização de sistemas e técnicas de cultivo para melhorar a produtividade de cianotoxinas e relação custo-benefício

Atualmente, a *Microcystis aeruginosa* e as suas toxinas, microcistinas (MCs), constituem uma ameaça para seres humanos e animais. Esta cianobactéria tem-se disseminado um pouco por todo o globo, o que acarreta sérios riscos de exposição às MCs nomeadamente através do consumo de água ou de contacto através de atividades aquáticas recreativas. Apesar do impacto negativo que as MCs podem ter – já foram inclusive responsáveis por várias mortes de pessoas e animais –, estes compostos apresentam também um enorme potencial biotecnológico, uma vez que são utilizados para investigação científica e em estudos de avaliação de risco ambiental. Além disto, estas toxinas foram também apontadas como muito promissoras para tratar ou reduzir sintomas associados a cancro ou à doença de Parkinson. Consequentemente, este trabalho focou-se no aumento do custo-benefício do processo produtivo da MC através da otimização de: i) fatores ambientais que afetem o crescimento celular e a produção de toxina; ii) diferentes estratégias de crescimento para melhorar a produtividade da toxina; e iii) processamento a jusante.

As interações sinergísticas entre intensidade de luz, concentração de CO_2, temperatura e pH revelaram ter um impacto significativo no crescimento de *M. aeruginosa* e correspondente toxicidade. A utilização combinada de intensidades de luz (50–120 μmol fotões.m^{-2}.s^{-1}) e concentrações de CO_2 (1–6 % v/v) baixas a intermédias levou a maiores concentrações celulares, enquanto que a taxa específica de crescimento e a produtividade de biomassa foram favorecidas por intensidades de luz (110–190 μmol photons.m^{-2}.s^{-1}), concentrações de CO_2 (4–9.5 % v/v) e temperaturas (29–39 °C) intermédias a altas. Relativamente à produção de toxina, as maiores concentrações foram alcançadas utilizando baixas intensidades de luz e concentrações de CO_2. Pelo contrário, concentrações de MCs cerca de 2000 vezes inferiores foram obtidas através do uso simultâneo de altas intensidades de luz (> 140 μmol fotões.m^{-2}.s^{-1}) e concentrações de CO_2 (> 6 % v/v).

O crescimento de cianobactérias foi bastante influenciado pelo tipo de fotobioreator utilizado que, por sua vez, afeta a exposição das células à luz e a sua distribuição no interior do reator, o padrão do fluxo da cultura e também todos os parâmetros hidrodinâmicos e de transferência de massa. A coluna de bolhas e o reator de placas

atingiram concentrações celulares superiores às observadas no *airlift* – 76 e 98 %, respetivamente. Este facto é bastante interessante, tendo em conta que a superfície continuamente iluminada apresentada por ambos os fotobioreatores é menor em cerca de 20 % da correspondente ao *airlift*. Quanto à produtividade máxima de biomassa (P_{max}) e à taxa específica de crescimento máxima (μ_{max}), o reator de placas voltou a apresentar os melhores resultados – até cerca de 40 e 57 % maiores, respetivamente.

Implementou-se também uma estratégia de crescimento em duas fases de forma a otimizar a produtividade de toxina. Na primeira fase, as condições de crescimento foram definidas no sentido de maximizar o crescimento celular, seguindo-se uma segunda fase onde as condições foram alteradas de modo a induzir a acumulação de MCs. Como esperado, a primeira fase apresentou os valores de P_{max} (≈ 0.088 $g.L^{-1}.d^{-1}$) e de μ_{max} (≈ 0.025 d^{-1}) mais elevados, enquanto que na segunda fase foi registado um aumento de 67 % da concentração de MCs quando comparada com a do controlo.

O crescimento de *M. aeruginosa* e a acumulação de MCs foram consideravelmente afetados pela presença de filtrados e extratos de outros microrganismos fotossintéticos. O maior incremento no crescimento, cerca de 17 %, foi obtido com 20 % de extrato da estirpe não-tóxica de *M. aeruginosa* (NTMASF20). No entanto, neste caso a produção de toxina manteve-se semelhante à do controlo. Nas culturas em que foram adicionados 20 % de filtrado de *S. obliquus* (SOF20) e de extrato de *C. vulgaris* (CVSF20) houve uma inibição do crescimento de aproximadamente 13 e 12 %, respetivamente. O impacto na produção de MCs foi inexistente para CVSF20, enquanto que SOF20 resultou num aumento de 37 %. Ainda assim, as condições que resultaram na maior produção de MCs foram a utilização de 20 % de filtrado (CEF20) e 10 % de extrato de *C. emersonii* (CESF10), com as concentrações a ultrapassarem o controlo em cerca de 49 e 58 %, respetivamente.

Testaram-se, otimizaram-se e compararam-se vários métodos de colheita. De entre os testes de floculação induzida por pH realizados, as maiores eficiências de colheita ocorreram a pH 3 e 4 – eficiências > 90 % após 8 h. O ajuste dos valores de potencial zeta a valores compreendidos entre -6.7 e -20.7 mV aumentou significativamente as taxas de sedimentação usando agentes floculantes. Os melhores resultados foram obtidos com $FeCl_3$ onde a eficiência de colheita registou subidas até 88 %, atingindo eficiências de 92 % em 4 h. A utilização de micropartículas magnéticas de óxido de ferro provou ser uma alternativa promissora aos métodos convencionais, uma vez que

possibilitou a obtenção de eficiências de colheita de 93.6 % em apenas 4 min. As condições ótimas definidas para esta metodologia foram a utilização de pH 12 e um rácio partículas:células (g/g) de 0.8:1.

Testaram-se, otimizaram-se e compararam-se também diferentes métodos de rutura celular. As condições ótimas definidas para cada um dos métodos de rutura celular testados foram as seguintes: i) 20 % de esferas de vidro e 7 min de tratamento (moinho de bolas); ii) 800 W durante 1.5 min (microondas); iii) três ciclos de congelamento de 12 h a -20 ˚C (congelamento-descongelamento); iv) 15000 rpm durante 7 min (homogeneização de alta velocidade); v) 40 kHz durante 10 min (sonicação). A sonicação e o congelamento-descongelamento seguido de sonicação revelaram ser os métodos mais eficientes para a extração de MCs e para a libertação de matéria orgânica intracelular. Contudo, o impacto na viabilidade celular foi mais evidente no caso do congelamento-descongelamento, congelamento-descongelamento seguido de sonicação e microondas, onde apenas 0.3, 0.05 e 0.9 % das células da cultura inicial, respetivamente, se mantiveram viáveis após serem tratadas. Por outro lado, a sonicação e o moinho de bolas reduziram a viabilidade da cultura original para 5 e 15.5 %, respetivamente, enquanto que a homogeneização de alta velocidade não apresentou alterações significativas quando comparada com o controlo.

Os resultados obtidos nesta tese, assim como a otimização de várias etapas do processo de produção de MCs através de *M. aeruginosa*, permitiram concluir que é possível aumentar o custo-benefício do processo e que uma utilização alargada destas toxinas em múltiplos sectores biotecnológicos pode tornar-se uma realidade no futuro.

TABLE OF CONTENTS

List of publications

This thesis is based on the following original publications:

Book chapters:

Geada, **P**., Gkelis, S., Teixeira, J.A., Vasconcelos, V., Vicente, A.A., Fernandes, B., 2017. Chapter 17 - Cyanobacterial toxins as a high added value product, In Muñoz, R. and Gonzalez, C. (Eds). Microalgae-Based Biofuels and Bioproducts, Woodhead Publishing, Cambridge, UK, pp. 405-432.

Geada, **P**., Vasconcelos, V., Vicente, A.A., Fernandes, B., 2017. Chapter 13 - Microalgal Biomass Cultivation, In Rastogi, R.P., Madamwar, D. and Pandey, A. (Eds). Algal Green Chemistry: Recent progress in Biotechnology (Biotechnology series), Elsevier, Amsterdam, Netherlands, pp. 257-284.

Papers in peer-reviewed journals:

Geada, **P**.; Pereira, R.N.; Vasconcelos, V.; Vicente, A.A.; Fernandes, B.D., 2017. Assessment of synergistic interactions between environmental factors on *Microcystis aeruginosa* growth and microcystin production, Algal Research, 27, 235-243.

Geada, **P**., Loureiro, L., Escobar, L., Jewkes, A., Teixeira, J.A., Vasconcelos, V., Vicente, A.A., Fernandes, B., *Microcystis aeruginosa* disruption/permeabilization methodologies study and comparison in order to obtain high yields of microcystin release. Submitted to Bioresource Technology.

Geada, **P**., Oliveira, F., Loureiro, L., Esteves, D., Teixeira, J.A., Vasconcelos, V., Vicente, A.A., Fernandes, B., Comparison and optimization of different methods for *Microcystis aeruginosa*'s harvesting and the role of zeta potential on its efficiency. Submitted to Algal Research.

LIST OF GENERAL NOMENCLATURE

Symbols

D – Dilution rate

F – Volumetric flow

$L{:}D$ – Ligh:dark

P_{max} – Maximum productivity

$[T]$ – Toxin concentration

u_s – Superficial gas velocity

X_{max} – Maximum biomass concentration

μ_{max} – Maximum specific growth rate

Abbreviations

AChE – Acetylcholinesterase

Adda – 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid

ALS – Amyotrophic lateral sclerosis

AMPA – Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

ANOVA – Analysis of variance

APX – Aplysiatoxin

ATX – Anatoxin-a

ATX-s – Anatoxin-a(S)

ATP – Adenosine triphosphate

BMAA – β-Methylamino-L-alanine

CCD – Central composite design

Ch – Chitosan

CIIMAR – Interdisciplinary Centre of Marine and Environmental Research

CP – Central point

CyanoHABs – Cyanobacterial harmful algal blooms

DHA – Docosahexaenoic acid

D-MeAsp – D-erythro-β- methylaspartic acid

DW – Dry weight

GTX – Gonyautoxins

HCB – Harmful cyanobacterial bloom

HE – Harvesting efficiency

LPS – Lipopolysacharides

LTX – Lyngbyatoxin

MC - Microcystin

Mdha – N-methyldehydroalanine

Mdhb – 2-(methylamino)-2-dehydrobutyric acid

NMDA – N-methyl-D-aspartic acid

OATP – Organic anion transporting polypeptide

OCD – Optimal cell density

OD - Optical density

PBR – Photobioreactor

PKC – Protein kinase C

PSP – Paralytic shellfish poisoning

PUFAs – Polyunsaturated fatty acids

RMSE – Root mean square error

SPE - Solid-phase extraction

STX – Saxitoxin

TDI – Tolerable Daily Intake

WHO – World Health Organisation

Chapter 1
Motivation and Outline

1.1 Thesis motivation

Cyanobacteria, classified as Gram-negative bacteria but also frequently known as "blue-green algae", are a group of prokaryotic organisms comprising more than 2000 species (Gutiérrez-Praena *et al.*, 2013). Their evolution across billions of years enhanced the ability to quickly thrive both in extreme and continuously changing environments, in which cyanobacteria are exposed to seasonal fluctuations, natural cycles of flood and drought, as well as agricultural pollution (Neilan *et al.*, 2013). The accumulation of toxin-producer cyanobacteria might lead to the formation of bright green, yellow-brown, and red blooms – cyanobacterial harmful algal blooms (CyanoHABs) – that arise in numerous ecosystems including marine, freshwater, and terrestrial habitats (Neilan *et al.*, 2013; Pavagadhi and Balasubramanian. 2013). Recreational activities such as bathing in contaminated surface water and the consumption of unsuitably treated drinking water constitute a potential risk of exposure of human beings to cyanotoxins (Merel *et al.*, 2013). Additionally, many mollusks, crustaceans, fish, and plants are capable of living under the presence of cyanotoxins and accumulate them, posing a serious threat for animals higher up the trophic chain (Gutiérrez-Praena *et al.*, 2013). In freshwater systems, problems with toxin-producing phytoplankton are almost exclusively associated to cyanobacteria and CyanoHABs have emerged over the past three decades as a worldwide concern because of their increased occurrence with severity to animals, plants, and humans (Neilan *et al.*, 2013; Pavagadhi and Balasubramanian. 2013).

Cyanotoxins are harmful secondary metabolites such as cyclic peptides, alkaloids, and lipopolysaccharides (LPS) that are synthesized by over 40 species (Guzmán-Guillén *et al.*, 2012; Pavagadhi and Balasubramanian. 2013). For Gutiérrez-Praena and his colleagues (2013), these toxins can be classified into four different groups – dermatotoxins, hepatotoxins, neurotoxins, and cytotoxins – according to the toxic effects inflicted. Although the presence of such toxins has been reported throughout the world, hepatotoxins are the most frequent group (Corbel *et al.*, 2014; Robillot *et al.*, 2000), being microcystin (MC) commonly dominant in cyanobacterial blooms and often produced by *Microcystis aeruginosa* (Dai *et al.*, 2009; Davis *et al.*, 2009; Vasconcelos *et al.*, 1996).

Several reports have noticed that cyanobacterial blooms toxicity is variable according to sites, seasons, weeks, or even days due to the influence environmental factors, such as temperature, light intensity, nutrient concentration, and carbon dioxide (CO_2) concentration (Robillot *et al.*, 2000). Numerous poisonings of domestic animals, wildlife and humans have happened worldwide because of the use of drinking and recreational water containing *Microcystis aeruginosa* (Dai *et al.*, 2009). Long term exposure to MC has been associated to liver and colorectal cancers, but the most dramatic case occurred in Brazil (1996) where 60 dialysis patients died as consequence of the use of contaminated water during treatment procedure (Davis *et al.*, 2009; Jochimsen *et al.*, 1998; Vasconcelos *et al.*, 2011). In order to protect consumers from the adverse effects of this cyanotoxin, the World Health Organization (WHO) has proposed an upper limit in drinking water (1 $\mu g.L^{-1}$) and a tolerable daily intake (TDI) of 0.04 $\mu g.kg^{-1}$ of body weight (Gutiérrez-Praena *et al.*, 2013).

Despite all the problems involving microcystins, these compounds are currently used not only as laboratory standards for human and environmental risk assessment, but also as tools for molecular and cell biology studies, and for therapeutic purposes. However, their availability is still very low due to some constraints found in production and extraction processes, which obviously influence the final price reaching values as high as 28000 $Euros.mg^{-1}$.

Considering the potential impact of environmental factors on cyanobacterial growth and toxicity, the optimization of cultivation strategies as well as downstream processing techniques would allow microcystin production maximization and consequently higher cost-effectiveness of the process which, in turn, could lead to MC's widespread application in multiple biotechnological fields.

1.2 Research aims

The main objective of this thesis was the development and optimization of strategies to improve the growth of cyanobacteria and the accumulation of cyanotoxins. To reach these goals, the following objectives were proposed:

- Assessment of the effect of CO_2 concentration, light intensity, pH and temperature on *Microcystis aeruginosa* growth and toxin production;

- Evaluation of growth-related parameters using three different cultivation systems: bubble column, airlift, and flat-plate/panel photobioreactors (PBRs);
- Optimization of cultivation strategies (i.e. batch and two-stage cultivation strategy) in order to increase both cyanobacterial growth and toxin production;
- Determination of the influence of chemical cues from other microalgae/cyanobacteria on biomass growth and cyanotoxin production;
- Optimization of harvesting methodologies for *Microcystis aeruginosa* LEGE 91094;
- Optimization of disruption techniques to increase intracellular toxin (MC) release.

1.3 Thesis outline

This thesis was divided into seven chapters; besides the present Chapter (Chapter 1) there is an Introduction (Chapter 2), Results (Chapters 3, 4, 5, and 6) and Conclusions (Chapter 7).

Chapter 2 contains relevant information from literature to understand the framework of the work developed in this thesis.

Chapters 3 to 6 are organized by Abstract, Introduction, Materials and Methods, Results and Discussion, and Conclusions sections, being the topics addressed in each of those chapters the following:

- **Chapter 3** – Effect of environmental conditions on *M. aeruginosa* growth and toxin production: Assessment of the influence of synergistic interactions of four abiotic factors (light intensity, CO_2 concentration, pH, and temperature) on the growth of *M. aeruginosa* and production of its toxin.
- **Chapter 4** – Strategies to improve biomass and toxin productivities: Growth of *M. aeruginosa* was compared for different cultivation systems. Two-stage growth strategies and the use of chemical cues from other microorganisms were also tested to understand their impact on growth of cyanobacteria and production of toxin.
- **Chapter 5** – Harvesting methods for *M. aeruginosa*: Five different methods of harvesting were evaluated (flocculation induced by pH, $FeCl_3$, $AlCl_3$, and chitosan and the use of magnetic microparticles) and the influence of zeta potential on process efficiency was analysed.

- **Chapter 6** – Disruption processing to release microcystin: Comparison of five techniques used to promote cell disruption (bead milling, high-speed homogenization, freeze-thaw cycles, sonication, microwave).

Chapter 7 includes the main conclusions and perspectives for future work.

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Chapter 2
Introduction

Most of the information presented in this Chapter was adapted from:

Geada, **P**., Gkelis, S., Teixeira, J.A., Vasconcelos, V., Vicente, A.A., Fernandes, B., 2017. Chapter 17 - Cyanobacterial toxins as a high added value product, In Muñoz, R. and Gonzalez, C. (Eds). Microalgae-Based Biofuels and Bioproducts, Woodhead Publishing, Cambridge, UK, pp. 405-432.

Geada, **P**., Vasconcelos, V., Vicente, A.A., Fernandes, B., 2017. Chapter 13 - Microalgal Biomass Cultivation, In Rastogi, R.P., Madamwar, D. and Pandey, A. (Eds). Algal Green Chemistry: Recent progress in Biotechnology (Biotechnology series), Elsevier, Amsterdam, Netherlands, pp. 257-284.

2.1 Microalgae's group characterization

Microalgae are thallophytes (plants lacking roots, stems, and leaves) that have chlorophyll a as their primary photosynthetic pigment and no sterile covering of cells around the reproductive cells (Brennan and Owende, 2010). In a taxonomic approach, cyanobacteria cannot be considered part of the algae as they are prokaryotes. However, in applied phycology those microorganisms are included in the microalgae group because they share a significant number of characteristics (Pulz *et al.*, 2001). Some of the current systems of microalgae classification are based on type of pigments, chemical nature of storage products, and cell wall composition. Additional criteria take into consideration the following cytological and morphological characters: occurrence of flagellate cells, structure of the flagella, nuclear and cell division particularities, presence of an envelope of endoplasmic reticulum around the chloroplasts, and possible connection between the endoplasmic reticulum and the nuclear membrane (Tomaselli, 2004). There are two basic types of microalgae cells: eukaryotic and prokaryotic.

2.1.1 Microalgal eukaryotic cells

Autotrophic eukaryotic cells have organelles and cell walls with a microfibrillar layer of cellulose that can be delimited by an amorphous layer (Lee, 2008). However, some species are naked, lacking the cell wall. Often, there is a plasma membrane that bounds the cytoplasm, which contains the nucleus and different organelles (e.g., Golgi apparatus, chloroplast, mitochondria, lipid globules, vacuoles). In the nucleus it is possible to observe the nucleolus and several DNA molecules distributed among the chromosomes. The chloroplast, on the hand, comprises a series of thylakoids, presenting the chlorophylls and associated pigments.

Among eukaryotic microalgae, there is a wide variety of reproduction strategies, from vegetative reproduction by cell division to fragmentation and production of spores. Sexual reproduction also occurs in most of the species.

2.1.2 Microalgal prokaryotic cells (cyanobacteria)

Cyanobacteria are a ubiquitous and diverse group of Gram-negative bacteria comprising more than 150 genera and 2000 species with a widespread geographical and

environmental distribution (Raja *et al.*, 2014). These microorganisms, commonly known as blue-green algae, show similar features with both bacteria (prokaryotes) and algae (eukaryotes). This peculiar arrangement makes them the only prokaryotes presenting a plant-like oxygenic photosynthesis (Lau *et al.*, 2015; Malathi *et al.*, 2015). Autotrophic prokaryotic cells do not display membrane-bound organelles such as mitochondria, Golgi bodies, and flagella, and the DNA is not structured in chromosomes, lying free in the cytoplasm (Tomaselli, 2004). They have a four-layered Gram-negative cell wall, with a lipopolysaccharide layer involving a murein (peptidoglycan) layer. The lack of cellulose in cyanobacteria is responsible for a high digestibility of these cells (e.g., *Spirulina*) when compared to eukaryotic cells (e.g., *Chlorella vulgaris*). The cell wall may be delimited by mucilaginous envelopes (glycocalyx, sheath, capsule, or slime), perforated by small pores or having appendages such as fimbriae and pili (Tomaselli, 2004). The plasma membrane is ≈8 nm thick.

In prokaryotic cells, the photosynthetic apparatus is enclosed in the thylakoid, which is a membrane system that may be organized in concentric rings, parallel bundles or simply dispersed in the cytoplasm. Glycogen, polyphosphate, and cyanophycin granules, as well as lipid droplets, carboxysomes, gas vacuoles, and ribosomes are some of the most common cell inclusions of cyanobacteria.

Cell division can occur through binary fission, multiple fission, and fragmentation (hormogonia); additionally, some filamentous genera produce akinetes. Genetic recombination by transformation or conjugation can occur despite the absence of evident sexual reproduction (Tomaselli, 2004).

2.1.3 Biodiversity and adaptation

Microalgae and cyanobacteria are present in all existing Earth ecosystems from deserts to polar seas living in a wide range of environmental conditions because they have developed different physiological systems, which allowed them to adapt to various, some of them extreme, conditions, e.g., temperature, pH, osmotic stress, salinity, nutrients starvation, anaerobiosis or light intensity. Although they are mostly present in aquatic environments (freshwater and salt water), they are also found in the surface of soils and stone both as free-living and in symbiotic association.

Although the mechanism of photosynthesis in microalgae/cyanobacteria is similar to that of higher plants, they are generally more efficient converters of solar energy because of their simple cellular structure. In addition, because the cells usually grow in aqueous suspension, they have more efficient access to water, CO_2, and other nutrients (Chisti, 2007).

It is estimated that more than 50000 species exist, but only a limited number (around two-thirds) have been studied and analysed and kept in collections all over the world. Some of the largest collections of microalgae/cyanobacteria are the Collection of Freshwater Algae at the University of Coimbra (Portugal), the Culture Collection of Algae of the Göttingen University (Germany), the Culture Collection of Algae in the University of Texas (USA), the National Institute for Environmental Studies (Japan), and the LEGE-CIIMAR Culture Collection (WDCM 1089) from University of Porto (Portugal) (Duong *et al.*, 2012; Mata *et al.*, 2010).

Despite of these numbers, only a limited number is cultivated at industrial scale for commercial purposes.

2.1.4 Biochemical composition

The diversity of microalgae and cyanobacteria is reflected also in their biochemical activity which includes the production of a wide range of metabolites with different properties, such as carbohydrates, lipids, and proteins, which in many cases are of commercial interest. In addition to a remarkable diversity of biomolecules, there is an extraordinary metabolic plasticity allowing the manipulation of the biochemical composition of those microorganisms through the control of growth conditions (cf. Chapter 3). However, the outstanding qualities of adaptability and metabolic plasticity exhibited might represent either a serious environmental risk or a huge biotechnological potential, as described in Sections 2.6 and 2.6.3, respectively.

In most of the microalgae species and some cyanobacteria, starch or starch-like compounds are the typical carbohydrate storage product. They are able to produce lipids as storage products being frequently observed as oil droplets in cells, mainly in the form of polyunsaturated fatty acids (PUFAs), including arachidonic acid, docosahexaenoic acid (DHA), and eicosapentaenoic acid. Fatty acids and sterols are also found in cellular membranes, being more difficult to extract than the storage lipids.

Microalgal proteins are exceptionally diverse and are present in high concentrations both in prokaryotic (e.g., 40-60% of *Spirulina*'s dry weight) and eukaryotic (e.g., 30-60% *Porphyridium*'s dry weight) cells, making these an excellent alternative protein source. In opposition to carbohydrates and lipids, that reach their maximum concentration at stationary growth phase, protein-rich cells are usually in the exponential growth phase (i.e. actively growing cells).

2.2 Nutritional needs of cyanobacteria

Many elements have to be provided for the growth of cyanobacteria, such as carbon (C), oxygen (O), hydrogen (H), nitrogen (N), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), sulfur (S), phosphorus (P), and trace elements such as copper (Cu), manganese (Mn), selenium (Se), or zinc (Zn).

In autotrophic growth, while C, O, and H are usually obtained from water and air, N, P, and K have to be absorbed from the culture medium (Zhu *et al.*, 2013); their uptake is therefore highly dependent on various factors such as species, nutrient ratios, temperature, or pH. These variations in the uptake rate led to a great amount of studies to determine the optimal nutrient concentrations for various species under different growth conditions. C, N, and P are usually regarded as the most important nutrients for microalgae cultivation.

Carbon (C) – Since atmospheric CO_2 cannot satisfy the carbon requirements of intensive autotrophic cyanobacterial cultivations, the supply of CO_2 and HCO_3 is of utmost importance. Additionally, it is common to reach pH values as high as 11 throughout autotrophic cultivations due to gradual accumulation of OH during active photosynthesis. The pH values can be controlled via direct sparging of CO_2 into the culture.

Nitrogen (N) – Although the N content of the biomass varies (1 to >10%) according to the supply and availability, its importance and influence is critical in cyanobacterial cultures, being considered the second most important nutrient (after carbon) for its cultivation (Richmond, 2013). The N concentration, mainly its limitation, is often used as strategy to control cell composition, usually resulting in the accumulation of PUFAs, polysaccharides, and carotenoids and the decrease in chlorophylls, leading to cell discoloration (Fernandes *et al.*, 2013). For other purposes, to ensure that N never

becomes the rate-limiting factor, an adequate supply is vital and consequently culture media are often formulated with an excess of N. This oversupply should be controlled to avoid toxic effects. By using the enzyme nitrogenase, some cyanobacteria are capable of utilizing elemental nitrogen reducing N_2 to NH_4^+; however, N is typically supplied as nitrate (NO_3), ammonia (NH_4^+), and urea. When used isolated, ammonia leads to a pH decrease and nitrate to a pH increase. Ammonia is often lost during the cultivation due to volatilization phenomena.

Phosphorus (P) – Due to its role in cyanobacterial growth and in different cellular processes of energy transfer and DNA biosynthesis, P (preferentially in the form of orthophosphate) is one of the key nutrients in its cultivation. It is very common to verify the precipitation of P because it bonds very easily to other ions (e.g., iron). This makes P unavailable for uptake and therefore P is frequently referred as one of the most common growth-limiting factors of cyanobacteria. This problem is usually overcome by the addition of metal chelators such as EDTA disodium salt and EDTA. Similarly to N, P availability influences biomass composition, especially the carbohydrate and lipid content (Grobbelaar, 2013). Another parameter to consider in order to maximize the biomass productivity is the N:P ratio. Microalgae have the ability to store P in polyphosphate bodies to be used when the supply of this nutrient is a limiting factor (Grobbelaar, 2013).

2.3 Cultivation modes

2.3.1 Batch cultures

Before starting batch cultures, all the nutrients (except the carbon source in autotrophic cultivations) needed for growth and the desired product formation are added to the cultivation medium.

During these cultivations, growth ceases due to limiting substrate depletion and/or growth-inhibiting products accumulation. The main consequences from these continuous changes in the cultivation environment and also the cell aging are: (1) the variation in cell composition during the growth and (2) the existence of different growth phases (i.e., lag, exponential, stationary, and death phases) with consequent productivity fluctuations. The biomass productivity of a batch cultivation is determined by dividing the total amount of biomass/product obtained by the total time of batch operation

(cyanobacteria cultivation plus turnaround time, i.e., draining the cultivation broth, cleaning the bioreactor, sterilizing the bioreactor, and refill with fresh culture medium).

Most of the current cyanobacteria mass cultivations are based on batch cultivation systems mainly due to its simplicity, reduced risk of contamination (when compared with continuous cultivations), and reduced need for maintenance.

2.3.2 Fed-batch cultures

Within the continuous flow cultivation techniques, fed-batch is probably the most used at industrial scale (Richmond, 2004). In these fed-batch cyanobacterial cultivations, fresh medium is continuously or discontinuously added; however, in opposition to continuous cultivations, the cells and the products remain in the bioreactor. The culture is harvested periodically at the end of the cultivation cycle, which results in a cultivation system with variable volume and dilution rate (D) (see definition in Equation 2.1, Section 2.3.3). Fed-batch, by definition, is placed between continuous and batch modes (Figure 2.1) and is especially suited to produce metabolites that are not associated with cyanobacterial growth or to avoid substrate inhibition.

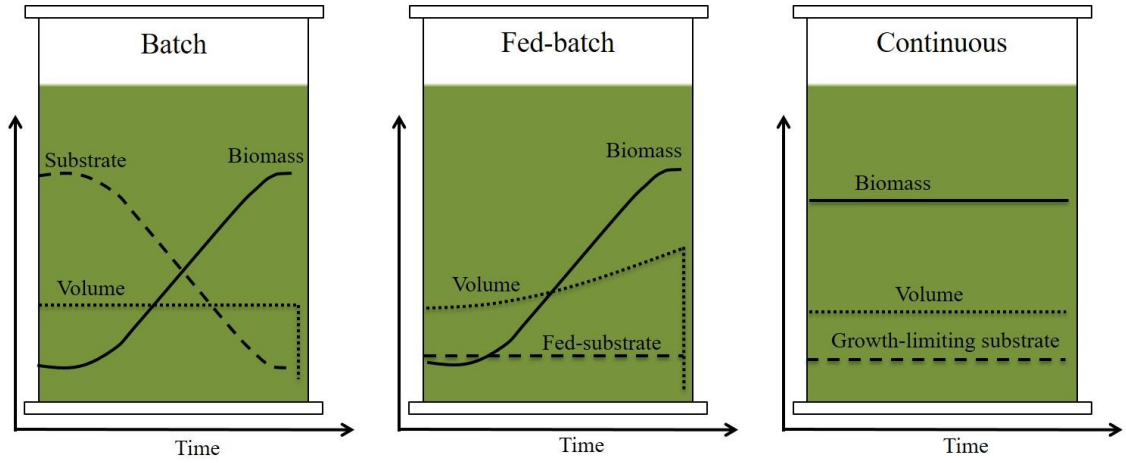


Figure 2.1 - Indicative variations in working volume, biomass, and substrate concentrations under different operation modes.

One possible strategy to obtain specific products is to increase the biomass concentration until a desirable value (using a batch mode) and then change the metabolism of cyanobacteria by feeding product precursors or just nutrients to meet the demand for maintenance and product synthesis.

When a state is achieved where parameters vary in a cyclic pattern, it is considered that a quasi-steady state is reached (Richmond, 2004). Fed-batch mode is used under different approaches. For example, it can be used with or without feedback/feedforward control, in a variable volume (most common) approach or, in opposition, in a "fixed" volume mode, which is achieved by adding a very concentrated nutrient stream that creates a negligible volume variation. With minor changes in the equipment, when compared with batch cultivations, it is possible to obtain an enhanced control over substrate and biomass concentration by simply adopting the fed-batch technique.

2.3.3 Continuous cultures

In opposition to batch cultivations, in continuous systems there is a control of cultivation conditions/environment, which enables obtaining tailor-made biomass composition at a fixed rate (Fernandes *et al.*, 2015; Klok *et al.*, 2013; Zhu and Jiang, 2008). Continuous cultivation of cyanobacteria has been extensively described by Fernandes *et al.* (2015), who pointed out other advantages assigned to continuous system such as higher volumetric productivities, reduced space required, lower labor, investment, and operational costs, and reduced "unprofitable" periods. In continuous cyanobacterial cultivations, the productivity is calculated by multiplying the biomass/product concentration in the outflow stream by the dilution rate (D), which is reciprocal of the residence time (average time that an element of volume spends inside the bioreactor). Comparing with batch cultivations, in continuous mode the turnaround time represents a much lower fraction of the total time (Fernandes *et al.*, 2015).

Continuous cultivation systems are open systems with continuous feeding of fresh medium and continuous culture broth removal, usually with an unchanged volume of medium inside the bioreactor and a constant cultivation environment that maintains cells (Figure 2.1), after some generations, in a balanced growth state or steady state (Guedes *et al.*, 2014). In theory, and due to the relationship between the availability of limiting substrate and microbial growth, it should be possible to keep the culture indeterminately in the exponential phase. However, in reality, cell aging-related problems will eventually stop the growth (Fernandes *et al.*, 2015).

In a bioreactor operating under continuous mode and assuming steady-state conditions, no biomass in the substrate inflow, and constant volume, it is considered that:

$$\mu = F / V = D \qquad \text{(Equation 2.1)}$$

where μ – specific growth rate (h^{-1}), F – volumetric flow ($m^3.h^{-1}$), V – working volume of the bioreactor (m^3), and D – dilution rate (h^{-1}). In Equation 2.1 the specific death rate is considered to be negligible comparing to μ. If $D > \mu_{max}$, the biomass concentration will tend to zero due to the so-called washout process.

However, photoautotrophic continuous cultivation of microalgae and cyanobacteria greatly differs from the cultivation of other microorganisms under the same cultivation mode as consequence of light-related constraints (Cuaresma *et al.*, 2011a; Grima *et al.*, 1999; Sforza *et al.*, 2014). Therefore, in cyanobacterial continuous cultivations, maximum productivities occur at high cell densities and low to moderate μ at light-limited conditions (all energy is absorbed by the biomass during a defined short time interval). Maximum growth rate (μ_{max}), on the other hand, is regularly achieved in the absence of light-limited conditions and in the presence of low cell concentrations (Fernandes *et al.*, 2015; Richmond, 2013).

The concept of optimal cell density (OCD) is often used in microalgal and cyanobacterial continuous cultivations. The OCD is the cell concentration that allows maximum biomass productivity being μ, under these conditions, far (around 50%) from μ_{max} (Myers and Graham, 1959; Richmond, 2013).

2.4 Cultivation systems

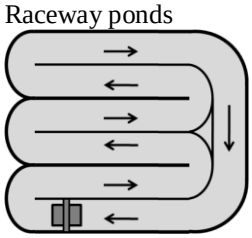
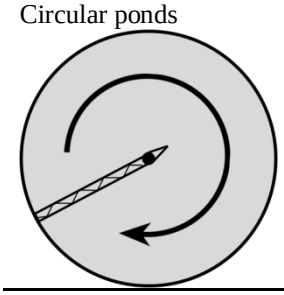
All the process details, from biomass production to product extraction and purification must be considered so that a viable commercialization strategy is achieved. As a result, the choice of an adequate cultivation method, whether by open or closed systems, must be intimately related to the product of interest and its application.

2.4.1 Open systems

Despite being intensively studied in later years of the 20th century, open-air cultivation systems have been used since 1950s and include lakes and natural, circular and raceway ponds, and inclined or cascade systems. These are the most common and widespread systems applied for large-scale commercial growths because open-air culture structures involve lower costs, are easier to build, and usually present longer lifetime and larger

production capacity when compared to closed systems. Still, several drawbacks arise from the use of open-air systems, as shown in Table 2.1.

Table 2.1 – Summarized advantages and limitations of open cultivation systems (adapted from Brennan and Owende (2010) and Dragone *et al.* (2010))

Open Systems	Advantages	Limitations
Raceway ponds 	- Relatively economical - Easy maintenance and cleaning - Low energy consumption	- Poor control over culture parameters - Ineffective light and CO_2 utilization - Difficult to grow cultures for long periods - Poor mixing - Low productivity - Limited to few strains - High risk of contamination
Circular ponds 		

For instance, open ponds are more susceptible to regional climate conditions such as annual rainfall and rainfall pattern when compared to closed systems, representing significant technical challenges for a proper control of crucial growth parameters such as evaporation. Another disadvantage of the open culture systems concerns to the lack of a sterile or controlled environment, which may give rise to contaminations by predators and other fast-growing heterotrophs. In that sense, commercial production of algal and cyanobacterial species using natural and artificial ponds is limited to fast-growing, naturally occurring, or extremophilic organisms such as *Dunaliella* (adaptable to very high salinity), *Spirulina* (adaptable to high alkalinity), and *Chlorella* (adaptable to nutrient-rich media) thus ensuring the existence of a monoculture (Das *et al.*, 2011; Spolaore *et al.*, 2006). Due to these constraints, the most interesting applications for growths carried out in open-air systems are aquaculture feed, biofuels production, and wastewater or flue gas treatment. However, successful examples employing grown in open systems in other biotechnological fields are also known.

Among open-air cultivation systems, circular ponds are widely used in Asia. They are usually characterized by a central rotating agitator and a depth around 0.3 m. Due to

mechanical restrictions found in rotating arm design, the area of such systems must not exceed 10000 m^2. The additional concrete construction costs and high energy input for mixing make circular ponds an unfeasible primary option for commercial plants (Borowitzka, 2005).

Raceway ponds are the most commonly used artificial systems presenting an oval-shaped closed loop, generally between 0.2 and 0.5 m deep. Mixing is induced by the continuous movement of a paddle wheel, which avoids algae sedimentation. Using low-depth (0.15-0.2 m) raceway ponds, productivities of 10-25 $g.m^{-2}.day^{-1}$ and biomass concentrations up to 1 $g.L^{-1}$ might be achieved (Pulz, 2001). The largest facility using this kind of ponds, 440000 m^2, is used to grow *Spirulina* and is located in California, USA (Demirbas, 2010; Spolaore *et al.*, 2006).

Cascade systems are the only open-air systems able to reach high cell densities (up to 10 $g.L^{-1}$) and are suitable for repeated pumping-tolerating cyanobacteria. Culture suspension flows throughout a sloping surface in which turbulence is generated by gravity (Marchin *et al.*, 2015; Šetlík *et al.*, 1970). This highly turbulent regime allows the existence of very thin culture layers (<2 cm), which provide higher cell concentrations and surface-to-volume ratios when compared to raceway ponds.

2.4.2 Closed systems

The term "photobioreactor" (PBR) is sometimes applied to describe open ponds and channels, but in this thesis this expression will be used to refer to closed systems. Due to a good regulation and control of nearly all the important biotechnological parameters, the risk of contamination inside PBRs is low thus allowing the growth of monoseptic cultures. Furthermore, PBRs are also characterized by a flexible technical design, no major CO_2 losses, reproducible cultivation conditions, and reliable control over temperature and hydrodynamics (Fernandes *et al.*, 2015; Pulz, 2001).

Despite the relative success of open systems, enclosed PBRs present the following advantages: (1) better control over pH, temperature, light, CO_2 concentration, and gas transfer; (2) large surface-to-volume ratio; (3) low or none CO_2 loss and reduced growth medium evaporation; (4) more uniform temperature; (5) reduced outside contamination risk; (6) high cell densities; and (7) allows the production of complex biopharmaceuticals (Chen, 1996; Singh and Sharma, 2012). Because some of the new

cyanobacteria and cyanobacterial high-value products are employed in pharmaceutical and cosmetics industries, biomass growth in PBRs becomes mandatory to reach a culture free of pollution, as required in those biotechnological fields. Furthermore, some cyanobacteria need a culture environment that is not highly selective, being the use of closed systems a safe manner to avoid potential contaminants (such as other microorganisms) without compromising the final product quality (Benson *et al.*, 2014).

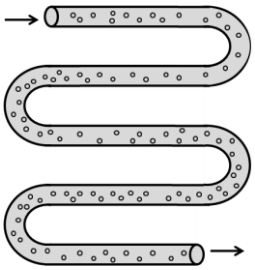
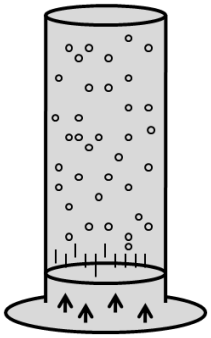
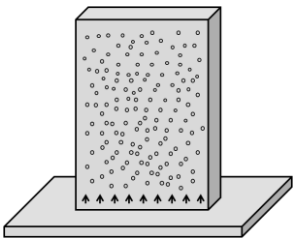
Despite the great variety of designs developed so far, there are three main PBR categories: (1) tubular (e.g., helical, manifold, serpentine, and α-shaped); (2) flat (e.g., alveolar panels and glass plates); and (3) column (e.g., bubble columns and airlift). Table 2.2 summarizes the advantages and limitations related to these systems.

Closed horizontal continuous-run tubular loop PBRs are generally applied in commercial monocultures. Biomass circulation throughout the tubes is promoted by a pump system or preferably using airlift technology. These are the largest closed PBRs, as the 25 m^3 plant from Mera Pharmaceuticals, Hawaii (USA), and the 700 m^3 plant located nearby Wolfsburg (Germany) can prove it (Fernandes *et al.*, 2015; Olaizola, 2000; Pulz, 2001). Maximum productivities between 25 and 30 $g.m^{-2}.day^{-1}$ have been achieved using serpentine and two-plane tubular PBRs (Torzillo *et al.*, 1993; Torzillo *et al.*, 1986). Despite having notable advantages such as relatively low costs associated, large illumination surface area, and fairly reasonable biomass productivities, tubular systems also comprise serious limitations. Large land surface occupation, difficult temperature control, fouling and foaming formation, dissolved O_2 and CO_2 gradients along with pH gradients that jointly cause growth inhibition, and the need for frequent recarbonation of the cultures are some of the disadvantages that make these PBRs only justifiable for the production of high-value products (Mirón *et al.*, 2002; Weissman *et al.*, 1988). To overcome these constraints, the use of vertical PBRs might pose a good alternative.

Vertical orientation has been proposed to enhance productivity because sunlight incidence is spread over a larger reactor surface area, which results in the exposure of cells to lower light intensities and consequently a maximized photosynthetic efficiency. On the other hand, a reduced photosaturation effect is also observed (Cuaresma *et al.*, 2011b; Posten and Schaub, 2009). Vertical column PBRs are usually cylinders characterized by a diameter up to 0.4 m and a height up to 4 m. To increase the surface-volume ratio, these PBRs should present relatively small diameters. Height restriction

must be taken into account to avoid gas transfer limitations that might result in CO_2 and pH gradients and O_2 accumulation. Additionally, it is imperative to choose a resistant transparent material suitable for building these PBRs.

Table 2.2 – Summarized advantages and limitations of closed cultivation systems (tubular, column, and flat PBRs) (adapted from Brennan and Owende (2010) and Dragone *et al.* (2010))

Closed Systems	Advantages	Limitations
Tubular PBRs 	- Reasonably economic - Great illumination surface area - Suitable for outdoor cultures - Good biomass productivities	- pH, dissolved O_2 and CO_2 gradients generation - Fouling formation - Considerable wall growth - Makes use of large land space - Risk of photoinhibition effect
Column PBRs 	- Low energy consumption - Good mass transfer and mixing - Best exposure to light-dark (L:D) cycles - Low shear stress, photoinhibition and photo-oxidation effects - High potential for scalability - Easy to sterilize - High photosynthetic efficiency	- Low illumination surface area - Expensive and sophisticated construction materials - Possible shear stress to cultures under certain growth conditions - Decreasing illumination surface area associated with scale-up - Scalability
Flat PBRs 	- Relatively cheap - Easy to clean up - Large illumination surface area - Reduced power consumption - Good biomass productivities - Good light path - Low oxygen build-up	- Difficult to scale-up - Difficult temperature control - Moderate wall growth - Hydrodynamic stress to some strains - Low photosynthetic efficiency

Column PBRs are characterized by high volumetric gas transfer coefficients. They have a constant bubbling of gas from the bottom of the reactor that enables an efficient CO_2 utilization and optimal O_2 removal (Wang *et al.*, 2012). Furthermore, gas bubbles provoke a gentle culture mixing with little shear stress for cells and thus almost

inexistent cell damage is associated with these PBRs, except when high superficial gas velocities are employed (Vega-Estrada *et al.*, 2005; Wang *et al.*, 2012). However, cyanoabacterial growth is often limited by other parameters such as efficient utilization of light. Airlift PBRs are probably the only vertical reactors that are capable of providing regular L:D cycles and sustaining better biomass production of different microalgae and cyanobacteria, probably due to the excellent and non-chaotic mixing they can offer (Wang *et al.*, 2012). The efficient mixing of swirling flows ensures a good homogenization of nutrients inside the culture as well as the cells displacement along the column. In addition, high shear stress generated at walls restricts the biofilm formation at the PBR surfaces (Loubière *et al.*, 2009; Pruvost *et al.*, 2004; Pruvost *et al.*, 2002). The existence of some other advantages like low power input, well-defined fluid flow patterns, simplicity in design and construction, and low capital and operating costs make these the most required vertical PBRs (Fernandes *et al.*, 2010; Loubière *et al.*, 2011). Some limitations such as difficult temperature control and large fraction of dark zones inside the reactor, mainly due to the presence of the internal column that limits light penetration, could though be found when performing cynobacteria growth using airlift PBRs.

Some of the first closed systems' forms were flat PBRs that have received special attention mainly due to the large light exposure surface area and high cell densities, higher than 80 $g.L^{-1}$, revealed during photoautotrophic growth (Hu *et al.*, 1998; Ugwu *et al.*, 2008). A thin layer of very dense culture is mixed or moved across a flat transparent panel allowing radiation absorbance in the first few millimeters near the PBR surface. These panels are usually irradiated by direct sunlight from one side and can be placed either vertically or inclined at an optimum incident angle to present the most efficient energy absorption possible from the sun. Due to the low accumulation of dissolved oxygen and high photosynthetic efficiency obtained comparing to tubular PBRs, flat designs are suitable for large cultures (Brennan and Owende, 2010). Nevertheless, cyanobacteria cultivation using flat PBRs might face some limitations including difficult temperature control of the culture, certain degree of wall growth, possible hydrodynamic stress to some strains, and the fact of implying many compartments and support materials when scale-up is attempted.

2.5 Strategies to increase cost-effectiveness of cyanobacteria cultivation

Natural resources' management, such as the use of microorganisms to fulfil human needs, is based on a key principle: sustainability. To reach such equilibrium, there must be a continuous seek for efficient operational conditions, minimum environmental risk, and the respect of socioeconomic considerations (Brennan and Owende, 2010). Despite of the increasing interest in cyanobacteria as a source of multiple products of interest, companies will not be interested to proceed with large investments unless improved productivity (either for biomass and/or metabolites) and/or process cost reduction are achieved (Singh and Gu, 2010). Numerous variables such as growth conditions applied (Chapter 3), cultivation system selection (Chapter 4.1), nutrients supply and cultivation strategy implementation (Chapters 4.2 and 4.3), final products' concentration, and downstream processing efficiency (Chapters 5 and 6) should be taken into account so that we manage to attain high-productivity cyanobacterial growth in a cost-effective way.

2.6 Cyanobacterial toxins

The potential applications of cyanobacteria in diverse bioindustrial processes such as food, feed, aquaculture, pharmaceutics, and biofuels are strongly supported by the simple growth requirements, amenability to genetic manipulation, and ability to convert solar energy and atmospheric carbon dioxide directly into industrial products that can be commercially exploited (Lau *et al.*, 2015; Raja *et al.*, 2014). Among the broad spectrum of biologically active metabolites – namely, secondary metabolites – displayed by these microorganisms, one can highlight the cyanotoxins, pigments, vitamins, amino acids, fatty acids, and enzymes (Lau *et al.*, 2015; Malathi *et al.*, 2014; Mostafa, 2012).

Worldwide occurrence of cyanobacterial harmful algal blooms (or CyanoHABs) has recently increased due to global warming along with intensive modern agriculture, which leads to excess of nutrients (e.g., nitrogen and phosphorous) in water bodies; this combination of factors makes them one of the main health risks for global water resources since humans and animals can be exposed to cyanotoxins via drinking water, aquaculture, and recreational activities (Malathi *et al.*, 2015). According to the WHO, humans and animals might be affected by the toxicological effects potentially arising from these compounds including neurotoxicity, hepatotoxicity, cytotoxicity, and dermatotoxicity (Chu, 2012). The worst recorded event involving cyanobacterial toxins

occurred in Brazil where 52 patients died after dialysis treatment using water contaminated with microcystins (Jochimsen *et al.*, 1998).

Although the economic impact of CyanoHABs is frequently imprecise and difficult to predict, there are four common issues usually related to these episodes: existence of tastes and odours, production of cyanotoxins, depletion of dissolved oxygen, and water turbidity (Hamilton *et al.*, 2014). Despite the significant direct costs associated with human and animal health as well as with water treatment, some other indirect effects such as interdiction of fisheries and recreational area exploitation or the bioaccumulation of cyanotoxins in seafood must be considered too (Woodhouse *et al.*, 2014).

Species and strains of all the common planktic cyanobacterial genera including *Dolichosperum*, *Anabaena*, *Aphanizomenon*, *Microcystis*, *Nodularia*, *Nostoc*, *Oscillatoria* (Carmichael, 1992), *Anabaenopsis*, *Lyngbya*, *Schizothrix*, *Planktothrix*, *Cylindrospermopsis*, *Umezakia*, and the terrestrial *Hapalosiphon* (Sivonen and Jones, 1999) produce toxins (Figure 2.2). Other genera such as *Coelosphaerium*, *Fischerella*, *Gleotrichia*, *Gomphosphaeria*, *Microcoleus*, *Scytonema*, *Symploca*, and *Tolypothrix* have been found to be toxic, but no toxin has been characterized yet from these genera. Nevertheless, as further surveys are carried out, more toxic cyanobacterial blooms and new toxic species are discovered.

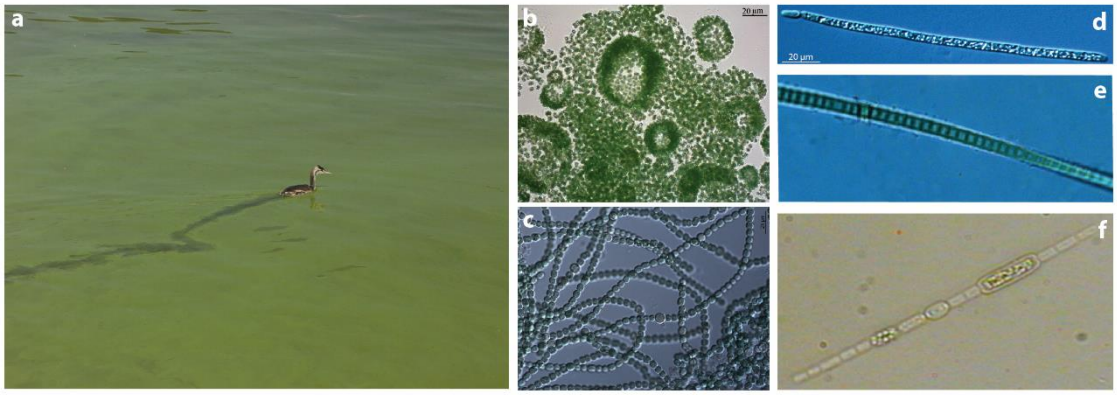


Figure 2.2 - Representative pictures of toxin-producing cyanobacteria. (a) Bloom of *Microcystis* at Lake Pamvotis, Greece, (b) colony of *Microcystis aeruginosa*, (c) filaments of *Dolichospermum* sp., (d) filament of *Cylindrospermopsis raciborskii*, (e) filament of *Planktothrix agardhi*, (f) filament of *Aphanizomenon ovalisporum*. Pictures A and F were taken by S. Gkelis on August 2009 and July 1999, respectively; pictures B–E from Gkelis and Panou (2016).

Not all the species of a cyanobacterial genus produce toxins. Even within a single-species bloom, there may be a mixture of toxic and nontoxic strains. Some strains are much more toxic than others, sometimes more than three orders of magnitude. Toxic and nontoxic strains from the same cyanobacterial species cannot be separated by microscopical examination. To confirm that a specific strain is a toxin producer, it is important to isolate a pure culture of that strain, preferably free of other bacteria (Sivonen and Jones, 1999).

2.6.1 Most common and well-studied toxins

CyanoHABs (Figure 2.2) represent one of the most conspicuous waterborne microbial hazards to human and agricultural water supplies, fishery production, and freshwater and marine ecosystems due to the production of cyanotoxins – harmful secondary metabolites such as microcystins, saxitoxins (STXs), and cylindrospermopsins (CYNs) that can have deleterious effects within reservoirs and in downstream-receiving water systems.

Despite numerous studies pointing to the possible ecological and physiological roles of cyanotoxins, a truly consensual definition of the word has not been reached yet. Nevertheless, referring to the term "toxin" as a biologically active metabolite responsible for negative effects on humans and environmental health as well as on potential consumers and sympatric competitors seems to be a widely accepted description (Berry *et al.*, 2008).

Generally, toxigenic cyanobacteria are identified as organisms capable of producing neurotoxic, dermatotoxic, and hepatotoxic substances (Fristachi *et al.*, 2008). However, if one also considers compounds with a protease inhibition effect such as micropeptins, cyanopeptolins, microviridins, oscillapeptins, oscillamides, nostopeptins, aeruginosins, aeuginopeptins anabaenopeptilides, anabaenopepins, and the cytotoxic compounds from marine origin, the number of toxin-producing cyanobacteria will be somewhat extended (Fristachi *et al.*, 2008). Cyanotoxins fall into three broad groups according to their chemical structure: cyclic peptides, alkaloids, and amino acids and are subdivided also according to their toxicity: hepatotoxins, neurotoxins, cytotoxins, and dermatotoxins (Figure 2.3 and Table 2.3).

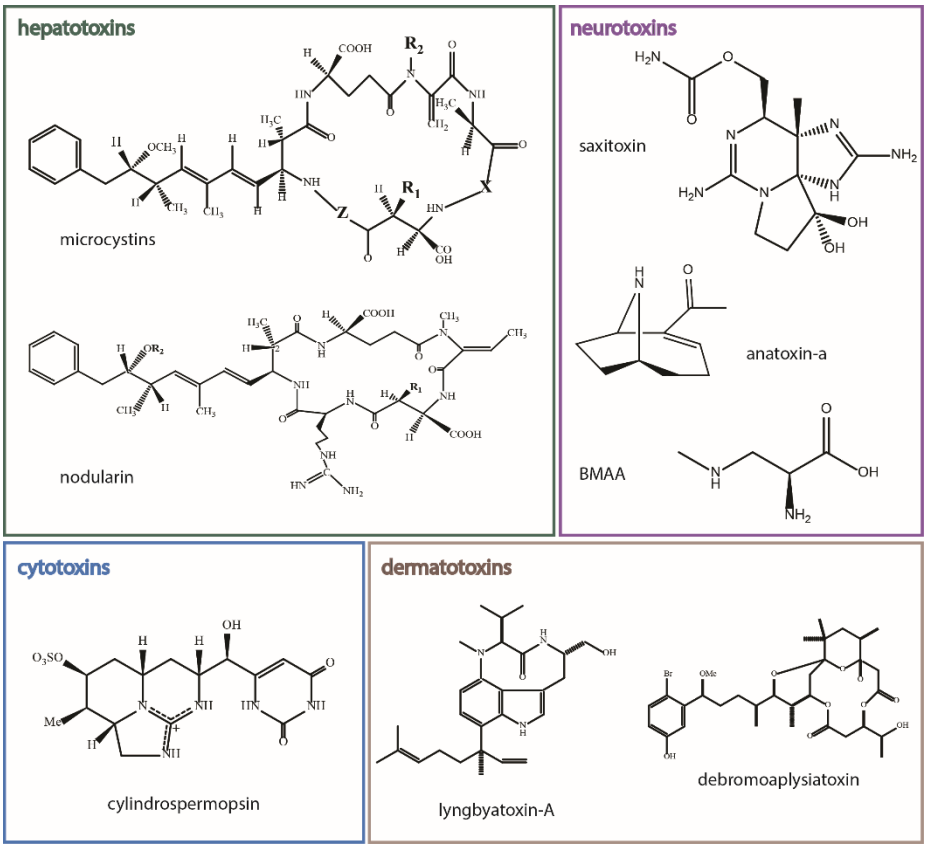


Figure 2.3 - Representative chemical structures for cyanobacterial toxin families.

Table 2.3 summarizes the most common existing cyanotoxins as well as the potential producers, toxicity, and genes responsible for their biosynthesis.

2.6.1.1 Microcystins

The most frequently reported cyanobacterial toxins are the hepatotoxic microcystins (MCs). MCs are peptides with a molecular weight ranging from 900 (Botes *et al.*, 1984) to 1100 Da (Sivonen *et al.*, 1992). They consist of seven amino acids of which the two terminal amino acids of the linear peptide are condensed to form a cyclic compound (Figure 2.3). These compounds were isolated first from the cyanobacterium *Microcystis aeruginosa* and therefore the toxins were named microcystins (Carmichael *et al.*, 1988). The general structure of the microcystins is:

$$\text{cyclo}(\text{–D–Ala}^{1}\text{–X}^{2}\text{–D–MeAsp}^{3}\text{–Z}^{4}\text{–Adda}^{5}\text{–D–Glu}^{6}\text{–Mdha}^{7}\text{–})$$

where X and Z indicate different L-amino acids, D-MeAsp is D-erythro-β-methylaspartic acid, and Mdha is N-methyldehydroalanine (Carmichael *et al.*, 1988).

Table 2.3 – Summary of some existing cyanotoxins, their corresponding synthetizing gene, toxicity, biological activities, and potential producers (adapted from Abed *et al.* (2009), Fristachi *et al.* (2008), Gupta *et al.* (2013), Metcalf *et al.* (2006), and Sivonen and Jones (1999))

Toxin group	Biosynthesis (genes)	Toxicity	Producing taxa
Cyclic peptides			
Microcystins	*mcyA-J*	Hepatotoxins: Inhibition of protein phosphatases (PP1 and PP2A).	*Microcystis* spp., *Dolichospermum* (*Anabaena*) spp., *Planktothrix* spp., *Nostoc* spp., *Hapalosiphon*, *Anabaenopsis* spp.
Nodularins	*ndaA-I*		*Nodularia spumigena*
Alkaloids			
Anatoxin-a	*anaA-H*	Neurotoxin: Binding irreversibly to the nicotinic acetylo-choline receptors.	*Dolichospermum* (*Anabaena*) spp., *Cuspidothrix* (*Aphanizomenon*) *issatschenkoi*, *Cylindrospermum*, *Phormidium*, *Tychonema bourrelly*, *Hydrocoleum lyngbyaceum*
Anatoxin-a (S)	*unknown*	Neurotoxin: Inhibition of acetylo-cholinesterase activity.	*Dolichospermum* (*Anabaena*) *lemmermannii*, *D. crassum*, *D. flos-aquae*, and *D. spiroides*
Cylindrospermopsin	*cyrA-O* *aoaA-C*	Hepatotoxin/Cytotoxin: Inhibitor of protein biosysnthesis; cyto-genetic damage on DNA.	*Cylindrospermopsis raciborskii*, *Rhaphidiopsis curvata*, *Aphanizomenon ovalisporum*, *Aph. klebahnii*, *Anabaena planctonica*, *An. lapponica*, *Umezakia natans*
Saxitoxins	*sxtA-Z*	Neurotoxin: Binding and blocking the sodium channels in neural cells	*Dolichospermum* spp., *Dolichospermum sigmoideum* (*Anabaena circinalis*), *Aphanizomenon flos-aquae*, *Cylindrospermopsis raciborskii*, *Scytonema*, *Lyngbya wollei*, *Planktothrix* sp. and *Scytonema* cf. *crispum*
Lyngbyatoxin-a	*ltxA-D*	Dermatotoxin: Dermatitis; oral and gastrointstinal inflammation	*Moorea producens* (*Lyngbya majuscula*)
Aplysiatoxins	*unknown*	Dermatotoxin: Inflammation; protein kinase C activator	*Moorea producens* (*Lyngbya majuscula*), *Schizothrix*, *Planktothrix*
Amino acids			
BMAA (β-methylamino-L-alanine)	*unknown*	Neurotoxin: Neurotoxic, motor neuron damage and loss	Many cyanobacteria

Adda, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid, is a unique structural feature of these toxins (Namikoshi *et al.*, 1989). Structural variations have been reported in all seven amino acids. However, the most frequent variations are the substitution of different L-amino acids at positions 2 and 4, and the demethylation of amino acids at positions 3 and/or 7. To date, about 90 structural microcystin variants are known (Welker *et al.*, 2004).

Microcystins are primarily hepatotoxins. After acute exposure to microcystins by intravenous or intraperitoneal injection, severe liver damage occurs, which is characterized by disruption of liver cell structure due to damage of the cytoskeleton, loss of sinusoidal structure, increases in liver weight due to intrahepatic hemorrhage, haemodynamic shock, heart failure, and death (Campos and Vasconcelos, 2010; Carmichael, 1992). Microcystins are also known to have biological activity against bacterioplankton and zooplankton.

2.6.1.2 Nodularins

Nodularins (Table 2.3, Figure 2.3) consist of five amino acids, are closely related to microcystins and mostly possess the structure:

cyclo (D–MeAsp–L–Arg–Adda–D–Glu–Mdhb)

in which Mdhb is 2-(methylamino)-2-dehydrobutyric acid (Figure 2.3). Nodularins have been isolated from the cyanobacterium *Nodularia spumigena*, which regularly forms blooms in the Baltic Sea. Nodularin, like MCs, is a potent inhibitor of eukaryotic-type protein phosphatases 1 and 2A and reaches intraperitoneal LD_{50} values of 30-50 $\mu g.g^{-1}$ body weight of mice (Chorus *et al.*, 2000). As anticipated by their closely related structures and toxicity mechanisms, nodularin and MCs share a closely related biosynthetic pathway. There are very little chemical differences in nodularins with just six variants reported from the literature (Dittmann *et al.*, 2013). Nodularin has been reported as possessing strong antifungal and algicidal activities.

2.6.1.3 Anatoxins

Anatoxin-a (ATX) is a secondary amine bicyclic alkaloid (Figure 2.3) with a molecular weight of 165 Da. Its methylene homolog homoanatoxin-a differs by the presence of a propionyl instead of the acetyl group at C-2 (Testai *et al.*, 2016). Anatoxin-a(S) (ATX-

s) is an N-hydroxyguanidine methyl phosphate ester with a molecular weight of 252 Da and thus structurally unrelated to anatoxin-a. The "S" in ATX-s comes from viscous mucoid hypersalivation, a typical symptom induced by ATX-s in mammals. Anatoxin-a is a nicotinic agonist that binds to neuronal nicotinic acetylcholine receptors (Osswald *et al.*, 2009) and as such has been studied as a pharmacological probe in the investigation of diseases characterized by low acetylcholine levels, such as Parkinson's and Alzheimer's disease, muscular dystrophy, and myasthenia gravis.

The toxin affects signal transmission between neurons and muscles, eventually leading to respiratory arrest and death within a few minutes. ATX-s is a non-competitive irreversible inhibitor of AChE in the neuromuscular junctions with a mechanism similar to that of carbamate and organophosphorus insecticides; ATX-s causes acetylcholine accumulation leading to nerve hyperexcitability (Dittmann *et al.*, 2013, and references therein).

2.6.1.4 Cylindrospermopsins

CYN is a highly water-soluble cyclic sulfated guanidine alkaloid (Figure 2.3) causing general cytotoxic, hepatotoxic, and neurotoxic effects (Ohtani *et al.*, 1992). Structural variants include 7-epi-CYN (an epimer at the hydroxyl bridge) and 7-deoxy-CYN (lacking the hydroxyl group) (Banker *et al.*, 2001; Seifert *et al.*, 2007). CYN was identified in some reservoirs used for supplying drinking water and has been documented as being involved in at least two epidemical cases of human poisoning, one of them being the so-called Palm Island mystery disease in 1979 (Carmichael *et al.*, 2001; Griffiths and Saker, 2003). CYN has been shown to interfere with different metabolic pathways and induce a wide range of responses including oxidative stress, genotoxicity, immunosuppression, and abnormal function of hepatocytes (Rzymski and Poniedziałek, 2014, and references therein). CYN has been predominantly reported from tropical and subtropical waters; however, due to the wide geographical distribution of CYN producing cyanobacteria, the toxin occurs globally being increasingly detected in lakes of temperate climates such as in Greece (Gkelis and Zaoutsos, 2014), France (Brient *et al.*, 2009), Germany (Fastner *et al.*, 2007), and even boreal environments in Finland (Spoof *et al.*, 2006). CYN was found to have different biological activities such as hepatotoxicity, genotoxicity, cytotoxicity, and carcinogenic potential as well as protein and glutathione synthesis inhibition.

2.6.1.5 Saxitoxins

STXs are a group of carbamate alkaloid toxins (Figure 2.3) consisting of one tetrahydropurine group and two guanidine subunits. They are classified into nonsulfated molecules (STX and neo-STX), mono sulfated (gonyautoxins-GTX), doubly sulfated (C-toxins), and decarbamylated analogs (Pereira *et al.*, 2004). STX represent the principal toxins responsible for paralytic shellfish poisoning (PSP) known from marine environments; therefore, they are being studied as analgesic/anaesthetic and muscle relaxant compounds. STXs can reversibly bind to voltage-gated Na^+ channels. Animals treated with STX show typical neurological effects including nervousness, jumping, convulsions, and paralysis (van Apeldoorn *et al.*, 2007). STX is the most toxic variant of the STX family with an intraperitoneal LD_{50} value of 10 $\mu g.g^{-1}$ body weight of mice (Chorus and Bartram, 1999).

2.6.1.6 Lyngbyatoxins and aplysiatoxins

Lyngbyatoxin (LTX) and aplysiatoxin (APX) are both dermatotoxins (i.e., the causative agent of dermatitis after contact with the skin (Sivonen and Jones, 1999)). The different congeners of LTX are characterized by an indolactam ring and contain prenyl side chains whereas APX and debromoAPX are phenolic bislactones (Figure 2.3; Dittmann *et al.*, 2013, and references therein). Both LTX and APX are potent tumor promoters that operate by competitively binding to protein kinase C (PKC) and are produced by the benthic marine cyanobacterium *Lyngbya majuscule* (now renamed *Moorea producens*). APX also has been reported to occur in *Schizothrix* and *Planktothrix* (Table 2.3; Sivonen and Jones, 1999).

2.6.1.7 BMAAs

β-Methylamino-L-alanine (BMAA, Figure 2.3) is a nonproteinogenic amino acid that has been connected with serious incidents of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and dementia on the island of Guam (Cox *et al.*, 2003). Diverse taxa of all known groups of cyanobacteria, including cyanobacterial symbionts and free-living taxa produce BMAA (Cianca *et al.*, 2012). BMAA production has also been shown in cyanobacteria of the genera *Nodularia* and *Aphanizomenon* that dominate massive blooms in the Baltic Sea and in the food chain

of this brackish water environment (Jonasson *et al.*, 2010).BMAA was also demonstrated to affect motor neurons with different mechanisms including direct agonist action on glutamate NMDA (N-methyl-D-aspartic acid) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, induction of oxidative stress, and depletion of glutathione (Banack *et al.*, 2010; Lobner *et al.*, 2007). BMAA has been hypothetically associated with the development of the ALS/Parkinsonism-dementia complex, a neurodegenerative disease (Cox *et al.*, 2003). The role of BMAA is still very controversial among the scientific community despite the increasing number of publications on the issue (Baptista *et al.*, 2011). Recently, it has been shown (Glover *et al.*, 2014) that insertion of BMAA into proteins may trigger protein malfunction, misfolding, and/or aggregation, the hallmark of neurodegeneration. A biosynthetic pathway toward BMAA has not yet been elucidated (Dittmann *et al.*, 2013).

2.6.2 Legislation

Recognizing the risk that cyanotoxins might present for living beings, international and national health and environmental agencies decided to set guidelines, particularly for drinking water, and to subject these toxic compounds to regulations for production, storage, packaging, and transportation, placing them in dangerous goods (DG) category (Metcalf *et al.*, 2006). Some examples of guidelines and legislation employed around the world are presented in Table 2.4.

Presently, the transport of some purified cyanotoxins or cyanobacteria biomass (cells or cell extracts) either for commercial (standards for analysis or use as reference material) or research purposes is no longer allowed by air mail or in personal air travel baggage (Metcalf *et al.*, 2006). Due to the DGs labelling, these substances must be transported and packed according to strict requirements determined by the United Nations, the International Civil Aviation Organisation, the International Air Transport Association, and specific national government regulations. Therefore, only registered courier services are authorized to transport DGs (Metcalf *et al.*, 2006).

2.7 Potential applications of cyanotoxins

As discussed previously, most cyanotoxins are known as potent toxicants; however, current research on cyanobacteria, and more specifically their cyanotoxins, may change this perspective since some of them have a considerable potential for drug discovery

and thus can be used, for example, in therapeutic applications. Cyanotoxins applications can be subdivided in two major categories: (1) pure, as commercial cyanotoxins standards and (2) exploitation of their biological activities.

Table 2.4 – Examples of guidelines for maximum amount of cyanotoxins allowed and legislation currently applied (Burch, 2008; Metcalf *et al.*, 2006; Woodhouse *et al.*, 2014)

Guidelines/Legislation	Toxin	Value ($\mu g.L^{-1}$)	Country
Drinking water	Microcystin-LR	1.0	Most of Europe, China, Japan, Korea, New Zealand, Brazil
		1.3	Australia
		1.5	Canada
	Saxitoxin	1.0	New Zealand
		3.0	Brazil
	Nodularin	1.0	New Zealand
	Cylindrospermopsin	3.0	New Zealand
		15.0	Brazil
	Anatoxin-a(S)	1.0	New Zealand
Recreational water	Microcystin	20	Netherlands
Shellfish (food)	Saxitoxin	80 µg per 100 g of mussel meat	European Union
Antiterrorism	Microcystins	—	United Kingdom, Czech Republic
	Saxitoxin	—	United Kingdom, Czech Republic, United States
	Anatoxin-a	—	Czech Republic

2.7.1 Cyanotoxins standards

Since cyanotoxins can pose a serious threat for animals and people, high-purity standards are also necessary to meet research needs, either on human and environmental risk assessment tests or as a tool for molecular and cell biology studies.

Despite several commercial cyanotoxins are provided by some suppliers and national institutions, there is still a lack of pure, reliable standards for main toxins such as MCs, anatoxins, and CYNs, and their prices are usually exorbitant. Furthermore, dramatic findings caused suspicion over results obtained by a number of scientific studies (Pegram *et al.*, 2008). The reason for such concern arose from the use of in-house made pure substances as well as other available cyanotoxins that do not meet quality control parameters or even do not have enough product to perform the tests (Meriluoto and Spoof, 2008; Pegram *et al.*, 2008). As an example, among three commercial standards classified as pure MC-RR, only one matched that description during the tests performed by Kubwabo *et al.* (2004). One of them was found to be a mixture of MC-RR and a variant of this cyanotoxin, and the other one was made just of its variant (Meriluoto and Spoof, 2008). Thus, a growing demand for quality reference materials must be fulfilled in the next years to overcome possible limitations not only in water-monitoring tests but also in the aforementioned risk assessment studies (Pegram *et al.*, 2008).

2.7.2 Exploitation of cyanotoxins' biological activities

Cyanotoxins present a considerable biotechnological potential, for example, in the development of pharmaceuticals or other biomedical applications (Berry *et al.*, 2008; Chu, 2012).

The therapeutic value of different cyanotoxins is currently under investigation (Ilić *et al.*, 2011; Mahdi and Fariba, 2012; Niedermeyer *et al.*, 2014; Ramos *et al.*, 2015; Singh *et al.*, 2001); however, it is already possible to take advantage of their potential ecological role or allelopathic activity whether to apply against other organisms in a controlled way or to develop drugs using specific active principles (Berry *et al.*, 2008; Lau *et al.*, 2015; Pulz and Gross, 2004).

Among the studied biological activities, one can identify antibacterial/antibiotic, antifungal, antiviral, antialgal, anticancer/antitumor, anti-inflammatory, immunosuppressant, neurotoxic, cytotoxic, insecticide/larvicide, antimitotic, anticoagulation, antimalarial, and herbicide effects (Berry *et al.*, 2008; Chu, 2012; Lau *et al.*, 2015) of some of the cyanotoxins as presented on Table 2.4. Similar to botulinum toxin, previously seen as a food poisoning agent and now as a versatile pharmaceutical (Dias *et al.*, 2015), several cyanotoxins such as STXs and their analogues have been studied for medical purposes. Based on muscle paralysis action provoked by PSP

toxins, it was found that when applied locally using small amounts, muscle relaxation is induced (Dias *et al.*, 2015). Several studies have shown that medical conditions, such as achalasia (Rodriguez-Navarro *et al.*, 2006), anal fissure (Garrido *et al.*, 2005), and tension-type headache (Lattes *et al.*, 2009) can be treated using PSP toxins such as neosaxitoxins and GTXs. Furthermore, neosaxitoxins were also successfully applied as anaesthetic agents (Rodriguez-Navarro *et al.*, 2007).

The neurotoxicity of cyanotoxins (e.g., kalkitoxin) is also suitable to be explored in developing drugs for neurodegenerative diseases or as a tool in studies performed to get further understanding of brain interactions (Chu, 2012; Umezawa *et al.*, 2012). Due to the protease inhibitory activity demonstrated against enzymes including thrombin, plasmin, trypsin, and chymotrypsin, several cyanotoxins such as aeruginosins, cyanopeptolins, and oscillapeptins might represent potential treatments for strokes, coronary artery occlusions, and pulmonary emphysema (Chu, 2012; Murakami *et al.*, 1995; Patterson, 1996; Singh *et al.*, 2005; Skulberg, 2004).

Although MC-LR is known to inhibit protein phosphate, it was found that the Adda group from this cyanotoxin can play a regulatory role on protein phosphatase 1 by increasing its activity and normalizing protein phosphorylation, thus treating/reducing some Parkinson's disease symptoms (Braithwaite *et al.*, 2012).

Cytotoxic activity revealed by several cyanobacterial toxins makes them interesting candidates for anticancer drugs (Mostafa, 2012). To get inside human liver cells, MCs need to be transported across the membrane by organic anion transporting polypeptides (OATPs), such as OATP1B1 and OATP1B3 (Niedermeyer *et al.*, 2014). Although the expression of such peptides in liver tumours is high, in some other types, such as lung, colon, breast, and pancreatic tumours, it is not observed for OATP1B1, the main transporter in liver cells (Niedermeyer *et al.*, 2014). Therefore, by selecting MC variants and analogues with higher affinity to OATP1B3, these cyanotoxins would just act on cancer cells, not presenting negative effects on healthy cells. As result of these findings, several studies have been pointing to MCs as promising anticancer drugs (Ilić *et al.*, 2011; Niedermeyer *et al.*, 2014; Zanchett and Oliveira-Filho, 2013). Also, ATX and nodularin have been mentioned to have the potential to conduct cancer treatment (Mahdi and Fariba, 2012).

Dolastatin 10, cryptophycins, and curacin A are among the cyanobacteria's secondary metabolites tested for treating cancer purposes (Malathi *et al.*, 2015). Some of the studied cyanotoxins are already in anticancer preclinical or clinical trials (Kubwabo *et al.*, 2004). Curacin A, for instance, was found to be a potent inhibitor for leukaemia L1210 cells, showing cytotoxic activity against colon, renal, and breast cancer cell lines as well (Chu, 2012; Zanchett and Oliveira-Filho, 2013). Similarly, apratoxin A, isolated from *L. majuscula*, was also found effective against colon LoVo carcinoma cells and epidermal KB carcinoma cells (Zanchett and Oliveira-Filho, 2013). An extensive list of cyanobacterial secondary metabolites and potential pharmacologic/medical applications can be found in Dias *et al.* (2015).

In a different field, cyanotoxins can be used to control CyanoHABs. Some of the bioactive compounds (e.g., cyanobacterin, enediyne, and hapalindoles) produced by several cyanobacteria genera as the case of *Scytonema*, *Fischerella*, or *Hapalosiphon* have shown great impact on microalgae growth mainly due to severe damage found in photosynthetic, respiratory, carbon uptake, and enzymatic activities; in addition, oxidative stress was induced on cells (Lau *et al.*, 2015). Singh *et al.* (2001) conducted a study that determined the algicidal effect of MC-LR produced by *M. aeruginosa*. Six days after adding 50 $\mu g.L^{-1}$ of the purified cyanotoxin to cultures of *Nostoc* and *Anabaena*, the growth was completely inhibited and cell disruption had occurred.

Since herbicides and insecticides are characterized as persistent and toxic, there is a high commercial potential for replacement with bio-products such as cyanobacterial toxins (Berry *et al.*, 2008). Despite some considerations, namely mammalian toxicity, ecosystem impact, and bioaccumulation, must be borne in mind, these compounds present an important alternative to reduce pest insects and consequently highly disseminated and mortal diseases carried by them such as malaria and dengue fever (Berry *et al.*, 2008). The successful use of cyanotoxins against these organisms is associated with the basis of mosquito larva diet, which consists of cyanobacteria (Berry *et al.*, 2008). Keeping this interesting feature in mind, ATX, MCs, CYNs, and pahayokolides were tested and revealed a significant larvicide effect, causing larva mortalities varying between 50 and 100 % (Berry *et al.*, 2008; Kiviranta *et al.*, 1993).

Some cyanotoxins also proved to be very effective when applied as antimicrobial agents, especially against Gram-positive and/or Gram-negative bacteria, as in the case of pahayokolide A produced by *Lyngbya* sp. that showed inhibition of *Bacillus* species

for concentrations of approximately 5 μg.mL^{-1} (Abed *et al.*, 2009; Berry *et al.*, 2004; Senhorinho *et al.*, 2015). MC-LR also presented antimicrobial activity against four different highly antibiotic resistant species of *Mycobacterium* ranging from the minimum inhibitory concentrations between 0.42 and 53 μM (Ramos *et al.*, 2015). Thus, toxin-producing cyanobacteria appear to be a promising source of novel antibiotics that might have the desired effect upon multi-resistant bacteria.

2.8 Specific features related to cyanotoxin production

To boost cyanotoxins market potential, in addition to the knowledge of their potentialities and most suitable growth conditions, there is also a need to develop appropriate infrastructures (e.g., cultivation systems and downstream processing units) and safe methodologies for their production, extraction, purification, certification, and distribution.

As mentioned in Section 2.6.2, cyanotoxins are subject to strict regulations that should be considered during their various production and distribution stages (storage, packaging, and transportation). On the other hand, their high market value allows the utilization of cultivation, cell harvesting, and cyanotoxin extraction and purification processes that, for economic reasons, cannot be considered in the cultivation of microalgae for other applications such as biofuels.

2.8.1 Biotic and abiotic factors affecting cyanotoxin production

Although the processes responsible for triggering cyanotoxin production are not completely understood, it is known that a diverse group of factors like temperature, pH, medium composition, light quantity and quality, and the duration of culture's growth can affect them (Hudnell and Dortch, 2008; Priyadarshani and Rath, 2012). Another aspect intimately associated with cyanotoxins production is the cyanobacterial growth phase. Under favourable conditions, the amount of cyanotoxin might reach its maximum during the exponential growth phase (e.g., ATX) or keep increasing throughout this stage until higher concentrations are achieved at a late exponential phase (e.g., MC and STX) (Neilan *et al.*, 2008; Sivonen and Jones, 1999). Thus, controlling growth conditions of these microorganisms seems to be a good way to manage cyanotoxin productivity.

Generally, cyanotoxin production rate is a strain-dependent feature. However, there are several common effects on cyanotoxin productivity caused by environmental factors.

In the case of MC, the influence of light is not fully understood since some studies suggest an increase under low light (12–44 $\mu mol_{photons}.m^{-2}.s^{-1}$) conditions (Sivonen, 1990), and others point to a maximum toxin content at high light quantities (142 $\mu mol_{photons}.m^{-2}.s^{-1}$) (Neilan *et al.*, 2008; Sivonen and Jones, 1999; van der Westhuizen and Eloff, 1985). Light quality seems to have impact on MC production process once growths performed using red light wavelengths showed increasing transcriptional responses when compared to those where blue light wavelengths were used instead (Kaebernick *et al.*, 2000; Neilan *et al.*, 2008). Comparing the influence of light on nodularin and STX concentrations, it appears to be completely different. While the nodularin amount, either intracellular or extracellular, was found to be higher under high light stress (up to 155 $\mu mol_{photons}.m^{-2}.s^{-1}$), STX (also frequently named as PSP toxin) production was limited under these conditions presenting an inversely proportional behaviour when compared to biomass growth rate (Lehtimaki *et al.*, 1997; Neilan *et al.*, 2008; Sivonen and Jones, 1999; Yin *et al.*, 1997).

Regarding the effect of temperature, nodularin, ATX, and MC production seems to be induced by temperature in several species grown at approximately 19–25 °C. It was also found that ATX levels are not dependent on cyanobacterial growth rate. Different responses were though obtained for STX with production rates higher at lower temperatures (15 °C) (Neilan *et al.*, 2008; Sivonen and Jones, 1999).

The presence/absence and concentration of macronutrients (e.g., nitrogen and phosphorus) and trace metals in the medium play an important role both in cyanobacteria growth and cyanotoxin productivity. Apparently, the availability of phosphorus and nitrogen is the most significant factor affecting the production of MC since it was previously demonstrated that a higher concentration of such nutrients is correlated with higher final toxin concentrations in *Oscillatoria* and *Microcystis* cultures (Neilan *et al.*, 2008). However, this correlation is not accepted because some data do not fully support this theory. For instance, in the experiments performed by Long *et al.* (2001), *M. aeruginosa* grown under a nitrogen-limited environment presented faster growth and greater intracellular MC content despite smaller cells were obtained. Also, *M. aeruginosa* was found to be more toxic when cells were grown in BG-11 medium without any source of phosphorus (Sivonen and Jones, 1999).

The use of larger amounts of nitrogen sources, like ammonium, to grow *N. spumigena* led to decreasing final concentrations of nodularin and reduced growth rates (Lehtimaki *et al.*, 1997; Neilan *et al.*, 2008). In spite of higher nodularin levels were achieved by applying higher phosphorus concentrations, some studies state that no effect was detected in total cyanotoxin values of *N. spumigena* cultures. Although the use of nitrogen-free media was associated with ATX production, variable results were obtained according to the strain and growth conditions utilized (Neilan *et al.*, 2008). As a result of several tests using different nitrogen conditions, CYN synthesis by *Cylindrospermopsis raciborskii* was found to be inversely correlated with biomass growth rate. Nitrogen-supplemented cultures reached intermediary values both for cyanotoxin production and growth rate while the maximum amount of CYN, and consequently the lowest cell growth, was observed in the absence of a nitrogen source. The highest growth rate was attained by supplying cultures with ammonia, resulting in the lowest concentration of toxin registered (Neilan *et al.*, 2008; Saker and Neilan, 2001). Conversely, the optimal concentrations of phosphorus verified for *Lyngbya wollei* growth have resulted in higher PSP production values (Neilan *et al.*, 2008; Yin *et al.*, 1997).

The effect of iron on cyanotoxin productivity is definitely one of the most widely studied among trace metals. However, the response of toxin-producer cyanobacteria in the presence of iron is still contradictory. Regardless of existing studies showing that lower iron concentrations induce a positive reaction in cyanotoxin production (Lukač and Aegerter, 1993), some others suggest completely different conclusions (Utkilen and Gjølme, 1995). These fluctuations might occur due to the strain-dependency of cyanotoxin biosynthesis, as in the case of MC, or because of the interference of external factors such as the amount of light available or the presence of organic compounds that indirectly affect growth rate (Neilan *et al.*, 2008; Sivonen and Jones, 1999).

2.8.2 Downstream processing

Despite significant expenses involved in cyanobacterial production process, the final cost of cyanotoxins is greatly dependent on downstream processing options. The harvesting step includes high energy consumption and, consequently, high costs, representing in some cases 20–30 % of the total production stage costs (Guedes *et al.*, 2014). The most common harvesting methodologies are: (1) centrifugation, (2)

flocculation, (3) sedimentation, (4) filtration, (5) electrophoresis/electric fields, and (6) flotation. In spite of the need for high energy amounts, centrifugation is probably the most widespread harvesting process at industrial scale. When applied in high value-added compounds production processes, high centrifugation costs are immediately overcome by the outstanding efficiencies presented at biomass recovery level (above 95 %) (Guedes *et al.*, 2014). Conversely, filtration is usually applied for small volumes. Given the microscopic dimensions of most cyanobacteria, this process is only technically feasible if microfiltration or ultrafiltration membranes are employed (Guedes *et al.*, 2014; Gupta *et al.*, 2013). Some limitations may derive from membrane filter clogging although the reverse-flow technique or the use of a filter scraper might mitigate this situation. Sedimentation driven by gravity force is a low-cost process regularly used in large-scale growths. Settling only occurs when cyanobacteria become somehow unstable. Stability is mainly dependent on cell size, density, and surface charge. By adding coagulant agents, for instance, cells lose stability and start agglomerating. Clump formation leads not only to sedimentation but also to an increasing settling velocity (Guedes *et al.*, 2014). Dissolved air flotation is also a cost-effective operation, consisting of the injection of air into a culture that results in cells entrapment in very small bubbles; this promotes biomass accumulation at the surface of the liquid from which it can be recovered. The addition of cationic surfactants makes the separation of cyanobacteria from water easier. In opposition to this type of flotation, froth flotation is a very costly method (Guedes *et al.*, 2014). The use of electric fields to promote harvesting of cells is a very promising option. Taking advantage of the negative charge on cyanobacteria cells' surface, it is possible to remove biomass by inducing an electrical field. If the electrolysis effect takes place, hydrogen molecules will be released and will guide cells to the upper part of the liquid. Besides its great selectivity, this operation does not involve any additional chemicals and is also efficient and environmental friendly.

After harvesting cyanobacterial cells, bioactive metabolites such as cyanotoxins need to be extracted from biomass. However, if these metabolites are intracellular, their extraction is possible only after cell disruption.

Disruption methods can be classified into three different groups: (1) mechanical/ physical (e.g., ultrasonication, microwave, bead milling, freeze-thaw cycles, and freeze-drying), (2) chemical, and (3) enzymatic (Michalak and Chojnacka, 2014).

Mechanical/physical methods are very effective but also require a significant amount of energy, which makes process costs higher. Additionally, those involving heat or shear stress might enhance the degradation or denaturation of active compounds (Michalak and Chojnacka, 2014). Conversely, enzymatic treatments are very selective, which is useful when unstable or fragile substances are the extraction target. Since cell-lysing enzymes are truly costly, these methods are not common on industrial scale (Michalak and Chojnacka, 2014). However, owing to the high commercial prices of cyanotoxins, this can be a solid option to apply in large-scale production processes. Another alternative pre-treatment refers to chemical cell disruption using, for instance, acids that will hydrolyse cell membrane, thus releasing the compounds of interest. However, chemical agents used need to be removed so that desired metabolites are not affected, which might impose additional costs to the process.

From all the previously mentioned methods and in opposition to what happens with microalgae, the most common techniques applied on cyanobacteria are freeze-thaw and freeze-drying, which can be supplemented with ultrasonication.

To sum up, in addition to the particularities of cyanobacteria downstream processes mentioned throughout this Section (2.6.4.2), one constant difference to microalgae downstream processing is the need to select and adapt techniques to protect the environment and mitigate the consequences of processing potentially toxic cells, metabolites, and effluents.

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Chapter 3

Effect of environmental conditions on *Microcystis aeruginosa* growth and toxin production

The information presented in this Chapter was adapted from:

Geada, **P**.; Pereira, R.N.; Vasconcelos, V.; Vicente, A.A.; Fernandes, B.D., 2017. Assessment of synergistic interactions between environmental factors on Microcystis aeruginosa growth and microcystin production, Algal Research, 27, 235-243.

3.1 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 $\mu mol_{photons}.m^{-2}.s^{-1}$), 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 $\mu mol_{photons}.m^{-2}.s^{-1}$) and CO_2 concentrations (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 $\mu mol_{photons}.m^{-2}.s^{-1}$), 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 and CO_2 concentration.

3.2 Introduction

Among CyanoHABs 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 (Bláhová *et al.*, 2013; Zhou *et al.*, 2013). 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 (Gutiérrez-Praena *et al.*, 2014; Jia *et al.*, 2014; Martins and Vasconcelos, 2009; Mohamed *et al.*, 2016; Mohamed *et al.*, 2015; Saker *et al.*, 2007; Saqrane *et al.*, 2008).

In order to avoid similar human lethality events as happened in Brazil (Jochimsen *et al.*, 1998), the World Health Organization (WHO) established a guideline value for MC-LR in drinking water, 1 $\mu g \cdot L^{-1}$, and a tolerable daily intake of 0.04 $\mu g \cdot kg^{-1}$ (WHO, 2003). Numerous laboratory analytical methods, including liquid chromatography, in vitro bioassays, and immunoassays, have been extensively used in MC detection and quantification (Lawton and Edwards, 2008). 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 (Meriluoto and Spoof, 2008; Pegram *et al.*, 2008). To overcome this issue as well as to 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 (Geada *et al.*, 2017).

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 CyanoHABs 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 (Deblois and Juneau, 2010; Yang *et al.*, 2012). Over the last years, many studies have been performed in order to assess the influence of light (Deblois and Juneau, 2012; Jähnichen *et al.*, 2011), CO_2 (Wang *et al.*, 2011; Yamamoto and Nakahara, 2005), nutrients (Jähnichen *et al.*, 2011; Liu *et al.* 2011), temperature (Bouchard and Purdie, 2011; Jähnichen *et al.*, 2011), and pH (Krüger *et al.*, 2012; Wang *et al.*, 2011) on *M. aeruginosa* growth and MC content. However, all these studies aimed at exploring the effect of each factor individually. One of the few exceptions is the study performed by Yang *et al.* (2012) where the combined effect of light intensity, temperature and nitrogen concentration on *M. aeruginosa* growth was evaluated, showing significant interactions affecting cyanobacterium growth. Still, the impact of such abiotic factors on toxin production was not determined in that 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 was to determine the impact of combined use of light intensity (10–190 $\mu mol_{photons}.m^{-2}.s^{-1}$), 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, in turn, will contribute to optimize culturing conditions and consequently decrease the high prices of analytical standards employed in control and

monitoring methodologies; ii) further understanding of the real impact of environmental conditions on *M. aeruginosa* growth and toxicity in order to improve CyanoHABs predicting mechanisms.

3.3 Materials and methods

3.3.1 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 (Kotai, 1972) under 10 $\mu mol_{photons}.m^{-2}.s^{-1}$ using a 12 h L:D 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 3.1. Initial biomass concentration was 0.05 $g \cdot L^{-1}$ (dry weight – DW) in all cultivations.

Table 3.1 – Range of tested environmental factors

Environmental factor tested	Tested values
Light intensity ($\mu mol_{photons}.m^{-2}.s^{-1}$)	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

3.3.1.1 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 3.1) and combining them, reaching a total of 18 different arrangements. The tested combinations are presented in Table 3.2. Following the experimental design, three central points (CP) were executed. The pH was kept at 8 by

adjusting its value with NaOH (0.1 mol.L^{-1}) or HCl (0.1 mol.L^{-1}) 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.

3.3.1.2 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 3.3.1.1 (Tables 3.1 and 3.4). The sampling and evaporation compensation was performed as described in Section 3.3.1.1.

3.3.2 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.

3.3.2.1 Biomass concentration

The optical density (*OD*) of cultures was measured at 670 nm and 750 nm (following the recommendations given by (Griffiths *et al.*, 2011) 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} dry weight - DW) was represented as function of *OD* (Equations 3.1 and 3.2), it was possible to follow biomass concentration during tests.

$$X = 0.821 \times OD\,(670\text{ nm}) + 0.014 \quad (R^2 = 0.995) \qquad \text{(Equation 3.1)}$$

$$X = 1.208 \times OD\,(750\text{ nm}) + 0.023 \quad (R^2 = 0.984) \qquad \text{(Equation 3.2)}$$

3.3.2.2 Determination of biomass productivity and specific growth rate

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

$$P = (X_t - X_0) / (t\text{-}t_0) \qquad \text{(Equation 3.3)}$$

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

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

$$\mu = [\ln(X_2) - \ln(X_1)] / (t_2 - t_1) \qquad \text{(Equation 3.4)}$$

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

3.3.3 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}, $\mu g_{toxins}.L^{-1}.d^{-1}$) was obtained from the following equation:

$$P_{toxin} = [T]_f \times P \qquad \text{(Equation 3.5)}$$

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

3.3.4 Statistical analysis

The experiments for quantification of MC 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.

3.4 Results and discussion

3.4.1 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 (μ, P and X) and toxin production ($[T]$ and P_{toxin}) was assessed through a CCD (Tables 3.2, 3.4 and 3.6). 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.

3.4.1.1 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 respective values obtained for each response variable, are shown in Table 3.2.

The results have shown a substantial variation of all dependent variables over the different combinations of light intensity and CO_2 concentration tested (Table 3.2). Namely, μ_{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 $g.L^{-1}$) and a 4.75-fold (0.040–0.190 $g.L^{-1}.d^{-1}$) variation, respectively.

In order to understand more in 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 (Figure 3.1 – data not shown for μ_{max} and X_{max}) built with the data described in Table 3.2.

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 3.3.

Table 3.2 – Experimental design including the combination of light intensity and CO_2 concentration and the respective responses of μ_{max}, X_{max}, and P_{max}

Run	CO_2 concentration (% v/v)	Light intensity ($\mu mol_{photons}.m^{-2}.s^{-1}$)	μ_{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)	5	100	0.0207	1.108	0.112
10 (CP)	5	100	0.0231	1.125	0.105
11 (CP)	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

Figure 3.1 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 μ_{max}, ($p < 0.05$), while X_{max} was not affected ($p > 0.05$) by any of these experimental independent variables. Regarding 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 μ_{max} (> 0.03 h^{-1}) can be obtained when high light intensities (130–190 $\mu mol_{photons}.m^{-2}.s^{-1}$) 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 Gonçalves *et al.* (2016) 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 (≈1.5 d^{-1}).

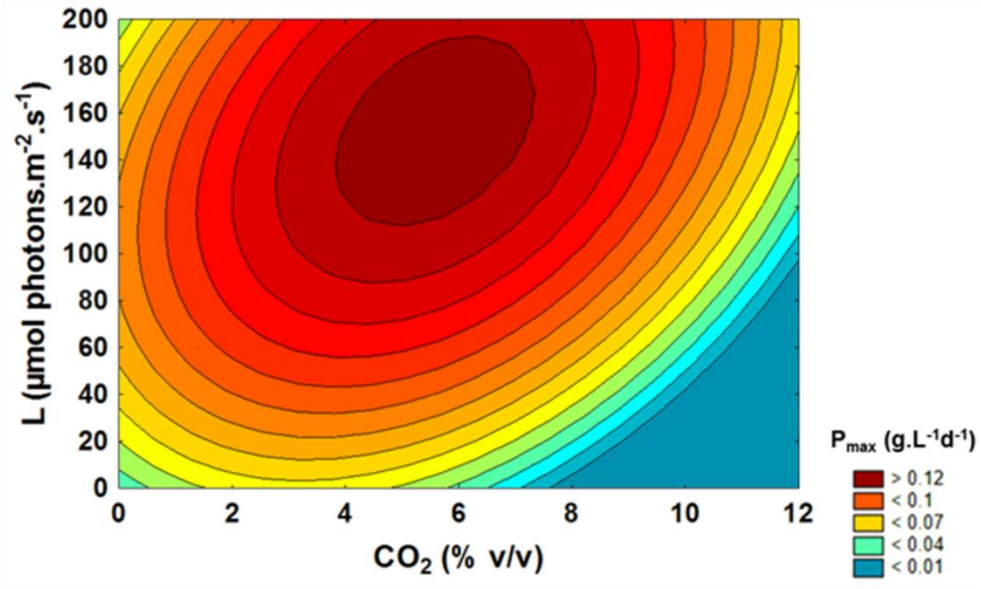

Figure 3.1 - Contour plot of maximum biomass productivity (P_{max}) determined for the set of light intensity (L) and CO_2 concentration combinations tested.

Differently to Gonçalves *et al.* (2016), who reached the highest biomass concentrations (≈ 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 $\mu mol_{photons}.m^{-2}.s^{-1}$) 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 μ_{max} and X_{max}, the results of Figure 3.1 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 $\mu mol_{photons}.m^{-2}.s^{-1}$) with medium to high concentrations of CO_2 (4–7% (v/v)). This response is in agreement with Gonçalves *et al.* (2016) achievements since productivity was nearly the same (≈0.136–0.154 $g \cdot L^{-1} \cdot d^{-1}$) under similar conditions of CO_2 (5–7% (v/v)).

According to Yang *et al.* (2012) and Jähnichen *et al.* (2011), increasing light intensity (up to 65 and 80 μmol photons·m^{-2}·s^{-1}, respectively) also results in higher specific growth rates and cell densities, which is confirmed by the results obtained in this work.

Table 3.3 – Effect estimates, standard errors, and *t*-test for the effect of combined use of light intensity (*L*) and CO_2 concentration (CO_2) on μ_{max}, X_{max}, and P_{max} according to the 2^2 full-factorial CCD. 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
			μ_{max}			
[a]CO_2	0.005	0.002	2.721	**0.020**		
[a]$(CO_2)^2$	-0.005	0.001	-3.348	**0.006**		
[a]L	0.007	0.002	2.879	**0.015**	0.004	0.810
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		
$(CO_2)^2$	-0.137	0.117	-1.170	0.267		
L	-0.054	0.191	-0.282	0.783	0.349	0.251
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		
[a]$(CO_2)^2$	-0.029	0.011	-2.736	**0.019**		
L	0.038	0.017	2.190	0.051	0.032	0.631
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.

Through second-order equations (Equations 3.6-3.8) resulting from response- surface methods (RSM) fitting of μ_{max}, X_{max} and P_{max}, 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.

$$\mu_{max} = 5.5\times10^{-3} + 3.1\times10^{-3}CO_2 - 3.7\times10^{-4}(CO_2)^2 + 1.7\times10^{-4}L - 8.7\times10^{-7}L^2 + 1.6\times10^{-5}CO_2L \quad \text{(Equation 3.6)}$$

$$X_{max} = 1.2 - 1.3\times10^{-3}CO_2 - 1.1\times10^{-2}(CO_2)^2 + 5.7\times10^{-3}L - 5.6\times10^{-5}L^2 + 1.0\times10^{-3}CO_2L \quad \text{(Equation 3.7)}$$

$$P_{max} = 3.3\times10^{-5} - 1.5\times10^{-2}CO_2 - 2.3\times10^{-3}(CO_2)^2 + 1.0\times10^{-3}L - 4.5\times10^{-6}L^2 + 7.5\times10^{-5}CO_2L \quad \text{(Equation 3.8)}$$

According to these equations, the maximum estimated value for μ_{max} (0.0312 h^{-1}) can be obtained at 168 $\mu mol_{photons}.m^{-2}.s^{-1}$ and 7.8 % of CO_2, while the maximum estimated for X_{max} (1.447 $g \cdot L^{-1}$) is obtained at 83 $\mu mol_{photons}.m^{-2}.s^{-1}$ and 3.7 % of CO_2 and P_{max} (0.146 $g.L^{-1}.d^{-1}$) is observed at 155 $\mu mol_{photons}.m^{-2}.s^{-1}$ 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 $\mu mol_{photons}.m^{-2}.s^{-1}$ 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 ± 0.027 $g.L^{-1}.d^{-1}$ – is in fact considerably higher than the "estimated P_{max}" value. The conditions (155 $\mu mol_{photons}.m^{-2}.s^{-1}$ 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 3.4.1.2).

3.4.1.2 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 3.4). As previously mentioned, the light intensity (155 $\mu mol_{photons}.m^{-2}.s^{-1}$) 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 μ_{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 Wang *et al.* (2011) 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 3.4, 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).

Figure 3.2 exhibits the contour plot of P_{max}, giving a clear idea about the combined effect of temperature and pH on this parameter.

Table 3.4 – Experimental design including the combination of temperature and pH and the respective responses of μ_{max}, X_{max}, and P_{max}

Run	pH	Temperature (°C)	μ_{max} (h^{-1})	X_{max} ($g.L^{-1}$)	P_{max} ($g.L^{-1}d^{-1}$)
21	6.5	15	NG	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)	8.0	30	0.0326	1.474	0.179
29 (CP)	8.0	30	0.0326	1.437	0.174
30 (CP)	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

*NG: No Growth.

Statistical analysis (summarized in Table 3.5) confirmed that linear term of temperature and quadratic term of pH have statistical significance on P_{max} ($p < 0.05$), while X_{max} and μ_{max} were only affected by the quadratic term of temperature ($p < 0.05$).

Response surfaces (data not shown for μ_{max} and X_{max}) allow concluding that the combination of temperatures ranging between 28 and 35 °C and culture media 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 Krüger *et al.* (2012) 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 Krüger *et al.* (2012) were carried out solely at 24 °C while our results rely on the combined use of higher temperatures with pH variation.

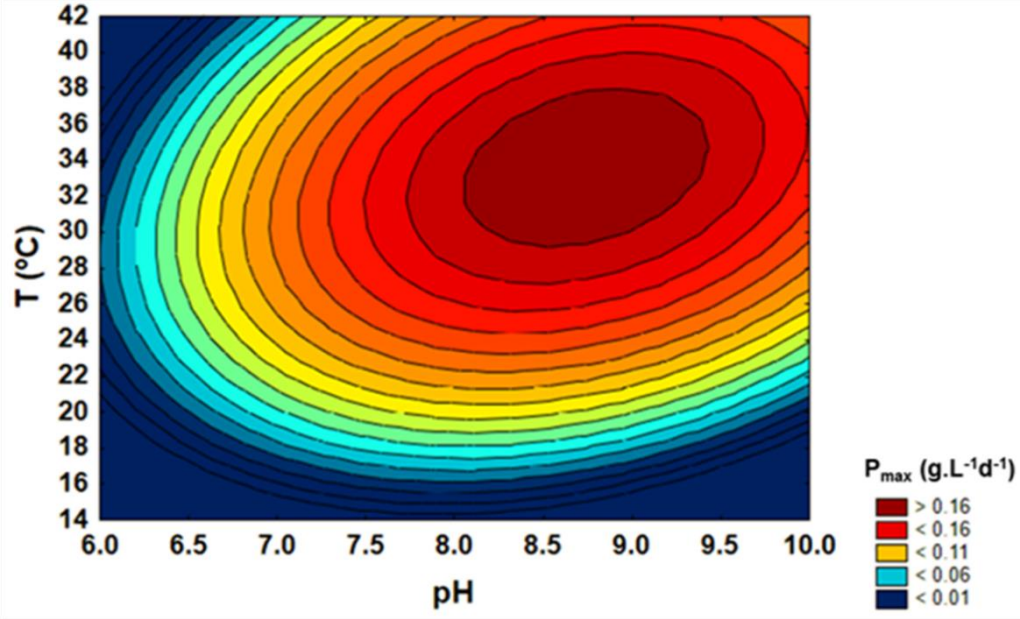

Figure 3.2 - Contour plot of maximum biomass productivity (P_{max}) determined for the set of temperature (T) and pH combinations tested. All growths were carried out at 155 $\mu mol_{photons}.m^{-2}.s^{-1}$and using a CO_2-rich atmosphere (5.5 % (v/v)).

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 μ_{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 Mowe *et al.* (2015). 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 μ_{max}, playing an important role in its variation, thus should not be neglected (Table 3.5). Furthermore, the μ_{max} achieved by Mowe *et al.* (2015) 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 $\mu mol_{photons}.m^{-2}.s^{-1}$) when compared to their study (60 $\mu mol_{photons}.m^{-2}.s^{-1}$).

Table 3.5 – Effect estimates, standard errors, and *t*-test for the effect of combined use of pH and temperature (*T*) on μ_{max}, X_{max}, and P_{max} according to the 2^2 full-factorial CCD. All growths were carried out at 155 $\mu mol_{photons}.m^{-2}.s^{-1}$ 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
			μ_{max}			
pH	0.011	0.006	1.793	0.107		
pH^2	-0.008	0.010	-0.762	0.466		
T	0.005	0.003	1.554	0.155	0.009	0.789
[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		
pH^2	-0.617	0.413	-1.493	0.170		
T	0.065	0.137	0.476	0.645	0.377	0.718
[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**		
pH^2	-0.090	0.045	-2.018	0.074		
T	0.023	0.015	1.523	0.162	0.041	0.815
[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.

Through the second-order equations (Equations 3.9-3.11) resulting from RSM fitting, it was possible to obtain the maximum estimated μ_{max}, X_{max}, P_{max}, respectively, and the conditions of pH and temperature under which they can be reached.

$$\mu_{max} = -1.5\times10^{-1} + 2.2\times10^{-2}pH - 1.7\times10^{-3}pH^2 + 5.0\times10^{-3}T - 1.2\times10^{-4}T^2 + 3.3\times10^{-4}pH.T \quad \text{(Equation 3.9)}$$

$$X_{max} = -10.9 + 2.1pH - 1.4\times10^{-1}pH^2 + 2.5\times10^{-1}T - 4.9\times10^{-3}T^2 + 6.5\times10^{-3}pH.T \quad \text{(Equation 3.10)}$$

$$P_{max} = -1.4 + 2.9\times10^{-1}pH - 2.0\times10^{-2}pH^2 + 1.8\times10^{-2}T - 4.9\times10^{-4}T^2 + 1.7\times10^{-3}pH.T \quad \text{(Equation 3.11)}$$

According to these equations, the maximum estimated value for μ_{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 3.4.1.1), 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 $g.L^{-1}.d^{-1}$).

3.4.1.3 Combined effect of environmental factors on MC 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 3.6.

According to the results presented in Table 3.6, MC concentration (2.2–4790.7 $\mu g \cdot g_{.cells}^{-1}$) and productivity (0.1–308.6 $\mu g.L^{-1}.d^{-1}$) 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 $\mu g.g_{cells}^{-1}$) and P_{toxin} (308.6 $\mu g.L^{-1}.d^{-1}$) were both achieved using the same growth conditions, i.e. 55 $\mu mol_{photons}.m^{-2}.s^{-1}$ 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 $\mu mol_{photons}.m^{-2}.s^{-1}$ and subjected to a CO_2-enriched environment containing 5 % (v/v) presented the lowest values both for *[T]* (2.2 $\mu g.g_{cells}^{-1}$) and P_{toxin} (0.1 $\mu g.L^{-}.d^{-1}$). All the lower *[T]* and P_{toxin} were obtained in the growths performed at high (≥5 %) CO_2 concentrations.

A clearer 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 (Figure 3.3) built with data from Table 3.6.

According to Figure 3.3A, and confirming the conclusions withdrawn from Table 3.6, higher concentrations of toxin are attained by coupling very low to medium light quantities (below 60 $\mu mol_{photons}.m^{-2}.s^{-1}$) with low levels of CO_2 available (0–1 % (v/v)). These results are corroborated by Jähnichen *et al.* (2011), where a sharp decrease was

observed for toxin production of *M. aeruginosa* cells grown under 8 $\mu mol_{photons}.m^{-2}.s^{-1}$ when compared to experiments performed at 65 $\mu mol_{photons}.m^{-2}.s^{-1}$.

Table 3.6 – 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 ($\mu mol_{photons}.m^{-2}.s^{-1}$)	*[T]* ($\mu g.g_{cells}^{-1}$)	P_{toxin} ($\mu g.L^{-1}.d^{-1}$)
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 (ºC)	***[T]*** ($\mu g.g_{cells}^{-1}$)	P_{toxin} ($\mu g.L^{-1}.d^{-1}$)
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

According to Figure 3.3B, P_{toxin} also follows the behaviour found for *[T]* reaching values > 160 $\mu g.L^{-1}.d^{-1}$ under similar growth conditions (light intensities below 80 $\mu mol_{photons}.m^{-2}.s^{-1}$ combined with CO_2 concentrations never exceeding 1 % (v/v)). Lower *[T]* and P_{toxin} are obtained under high light intensities (> 140 $\mu mol_{photons}.m^{-2}.s^{-1}$) or combining light intensities below 100 $\mu mol_{photons}.m^{-2}.s^{-1}$ 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 3.7).

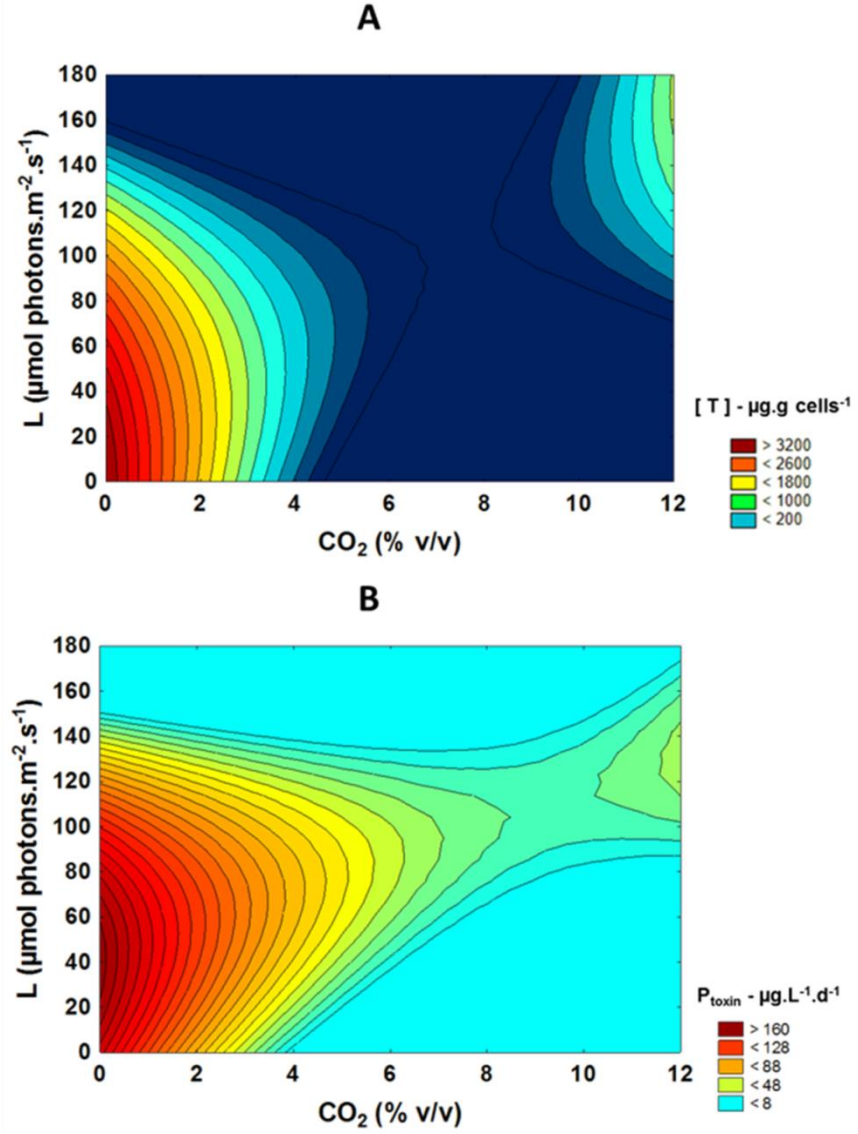

Figure 3.3 - Contour plot of A) toxin concentration (*[T]*) and, B) toxin productivity (P_{toxin}) determined for the set of light intensity (L) and CO_2 concentration combinations tested (n= 3).

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 closer to the natural environments might be more prone to improve toxin productivity rates.

Table 3.7 – 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 CCD. 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		
$(CO_2)^2$	1735.780	1237.350	1.403	0.198		
L	-728.980	573.789	-1.270	0.240	807.307	0.811
L^2	-505.450	518.396	-0.975	0.358		
$CO_2 \times L$	1653.090	892.231	1.853	0.101		
			$\boldsymbol{P_{toxin}}$			
CO_2	-115.908	63.756	-1.818	0.107		
$(CO_2)^2$	53.628	96.888	0.554	0.595		
L	-59.118	44.929	-1.316	0.225	63.215	0.690
L^2	-60.666	40.592	-1.495	0.173		
$CO_2 \times L$	100.675	69.864	1.441	0.188		

M. aeruginosa toxicity changes observed as a result of variation on temperatures and pH (Table 3.6) are in the same range as those obtained when high CO_2 concentrations were applied, maintaining values below 10 $\mu g.g_{cells}^{-1}$ and 1 $\mu g.L^{-1}.d^{-1}$ 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 $\mu mol_{photons}.m^{-2}.s^{-1}$ of light and 5.5% (v/v) of CO_2 being suitable to reach maximum P_{max} (Section 3.4.1.1), 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 order of magnitude as those observed when the influence of light intensity and CO_2 concentration was tested, the fact is that the linear and quadratic terms of pH were found to have a statistically significant effect on *[T]* and P_{toxin} (Table 3.8), respectively.

Figure 3.4 shows contour surfaces *[T]* and P_{toxin} under the effects of temperature and pH.

Table 3.8 – 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 CCD. All growths were carried out at 155 $\mu mol_{photons}.m^{-2}.s^{-1}$ 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.

Among the conditions tested, it is possible to infer through Figure 3.4A graph that the combination of high temperatures with high medium pH represent allow reaching greater toxin contents (> 8.75 $\mu g.g_{cells}^{-1}$). These results are not in agreement with other studies reporting that increases in temperature (15–36 °C) lead to a decrease of toxin production (Jähnichen *et al.*, 2011; Mowe *et al.*, 2015). On the other hand, Krüger *et al.* (2012) 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 Figure 3.4A.

The combined use of temperatures below 40 °C and pH values lower than 7.5 tends to have a negative impact over the amount of toxin produced (< 5 $\mu g.g_{cells}^{-1}$). Regarding P_{toxin}, there is a distinct region of conditions where high values can be attained (> 1 $\mu g.L^{-1}.d^{-1}$). 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 $\mu g.L^{-1}.d^{-1}$).

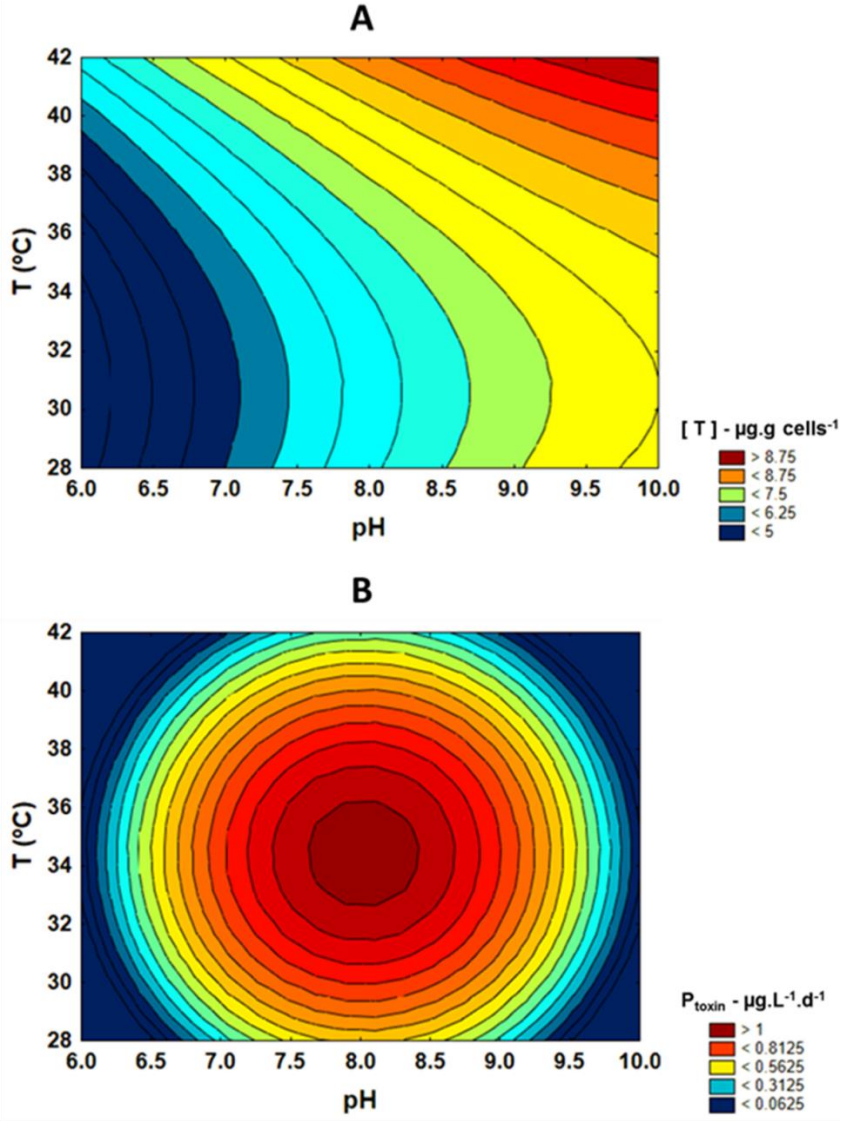

Figure 3.4 - Contour plot of A) toxin concentration (*[T]*) and, B) toxin productivity (P_{toxin}) determined for the set of temperature and medium pH combinations tested for cultures grown at 155 $\mu mol_{photons}.m^{-2}.s^{-1}$ using a CO_2-rich atmosphere (5.5 % (v/v)) (*n*=3).

3.4.1.4 Environmental factors as a tool to predict the behaviour of *M. aeruginosa* harmful blooms

As previously mentioned, the maximization of *M. aeruginosa* growth and MC content were the main objectives for this research, but we consider that these results provide good indications on how to predict *M. aeruginosa* thriving in natural environments, and consequently to avoid issues caused by their cyanotoxins. Environmental conditions that can minimize the appearance of *M. aeruginosa* harmful blooms were assessed by DFA tool (Derringer and Suich, 1980). DFA (Figure 3.5) allowed identifying the range of independent variables (light intensity, CO_2 concentration, temperature and pH) at which

the minimal values for the dependent variables (*[T]* and P_{toxin}) are obtained, in a way that is more precise than a simple consultation of the Figures presented above.

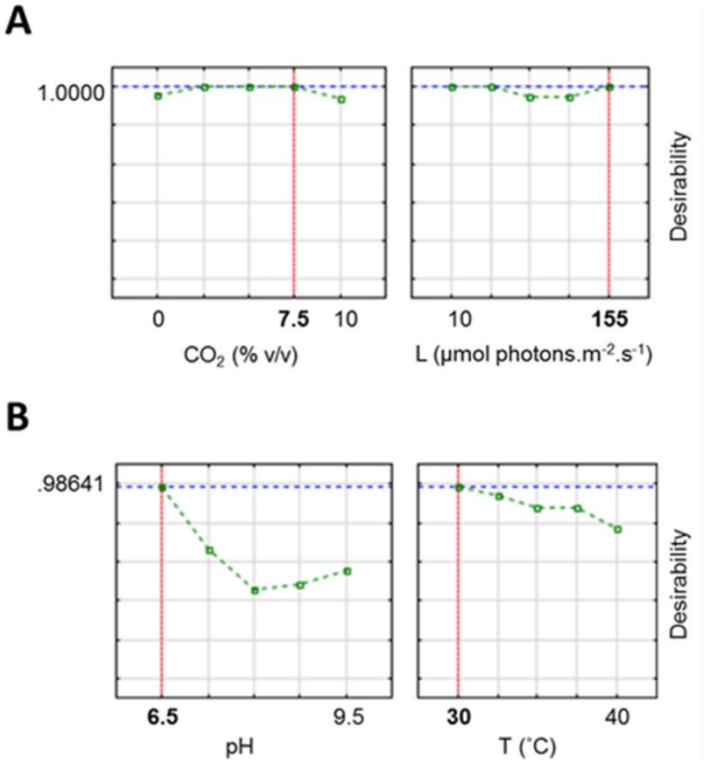

Figure 3.5 - Desirability profiles to obtain lower values of *[T]* and P_{toxin} associated with A) combined use of light intensity (*L*) and CO_2 concentration (CO_2) and, B) combined use of pH and temperature (*T*).

Taking into account the information presented in Figure 3.5, the environmental conditions (in the tested range) in which the lowest *[T]* and P_{toxin} were observed for *M. aeruginosa* cultures are: a) 155 $\mu mol_{photons}.m^{-2}.s^{-1}$ of light intensity; b) 7.5 % (v/v) of CO_2; c) temperature of 30 ˚C; and d) 6.5 as the value of pH. The results retrieved from DFA analysis (Figure 3.5) are in agreement with those obtained on section 3.4.1.3.

3.5 Conclusions

A great impact of the combined effect of light intensity, CO_2 concentration, temperature, and pH, was observed both on *Microcystis aeruginosa* growth parameters and MC production. Results suggest that maintaining cultures under light intensities below 80 $\mu mol_{photons}.m^{-2}.s^{-1}$ 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 $\mu mol_{photons}.m^{-2}.s^{-1}$ 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.

3.6 References

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Chapter 4

Strategies to enhance biomass and toxin productivities

Part of the information described in this Chapter was adapted from:

Geada, P., Gkelis, S., Teixeira, J.A., Vasconcelos, V., Vicente, A.A., Fernandes, B., 2017. Chapter 17 - Cyanobacterial toxins as a high added value product, In Muñoz, R. and Gonzalez, C. (Eds). Microalgae-Based Biofuels and Bioproducts, Woodhead Publishing, Cambridge, UK, pp. 405-432.

Geada, P., Vasconcelos, V., Vicente, A.A., Fernandes, B., 2017. Chapter 13 - Microalgal Biomass Cultivation, In Rastogi, R.P., Madamwar, D. and Pandey, A. (Eds). Algal Green Chemistry: Recent progress in Biotechnology (Biotechnology series), Elsevier, Amsterdam, Netherlands, pp. 257-284.

4.1 Comparison of cultivation systems on *M. aeruginosa* growth

4.1.1 Abstract

The study of the influence of the type of cultivation system on growth of microalgae and cyanobacteria is of utmost importance since it affects cell exposure to light and its distribution inside the vessel, the flow pattern of the culture, and all the hydrodynamics and mass transfer parameters. Therefore, we decided to compare the growth of *M. aeruginosa* on three types of PBR: a bubble column (BC), a split cylinder airlift (SCA), and a flat panel (FP). Although the continuously illuminated surface of BC and FP was approximately 20 % lower than SCA, both PBRs have attained biomass concentrations which were 76 and 98 % higher, respectively, than the ones registered in the SCA. Regarding maximum biomass productivity (P_{max}) and specific growth rate (μ_{max}), FP also showed the best results.

4.1.2 Introduction

Cultivation of cyanobacteria can be conducted either in open systems (e.g., raceway ponds) or closed systems (i.e., PBRs). A thorough comparison between both culture systems is described in Section 2.4. However, based on the information given throughout Section 2.6, it can be concluded that toxin-producing cyanobacteria growths should be carried out in highly controlled closed PBRs to reduce to a minimum the risk of contamination of the surrounding environment, as well as the contact of humans and animals with harmful metabolites. Also, the effluents derived from such cultures must be handled carefully because of their toxic content; the use of closed systems appears to be the most suitable option in order to manage them and restrict their inherent potential danger. If the objective is to optimize the productivity of useful metabolites collected from toxic cyanobacteria or of biomass itself, PBRs will allow a better control and adjustment of growth- and/or metabolites' production-affecting parameters. Contamination of growing cultures is also easier to prevent when growth occurs in PBRs. Despite the fact that closed PBRs seem to be the logical option to apply in toxin-producing cyanobacteria growth, they usually represent a significant capital investment (Guedes *et al.*, 2014). However, the need for strict manipulation conditions and the high commercial value of the product make these culture systems economically and operationally feasible.

In order to achieve high biomass yields, culturing systems must present a uniform light regime inside the vessel and allow an efficient mass transfer between the input air stream and cyanobacterial biomass. High performance is, however, difficult to maintain when PBRs scale-up process takes place since this encompasses significant changes in agitation patterns that necessarily affect illumination, gas transfer, pH, temperature, and nutrient distribution inside the reactor (Guedes *et al.*, 2014).

The aim of this work was therefore to compare the growth of *M. aeruginosa* LEGE 91094 using three different PBRs: a bubble column (BC), a flat-panel (FP), and a split cylinder airlift (SCA).

4.1.3 Materials and Methods

4.1.3.1 Microorganism and inoculum maintenance

Stock cultures of *Microcystis aeruginosa* LEGE 91094, kindly provided by the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR - Porto, Portugal) collection, were kept in Z8 medium (Kotai, 1972) under the following conditions: 10 $\mu mol_{photons}.m^{-2}.s^{-1}$ of light intensity, 12 h L:D cycles, and 20 °C. These cultures were renewed every month.

4.1.3.2 Culture conditions and PBRs description

The experiments were carried out in the same growth medium as in the inoculum applying light intensities of 155 $\mu mol_{photons}.m^{-2}.s^{-1}$ and 12 h L:D cycles. When necessary, the pH of the assays was adjusted twice a day to 8.7 through the use of NaOH 2M and HCl 2M. The three PBRs compared in this study with respect to the growth of *M. aeruginosa* are presented in Figure 4.1. The internal diameter of both BC and SCA was 0.090 m, being the height of the liquid 0.60 m, respectively. The separation between the riser and the downcomer of the SCA PBR was made using a baffle located 0.050 m above the bottom and 0.050 m below the surface of the culture. The length and width of the FP were 0.385 and 0.071 m, respectively, while the liquid height attained 0.14 m. The working volumes of circular PBRs were 3.7 L and in FP it was 4 L. In order to set a baseline on comparing the performances, a fixed superficial gas velocity (u_s) of approximately 0.0013 $m.s^{-1}$ was established.

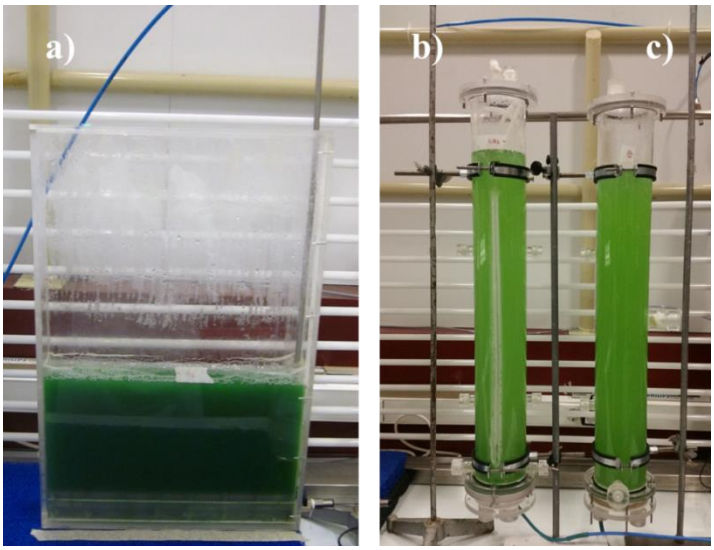

Figure 4.1 – PBRs used for growing *M. aeruginosa*: a) FP, b) SCA, and c) BC.

4.1.3.3 Growth kinetics

The biomass concentration (X) and productivity (P), and the specific growth rate (μ) obtained throughout this study were determined by the Equations 3.2 to 3.4, respectively, presented in Sections 3.3.2.1 and 3.3.2.2.

$$X = 1.208 \times DO\ (750\ \text{nm}) + 0.023 \quad (R^2 = 0.984) \qquad \text{(Equation 3.2)}$$

$$P = (X_t - X_0) / (t - t_0) \qquad \text{(Equation 3.3)}$$

$$\mu = [(\ln(X_2) - \ln(X_0)] / (t_2 - t_1) \qquad \text{(Equation 3.4)}$$

4.1.4 Results and discussion

The behaviour of *M. aeruginosa* growing in 3 different PBRs is shown in Figure 4.2.

According to these data, the FP promoted higher cell densities presenting an increment of approximately 12 % when compared to the BC. However, to attain maximum concentration on BC, cultures took roughly twice the time, which is reflected on lower biomass productivities (Table 4.1). On the other hand, SCA showed significant lower cell concentrations (nearly half) than the other two PBRs. Nevertheless, it appears to be irrelevant in terms of P_{max} since it was the same as determined for BC. Regarding μ_{max}, the FP has, once again, demonstrated to be the most suitable type to apply on *M. aeruginosa* growth (Table 4.1).

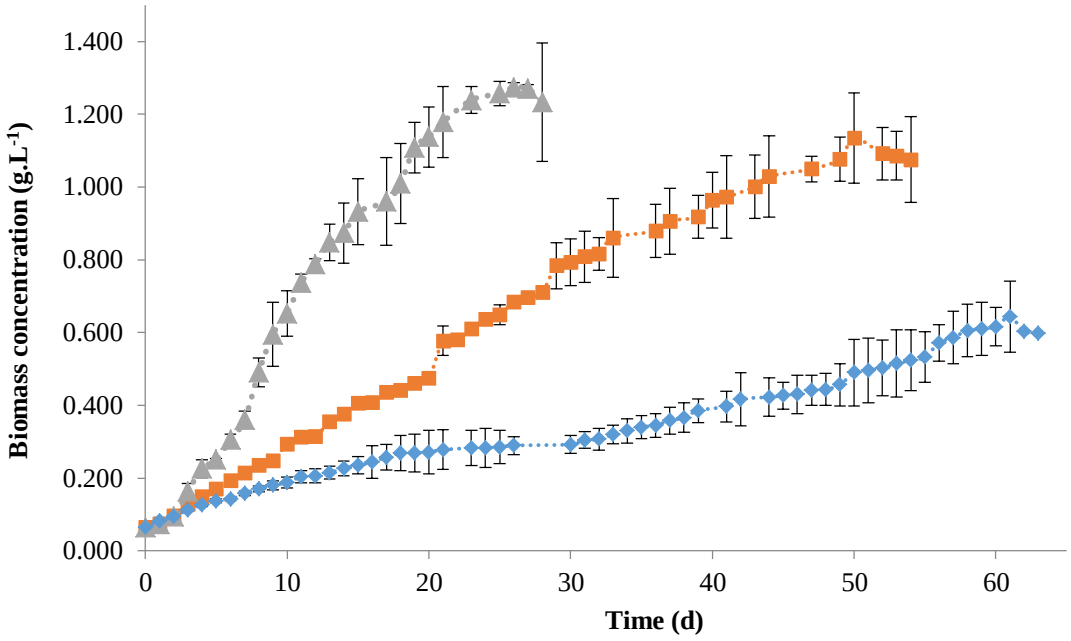

Figure 4.2 – Growths carried out in different PBRs: BC (■), FP (▲), and SCA (♦) (*n*=3).

Generally, FPs are associated with the highest cell densities in microalgae photoautotrophic growths. A better performance is associated with shorter light path and, consequently, greater areal and volumetric productivities are attained. However, the circular geometry allows a more efficient light penetration and higher fraction of illuminated volume inside the reactor, which can be beneficial in some cases (Fernandes *et al.*, 2010).

Table 4.1 – Maximum biomass concentration (X_{max}), productivity (P_{max}), and specific growth rate (μ_{max}) obtained for each of the PBRs tested (*n*=3)

Parameter	BC	SCA	FP
X_{max} ($g.L^{-1}$)	1.134 ± 0.125	0.643 ± 0.098	1.274 ± 0.013
P_{max} ($g.L^{-1}.d^{-1}$)	0.048 ± 0.001	0.047 ± 0.003	0.067 ± 0.002
μ_{max} (d^{-1})	0.281 ± 0.005	0.159 ± 0.002	0.440 ± 0.009

With respect to the differences between growths performed in the BC and the SCA, Merchuk *et al.* (2000) also reached the highest biomass concentration in the first reactor type when evaluating the best cultivation system for the microalga *Porphyridium sp*. The gap between growth yields presented for both types of PBR was though very small when compared to our results.

Using the same BC and SCA of this experiment, Fernandes *et al.* (2014) has demonstrated that higher biomass productivities of *C. vulgaris* could be obtained in SCA, which was justified by SCA's greater illuminated surface area (Table 4.2) and the regular flow pattern that provides a regular exposure of cells to L:D periods.

Table 4.2 – Continuously illuminated area for the different types of PBR

Type of PBR	Surface area illuminated (m^2)
FP	0.155
SCA	0.187
BC	0.157

Considering the surface area that is continuously illuminated in each PBR, our results seem somehow surprising. The differences found in our study might result from the higher light sensitivity of *M. aeruginosa* cells when compared to *C. vulgaris*, thus affecting the growth of the culture in SCA.

4.1.5 Conclusions

The FP PBR has shown to be the best option concerning the growth of *M. aeruginosa*. Maximum biomass concentration (1.274 ± 0.013 $g.L^{-1}$) and productivity (0.067 ± 0.002 $g.L^{-1}.d^{-1}$), and specific growth rate (0.440 ± 0.009 d^{-1}) were obtained using this type of PBR. Although the X_{max} obtained for BC (1.134 ± 0.125 $g.L^{-1}$) was not far from the observed in FP, the time needed to reach this value was the double. Despite of the low cell concentration determined for SCA (0.643 ± 0.098 $g.L^{-1}$), the fact is that the P_{max} attained (0.047 ± 0.003 $g.L^{-1}.d^{-1}$) was the same as the BC (0.048 ± 0.001 $g.L^{-1}.d^{-1}$).

4.1.6 References

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4.2 Influence of a two-stage cultivation strategy on *M. aeruginosa's* growth and toxin production

4.2.1 Abstract

Most of the high-value products obtained from cyanobacteria, namely secondary metabolites, does not reach their maximum concentration within the cell in conditions under which biomass growth is favoured. The study of strategies that allow the overproduction of valuable compounds is therefore a must nowadays. The aim of this work was testing a two-stage approach where biomass growth was firstly enhanced and MC accumulation was induced afterwards by changing the environmental conditions of the culture. As result, the first stage has presented the highest P_{max} (≈ 0.088 $g.L^{-1}.d^{-1}$) and μ_{max} (≈ 0.025 d^{-1}), whilst the maximum increment of MC concentration was reached in the second stage (66.7 %).

4.2.2 Introduction

Under natural photosynthetic conditions, many cyanobacteria have the ability to produce diverse metabolites including lipids, carbohydrates, proteins, pigments, toxins, and antioxidants in addition to biomass growth (Brennan and Owende, 2010; Geada *et al.*, 2017). Synthesis of structural compounds responsible for the maintenance of the normal metabolism and behaviour of the cells is thus favoured under these conditions. The optimization of growth-affecting parameters may lead to higher accumulation of products of interest, which are frequently secondary metabolites. However, this rationale is only valid when metabolites' productivity is correlated with biomass proliferation. In some cases, optimal growth conditions do not meet the proper environment to obtain the maximum product amount. To overcome this limitation while also balancing biomass growth and metabolites' accumulation, the implementation of a two-stage growth strategy arises as a promising alternative to conventional methods. The first stage is thus based on optimal conditions for cyanobacterial growth, while the second stage relies on a shift of cultivation conditions to decrease or even stop the primary metabolism. The use of carbon and energy is then directed towards the production of secondary metabolites, resulting in higher intracellular contents of those added-value products (Douskova *et al.*, 2008, Wang *et al.*, 2015). To achieve this overproduction state, which can induce an increased production (up to 10-fold), a

variety of strategies such as macronutrients limitation (Dragone *et al.*, 2011), high light intensity (Brányiková *et al.*, 2011), or the utilization of heterotrophic and mixotrophic culture conditions (Heredia-Arroyo *et al.*, 2010) might be applied.

Therefore, our study evaluated the influence of a two-stage strategy for the growth of *M. aeruginosa* and production of its toxin.

4.2.3 Materials and methods

4.2.3.1 Microorganism and stock culture

Microcystis aeruginosa LEGE 91094 was obtained from the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR - Porto, Portugal) culture collection and maintained under 10 $\mu mol_{photons}.m^{-2}.s^{-1}$ of light intensity and 20 °C in Z8 medium (Kotai, 1972) using 12 h L:D cycles. Stock solutions were renewed once a month.

4.2.3.2 Determination of toxin accumulation throughout the growth

The production and accumulation of MC was followed during the growth of *M. aeruginosa* in order to define the proper time for changing the conditions in the two-stage approach. These assays were performed in triplicate in Schott-flasks with a working volume of 1 L under culturing conditions determined as optimal to reach P_{max} in Chapter 3.

4.2.3.3 Experimental conditions

Based on the results from Chapter 3, we started by subjecting cells to 155 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 34 °C in a CO_2-enriched medium (5.5 %) with pH adjusted to 8.7. This first stage was meant to favour the quick growth of cyanobacteria (P_{max}). In order to enhance the accumulation of MC and attain its maximum concentration, also taking into account the results shown in Chapter 3, the second stage of growth was carried out applying the following growth conditions: 10 $\mu mol_{photons}.m^{-2}.s^{-1}$ of light intensity, pH medium adjusted to 9.5 and room temperature without addition of CO_2 to the air stream. Although there were numerous attempts to perform these tests in the FP PBR, described as the most suitable cultivation system for *M. aeruginosa* on Section 4.1.4, it revealed to be an impossible task. Thus, the experiments were done in triplicate using Schott flasks

with a working volume of 1 L. The u_s was kept the same as applied on Subchapter 4.1 for the comparison of the PBRs.

4.2.3.4 Determination of growth-related parameters

Biomass concentration (X) and productivity (P), and the specific growth rate (μ) obtained throughout this study were determined by Equations 3.2 to 3.4, respectively, presented in Sections 3.3.2.1 and 3.3.2.2.

$$X = 1.208 \times OD\,(750\text{ nm}) + 0.023 \quad (R^2 = 0.984) \qquad \text{(Equation 3.2)}$$

$$P = (X_t - X_0) / (t - t_0) \qquad \text{(Equation 3.3)}$$

$$\mu = [(\ln(X_2) - \ln(X_0)] / (t_2 - t_1) \qquad \text{(Equation 3.4)}$$

4.2.3.5 Cyanotoxin quantification

MC quantification was performed using the Microcystins-ADDA ELISA Kit (Abraxis, Inc., Pennsylvania, USA), as described in Section 3.3.3.

4.2.4 Results and discussion

4.2.4.1 Variation of MC concentration throughout culture's growth

The growth of *M. aeruginosa* and the respective concentration of toxin throughout the time are represented in Figure 4.3.

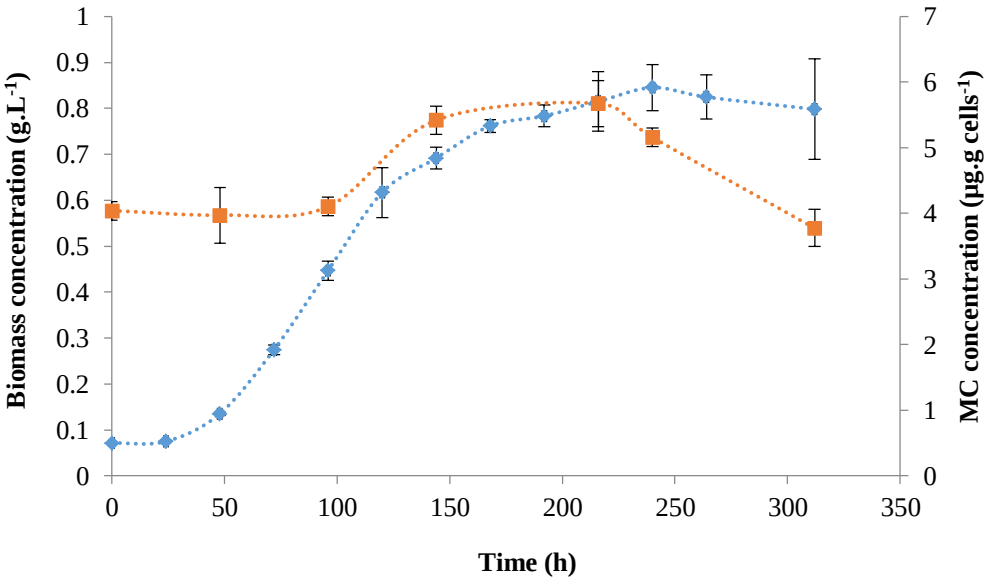

Figure 4.3 – Variation of MC content (■) throughout the growth of *M. aeruginosa* (♦) (n=3).

Analysing these data, it is possible to observe that MC's concentration remains stable until cells reach the intermediate stage of exponential growth phase. The maximum concentration of toxin was achieved at the end of the exponential phase, which is in agreement with the information found in literature (Sivonen and Jones, 1999). During the stationary phase of the culture, apparently the production of MC decreases significantly and its amount is kept almost unchanged. In the later phase of growth the concentration of cyanotoxin suffers a sharp reduction probably due to cell lysis and consequent release of the intracellular MCs (Sivonen and Jones, 1999), which become exposed to the external environment that might accelerate its degradation/denaturation process.

After evaluating the production of MC throughout the growth of *M. aeruginosa*, and considering the results of Figure 4.3, we decided that growth conditions shifting point should be at the late stage of exponential growth phase.

4.2.4.2 Two-stage approach in FP PBR

The two-stage growths were then performed in the selected PBR, FP. However, we had some problems in conducting such experiments, as shown in Figure 4.4. Cells usually started the exponential growth phase quickly, thus presenting great biomass productivities, but their concentration suddenly started to decrease after 4 to 6 days of growth.

Figure 4.4 shows that attempt 1 reached the late stage of exponential phase and, as consequence, we shifted environmental conditions according to the selected parameters in order to carry out the second stage of the culture. After this change, a continuous decrease of the amount of cells was observed. Although initially thought as being the major reason for this biomass loss, the fact is that the alteration of growth conditions seems not to be directly connected to cells' death. Analysing the growth curve of attempt number 2, for instance, one can see that biomass concentration sharply decreases even before achieving the late stage of exponential growth phase. Therefore, the shift of environmental conditions in order to induce MC accumulation was not possible to perform in this case.

This behaviour is more surprising and harder to explain considering that we had successful growths occurring under the conditions chosen to be applied on both stages

of a two-stage approach (Chapter 3), as well as using FP PBR (Subchapter 4.1). A possible explanation for turning these tests impractible can be associated with the nonlinearity of the scale-up (Silva and Reis, 2015) typically resulting in different mixing and flow patterns that affect the hydrodynamics of the culture (Borowitzka and Vonshak, 2017). These changes might also lead to photoinhibition (Borowitzka and Vonshak, 2017) and/or self-shading effect (Silva and Reis, 2015), influencing therefore some of the growth-affecting parameters observed in Chapter 3. It is important to point out that we moved from 40 mL test tubes to a 4 L FP PBR. Another change that might induce some stress over the cells is the injection of a CO_2-rich air stream (containing 5.5 %) into the PBR or the increase of temperature from room temperature to 34 °C, which did not happen in the case of Subchapter 4.1 experiments.

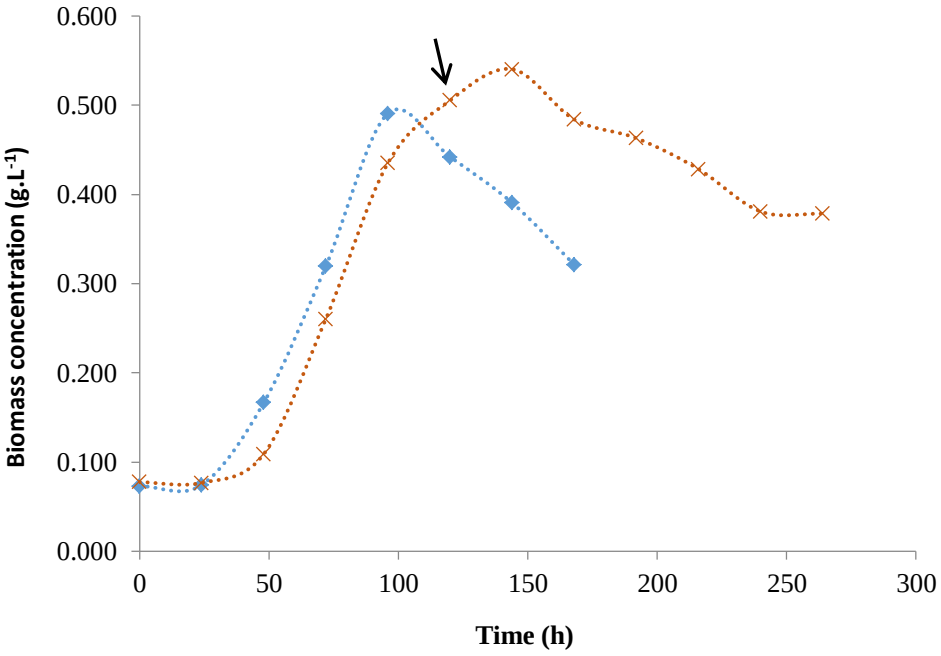

Figure 4.4 – Examples of two-stage growths using FP PBR: attempt 1 (×); attempt 2 (♦). Black arrow indicates the time at which the shift of environmental conditions took place in attempt 1.

To overcome this hurdle and proceed with the two-stage growth assessment, we decided to reduce the scale, performing the growths in Schott flasks with a working volume of 1 L, using the same environmental conditions previously selected for both stages.

4.2.4.3 Two-stage approach in Schott flasks

Figure 4.5 plots the growth curves obtained using the two-stage approach. According to the variation of MC concentration determined throughout the growth (Figure 4.3), we

defined four different sampling points: 1) at the beginning of growth; 2) when the shift of growth conditions occurred (late exponential phase); 3) when the amount of MC was estimated as maximum (at the middle of stationary phase); 4) at the end of growth.

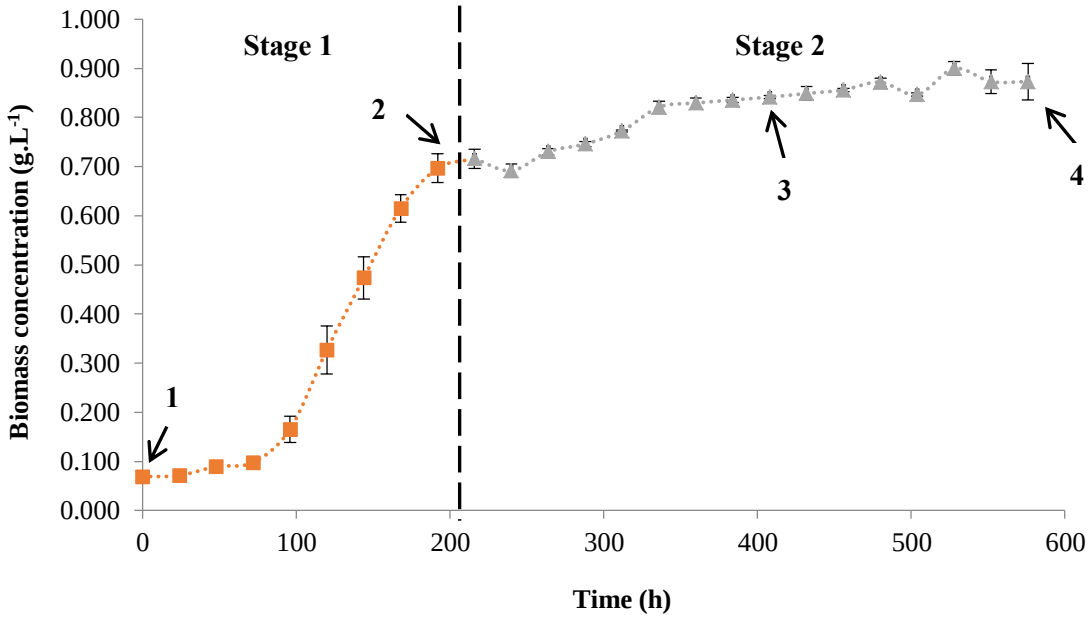

Figure 4.5 – Two-stage growth using Schott flasks: Stage 1 (■); Stage 2 (▲) (*n*=3). Black arrows indicate sampling points throughout the growth: 1) at the beginning of growth; 2) when the shift of growth conditions occurred (late exponential phase); 3) when the amount of MC was estimated as maximum (at the middle of stationary phase); 4) at the end of growth.

Figure 4.5 shows that the culture grew as expected, presenting a fast growth during the first stage and a steady but slow increase on the second stage. Additionally, when growth conditions were changed, there was an adaptation period of the culture that lasted ca. 2 days, where biomass concentration decreased slightly. The differences found in the culture's growth pattern between stages are very clear when evaluating some growth-related parameters (Table 4.3).

Table 4.3 – Maximum biomass concentration (X_{max}), productivity (P_{max}), and specific growth rate (μ_{max}) obtained for each stage of growth (*n*=3)

Parameter	Stage 1	Stage 2
X_{max} ($g.L^{-1}$)	0.697 ± 0.029	0.900 ± 0.014
P_{max} ($g.L^{-1}.d^{-1}$)	0.088 ± 0.004	0.021 ± 0.003
μ_{max} (d^{-1})	0.025 ± 0.004	0.002 ± 0.000

As shown here, the majority of growth occurred in Stage 1 during a short period of time, being P_{max} approximately four-times higher than the the corresponding value obtained for Stage 2. The value of μ_{max} also suffered a dramatic reduction (12- to 13-fold) from Stage 1 to Stage 2. As mentioned previously, a decrease in growth performance in Stage 2 was expected, since the goal of this phase was to enhance the production of toxin. Therefore, the focus should now be directed towards the variation of concentration of MC in the aforementioned sampling points, applying the two-stage approach (Table 4.4). Toxin's concentration at the starting point was determined from the culture that was used as inoculum and thus represents the control that must be used to compare the data. Since the inoculum was collected from this culture at the beginning of the stationary phase, we considered this as the maximum amount of MC achieved throughout the growth.

Table 4.4 – Variation of MC's concentration in the four selected sampling points using the two-stage approach and the respective increments (n=3)

Sampling point	MC concentration ($\mu g.L^{-1}$)	Increment (%)
1	0.18 ± 0.07	—
2	0.26 ± 0.04	41.7 ± 19.6
3	0.30 ± 0.00	66.7 ± 0.0
4	0.28 ± 0.01	52.8 ± 3.9

Results on Table 4.4 show that the use of a two-stage growth and, consequently, the application of two different growth conditions to the culture, allowed obtaining an increase of toxin accumulation inside cells. Although this increase was apparently significant in the first stage of growth, the variability in this point makes us suspecious of such great increment. On the other hand, Stage 2 conditions have shown a robust enhancement of MC production, reaching a maximum concentration which was 66.7 % higher than the control. Similarly to Figure 4.3, the later growth phase of the culture led to a decrease of toxin.

4.2.5 Conclusions

The assessment of MC concentration throughout culture's growth has shown to be in agreement with the values available in the literature (e.g., as in many other published works, also here its maximum was obtained in the late exponential growth phase).

The use of growth conditions found to be optimal (in Chapter 3) to promote both biomass productivity and MC accumulation revealed impossible to be applied in FP PBR. The scalability of the process might have been the constraint that forced us to use Schott flasks for two-stage growths, instead of the PBR.

Stage 1, as expected, has shown very high growth rates when compared to Stage 2, being P_{max} and μ_{max} four- and twelve- to thirteen-times greater, respectively. On the other hand, the accumulation of toxin in the second growth stage was 66.7 % higher than the control, while it did not exceed 42 % of increment in Stage 1.

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4.3 Influence of chemical cues of other microorganisms on *M. aeruginosa's* growth and toxin production

4.3.1 Abstract

The full understanding of the response of *M. aeruginosa* to other microorganisms or to remnants of their presence (such as chemical cues) is very important to predict the possible impact on growth as well as on the production of cells' metabolites. Therefore, this study aimed at evaluating the influence of filtrates and extracts of *C. vulgaris*, *C. emersonii*, *S. obliquus*, and a non-toxic strain of *M. aeruginosa* on growth of *M. aeruginosa* LEGE 91094 and production of MC.

The highest enhancement of growth was achieved using a culture with 20 % of extract of non-toxic *M. aeruginosa* (NTMASF20), with an increase around 17 %. This growth revealed however to have no influence over toxin production as it was the same as the control cultures. The application of 20 % of filtrate of *S. obliquus* (SOF20) and of 20 % of extract of *C. vulgaris* (CVSF20) led to an inhibition of toxic *M. aeruginosa* growth of approximately 13 and 12 %, respectively. The impact on MC accumulation was inexistent for CVSF20, while SOF20 resulted in an increase of 37 %. However, the most promising conditions to favour the production of MC were 20 % of filtrate (CEF20) and 10 % of extract of *C. emersonii* (CESF10), with cultures attaining concentrations 49.3 and 57.5 % greater than control, respectively.

4.3.2 Introduction

The interaction between different microorganisms is a mechanism known to induce changes on growth and metabolism of cells. This exposure may negatively affect the species involved by causing inhibition (Ma *et al.*, 2015a) or even death (Gumbo and Cloete, 2011); conversely, it can also enhance the production of interesting metabolites, such as MC (Jang *et al.*, 2003), or biomass growth itself (Ma *et al.*, 2015b; Żak and Kosakowska, 2015).

Much of the work done so far in this research area aimed at assessing the behaviour of microorganisms in co-culture (Gonçalves *et al.*, 2016; Zhu *et al.*, 2014). However, the use of exudates (Mello *et al.*, 2012; Wang *et al.*, 2017) and filtrates (Pineda-Mendoza *et*

al., 2014; Żak and Kosakowska, 2015) containing chemical cues and/or secondary metabolites is also a common strategy applied.

Despite of the numerous studies involving the use of *M. aeruginosa*, most of them make use of its ability to produce toxins to evaluate the impact on other microorganisms. Among the research work meant to analyse the influence of different species over this cyanobacterium, many focus only on growth (Mello *et al.*, 2012; Zhu *et al.*, 2014). Those where both growth of *M. aeruginosa* and production of MC are addressed are frequently performed with other microorganisms than microalgae or cyanobacteria (Jang *et al.*, 2003, Pineda-Mendoza *et al.*, 2014). One of the few exceptions is the work of Rzymski *et al.* (2014) that brought important insights regarding the effect of *Cylindrospermopsin raciborskii* and its toxin cylindrospermopsin on growth and MC production of *M. aeruginosa*.

Considering the facts reported previously, this study intended to assess the influence of *Chlorella vulgaris* (CV), *Scenedesmus obliquus* (SO), *Chlorella emersonii* (CE), and a non-toxic strain of *M. aeruginosa* (NTMA) on growth and MC production of *M. aeruginosa* LEGE 91094. To accomplish this goal, we utilized two different volumes (10 and 20 % of the total culture volume) of filtrates and extracts.

4.3.3 Materials and methods

4.3.3.1 Microorganisms and stock cultures

M. aeruginosa LEGE 91094 and *M. aeruginosa* LEGE 91341 (non-toxic strain) were provided by the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR - Porto, Portugal) while *C. vulgaris* P12, *S. obliquus*, and *C. emersonii* were obtained from the Culture Collection of Algal Laboratory (CCALA, Czech Republic). All cultures were maintained under 10 $\mu mol_{photons}.m^{-2}.s^{-1}$ of light intensity and 20 °C in Z8 medium (Kotai, 1972) using 12 h L:D cycles. Stock solutions were renewed on a monthly basis.

4.3.3.2 Screening method to select testing conditions

Considering all the microorganisms involved and the complexity of this study, there was a need to perform some preliminary tests in order to select the most interesting conditions (Figure 4.6). Thus, filtered and sonicated followed by filtration cultures

(extracts) of all four species were added to toxin-producer *M. aeruginosa* cultures in two different proportions (10 and 20 % of the total volume of the culture) (Table 4.5).

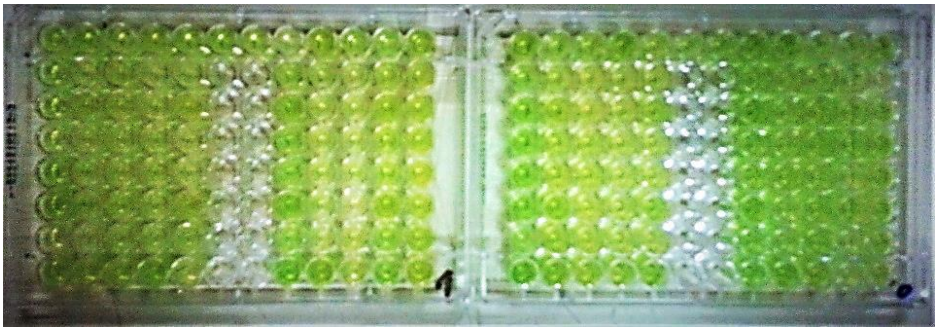

Figure 4.6 – Screening tests of *M. aeruginosa* growing in the presence of filtrates and extracts of other microorganisms.

Filtration was performed using syringe filters with 0.2 µm of porosity while sonication was conducted in an ultrasonic processor VCX 500 (Sonics & Materials, Inc., USA) at 40 kHz for 10 min. All the filtrates and extracts of *C. vulgaris*, *S. obliquus*, *C. emersonii*, and non-toxic *M. aeruginosa* presented the same *OD* at 750 nm (0.9) before being processed. Screening assays were done in 96-well microtiter plates under the same conditions mentioned in Section 4.3.3.1.

Table 4.5 – Designation set for each filtrate and extract tested

Microorganism	Filtration		Sonication + Filtration	
	10 %	20 %	10 %	20 %
C. vulgaris	CVF10	CVF20	CVSF10	CVSF20
C. emersonii	CEF10	CEF20	CESF10	CESF20
S. obliquus	SOF10	SOF20	SOSF10	SOSF20
Non-toxic *M. aeruginosa*	NTMAF10	NTMAF20	NTMASF10	NTMASF20

4.3.3.3 Experimental conditions

Upon the results of prelimary tests, selected conditions were tested under the same environmental conditions of previous sections in 50 mL Erlenmeyer flasks.

4.3.3.4 Determination of growth-related parameters

Biomass concentration (*X*) of *M. aeruginosa* LEGE 91094 obtained throughout this study was determined by the Equation 3.2 presented in Section 3.3.2.1.

$$X = 1.208 \times OD\ (750\ nm) + 0.023 \quad (R^2 = 0.984) \qquad \text{(Equation 3.2)}$$

4.3.3.5 Quantification of MC concentration

MC quantification was performed using the Microcystins-ADDA ELISA Kit (Abraxis, Inc., Pennsylvania, USA), as described in Section 3.3.3.

4.3.4 Results and discussion

4.3.4.1 Preliminary tests

The influence of all filtrates and extracts presented in Table 4.5 on growth of toxic *M. aeruginosa* is expressed in Figure 4.7.

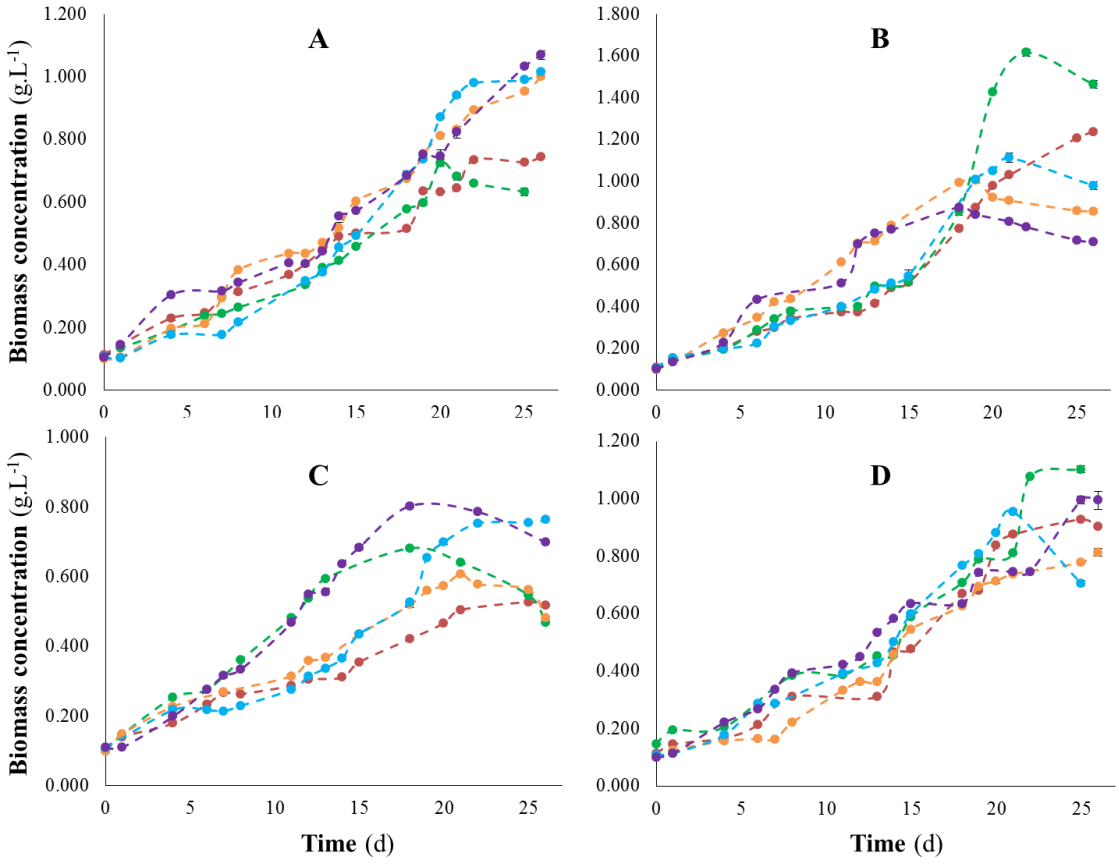

Figure 4.7 – Impact of filtrates and extracts of A) *C. emersonii*, B) *C. vulgaris*, C) non-toxic strain of *M. aeruginosa*, and D) *S. obliquus* on growth of toxin-producer *M. aeruginosa* (Control (●), F10 (●), F20 (●), SF10 (●), SF20 (●)). Error bars correspond to the standard deviation of the average value determined for triplicates.

Although most of the filtrates and extracts were found to positively or negatively affect the normal behaviour of *M. aeruginosa* LEGE 91094 cultures, as shown in Figure 4.7,

we had to choose those which were considered the most promising in order to proceed with our study.

Since at least one filtrate or extract of each microorganism tested should be selected so that a wide range of variables could be addressed in this assessment, SOF20 and CVSF20 were picked due to the inhibition demonstrated, whilst CVF10, CEF20, CESF10, and NTMASF20 were chosen as a consequence of enhancing biomass growth of toxin-producer *M. aeruginosa*.

4.3.4.2 Influence of selected conditions on growth of toxic *M. aeruginosa*

Probably due to the low reproducibility of the screening method or the scale-up introduced in the process, the variations expected to occur by putting in contact the selected filtrates and extracts with toxic *M. aeruginosa* were not, in general, fulfilled (Figure 4.8). In fact, several assays, such as CVF10, CEF20, and CESF10, presented an opposite behaviour when compared to the results obtained in Figure 4.7.

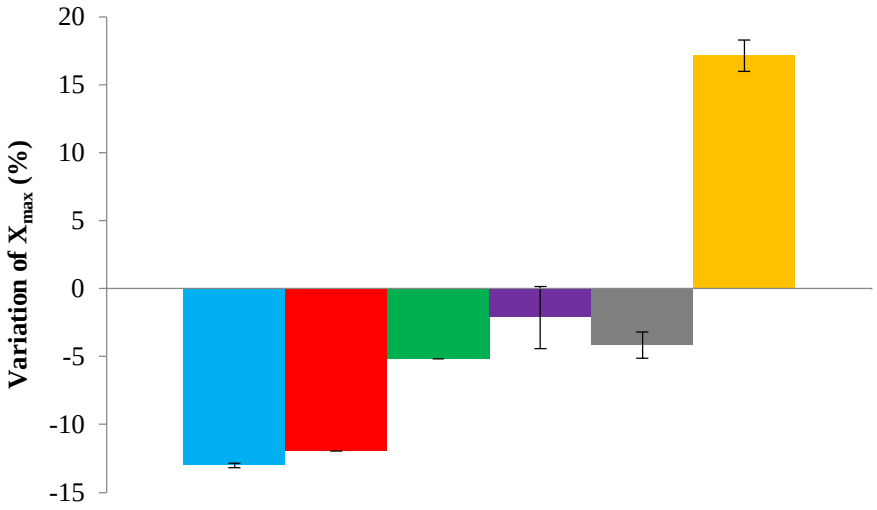

Figure 4.8 – Variation of X_{max} (%) of the assays carried out with SOF20 (■), CVSF20 (■), CVF10 (■), CEF20 (■), CESF10 (■), and NTMASF20 (■) in comparison to control. Error bars correspond to the standard deviation of the average value determined for triplicates.

According to Figure 4.8, NTMASF20 was the only test that enabled enhancing the growth of *M. aeruginosa*, reaching a biomass concentration 17 % higher than the control. In spite of the great increase found in this experiment, other studies have presented considerably higher increments (up to 80-90 %) using *Daphnia magna* (Pineda-Mendoza *et al.*, 2014) and *Synechocystis salina* (Gonçalves *et al.*, 2016).

However, there are some particularities that might contribute for this huge variability. Besides the strain of *M. aeruginosa* used, which influences significantly the behaviour throughout the whole study, the comparison with other studies is also difficult because of the diversity of growth conditions applied. This is the case of Gonçalves *et al.* (2016), where *M. aeruginosa* was co-cultured with *S. salina* and not simply exposed to filtrates during its growth.

Among the inhibitory solutions, SOF20 and CVSF20 were the most successful with a reduction of about 13 and 12 %, respectively, while CEF20 appeared not to have any influence over growth. In agreement to what was discussed for NTMASF20, despite of the great inhibition verified for SOF20 and CVSF20, Pineda-Mendoza *et al.* (2014) were able to limit the growth of *M. aeruginosa* in approximately 90 % using infochemicals released by *D. magna*. Using a co-culture of *Cyperus alternifolius* (plant) and *M. aeruginosa*, Zhu *et al.* (2014) also caused a decrease higher than 30 % in the growth of cyanobacteria. With a different approach, Rzymski *et al.* (2014) reached inhibition rates of up to 36.2 % when growing *M. aeruginosa* in medium containing cylindrospermopsin (CYN) produced by *C. raciborskii*. Nevertheless, the greatest inhibition of growth was observed when higher concentrations of CYN were used. When lower amounts of cyanotoxin were tested, the reduction of *M. aeruginosa* growth revealed to be lower than ours since it was in the range of 7-10.3 %. Furthermore, in our study no toxic substances were added to *M. aeruginosa* cultures, which makes this a more environmentally-friendly process to influence the growth of cyanobacteria.

4.3.4.3 Influence of selected conditions on MC production

Determination of MC accumulation in the cultures of *M. aeruginosa* grown in contact with the selected filtrates and extracts has revealed interesting findings (Figure 4.9).

Apparently, there is no direct relationship between higher biomass growths and greater MC concentrations since NTMASF20, which enhanced growth (Figure 4.8), did not present any significant impact on MC accumulation. On the other hand, SOF20 and CVSF20, that presented the highest inhibitory rates over *M. aeruginosa*, have shown to influence differently the production of MC. The first one caused an increase of more than 37 %, while the other one did not affect this parameter at all.

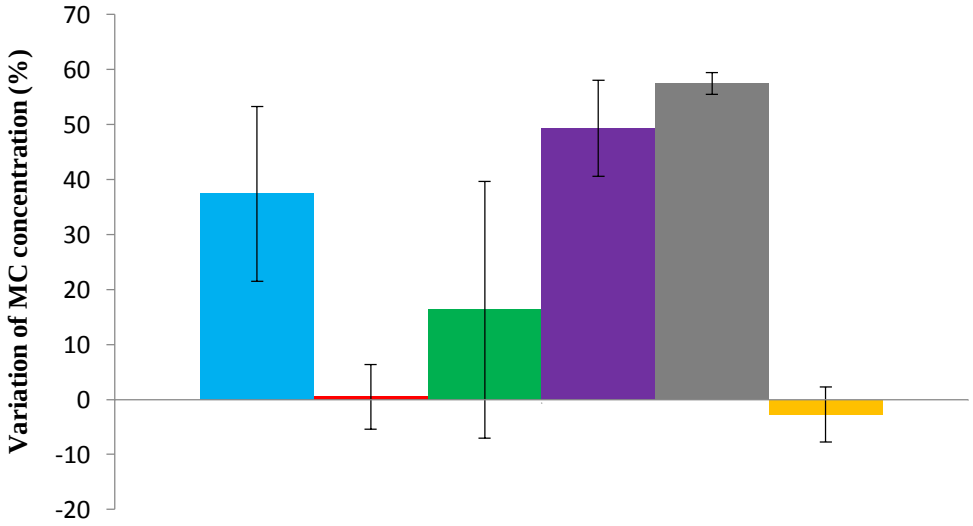

Figure 4.9 – Variation of MC concentration (%) of the assays carried out with SOF20 (■), CVSF20 (■), CVF10 (■), CEF20 (■), CESF10 (■), and NTMASF20 (■) in comparison to control. Error bars correspond to minimum and maximum values.

The most remarkable results were however observed for CEF20 and CESF10 since they were responsible for increasing MC concentration in approximately 49.3 and 57.5 %, respectively. Although these increments are very interesting the fact is that the use of *D. magna*, whether by exposing directly (Jang *et al.*, 2003) or by feeding its released infochemicals (Pineda-Mendoza *et al.*, 2014) to *M. aeruginosa*, allowed reaching 2-4 times higher concentrations of toxin comparing to control cultures, which is considerably better than our results. However, the connection between cyanobacteria growth and toxin production in those two works is the opposite. Generally, when compared to control Jang *et al.* (2003) have registered higher biomass concentrations for all experiments, while Pineda-Mendoza *et al.* (2014) found greater MC accumulation in cultures that suffered inhibition, as was detected in our study (Figure 4.8, Figure 4.9). The behaviour of *M. aeruginosa* when exposed to other microorganisms' cells or chemical cues is deeply dependent on the strain used, as shown by Jang *et al.* (2003) and Pineda-Mendoza *et al.* (2014), which might explain the huge variability observed between those two research studies and between them and our data (especially in the case of Jang *et al.*, 2003). Despite of the great increments presented by Pineda-Mendoza *et al.* (2014), these authors also obtained reductions of around 50 % of MC production. Similarly to NTMASF20, this inhibition was found in cultures where growth was significantly enhanced by *D. magna*.

4.3.5 Conclusions

Most of the filtrates and extracts evaluated were found to affect growth of toxin-producer *M. aeruginosa*. The screening method revealed however not to be robust and accurate since several selected conditions did not induce a similar behaviour on growth when we moved from preliminary to final tests. Scale-up of the process may also have contributed for this variability.

NTMASF20 was the only selected condition that enhanced the growth of toxic strain presenting an increment of 17 %. This increase was though not accompanied by higher production of MC, which kept unchanged. The greatest inhibition rates were determined for SOF20 and CVSF20, presenting reductions of the growth of cyanobacteria around 13 and 12 %, respectively. Still, distinct impacts were caused by those two solutions over toxin metabolism once, compared to control, the first one promoted its accumulation in 37 % and the last one did not result in any changes. The highest concentrations of MC were detected using CEF20 and CESF10 with increases of approximately 49.3 and 57.5 %, respectively. Curiously, these were the conditions that presented the lowest impacts on growth.

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Chapter 5

Optimization and comparison of different methods to harvest *M. aeruginosa*

Most of the information described in this Chapter was adapted from:

Geada, **P**., Oliveira, F., Loureiro, L., Esteves, D., Teixeira, J.A., Vasconcelos, V., Vicente, A.A., Fernandes, B., Comparison and optimization of different methods for *Microcystis aeruginosa*'s harvesting and the role of zeta potential on its efficiency. Submitted to Algal Research.

5.1 Abstract

The presence of cyanobacteria in water bodies represents a major problem for environmental and human health nowadays. This is especially relevant considering that some cyanobacteria, such as *Microcystis aeruginosa*, have the ability to produce intracellular toxins. In this case, water management and treatment present a great challenge since high removal efficiencies are required and cell integrity should be maintained to avoid the release of cyanotoxins.

This study has compared the harvesting efficiency (*HE*) of four flocculation methods, namely: induced by pH, $FeCl_3$, $AlCl_3$ and chitosan. Additionally, a novel harvesting technique involving the utilization of iron oxide magnetic microparticles (IOMMs) was studied. No changes were observed on *M. aeruginosa* cells for methods.

Flocculation assays performed at pH 3 and 4 have shown the best *HE* among the pH-induced tests, reaching values above 90 % after 8 h. The adjustment of zeta potential (ZP) to values comprised between -6.7 and -20.7 mV enhanced significantly the settling rates using flocculant agents, being $FeCl_3$ the best example where increments up to 88 % of *HE* were obtained. Although the four methods compared here have presented *HE*s above 91 % within the first 8 h after the optimization process, the highest performance was obtained using 3.75 $mg.L^{-1}$ of $FeCl_3$, that allowed reaching 92 % in 4 h. The use of IOMMs have shown to be a very promising alternative to conventional harvesting methods once it was possible to reach *HE*s of 93.6 % with just 4 min of treatment. The optimal conditions verified for the implementation of IOMMs was a IOMMs:cells (g/g) ratio of 0.8:1 and pH 12.

5.2 Introduction

The continuous deterioration of water supplies represents an emerging threat for all human beings which might, in a near future, lead to higher scarcity of potable water. Due to the extreme necessity for water, this dispute is somehow pointed as a potential triggering event to a World War III.

Owing to the presence of intracellular toxins such as MCs, the removal process of toxin-producer cyanobacteria should be carried out under mild conditions to prevent the release of toxic compounds from the cells and, consequently, the contamination of water

(Zamyadi *et al.*, 2012). However, some of the techniques currently applied in the treatment of water containing cyanobacteria might damage or at least compromise cells' membrane (Ma *et al.*, 2012; Mucci *et al.*, 2017; Sun *et al.*, 2012). Thus, supplementary methods may be needed to promote cyanotoxins' decomposition, which increases significantly the overall cost of the process (Geada *et al.*, 2017; Oberholster *et al.*, 2004). Although numerous studies have been performed to assess the removal efficiency of *M. aeruginosa*, including the use of magnetic particles (Jiang *et al.*, 2010; Lin *et al.*, 2015), coagulant/flocculant agents (Lürling *et al.*, 2017; Ma *et al.*, 2016; Qi *et al.*, 2016; Shi *et al.*, 2016; Wang *et al.*, 2015a; Yuan *et al.*, 2016; Zhou *et al.*, 2014), ultrasounds (Rodriguez-Molares *et al.*, 2014), and flotation (Yap *et al.*, 2014), none of those presented a comparison between techniques. One of the few exceptions, conducted by Teixeira and Rosa (2007), showed that the best harvesting efficiencies (*HE*s) were obtained using coagulation/flocculation/dissolved air flotation when compared to coagulation/flocculation/sedimentation process. Furthermore, the flocculation solely induced by the variation of pH is still poorly understood and might represent a promising technique since no additional compounds, such as coagulants, are required. The surface of *M. aeruginosa* cells generally presents a negative charge for a wide range of pH values (Hadjoudja *et al.*, 2010), which enables their suspension as individual cells. However, variations on the pH of the surrounding environment might lead to the destabilization of this membrane charge and, consequently, to the aggregation of cyanobacteria (and formation of flocs).

In contrast to the problematic presence of toxins in drinking water, some of these compounds are drawing attention of researchers from medical fields once several interesting bioactivities were found in these secondary metabolites (Geada *et al.*, 2017). MC, for instance, was demonstrated to treat/reduce some of the Parkinson's disease symptoms and to be a promising anticancer drug (Braithwaite *et al.*, 2012; Ilić *et al.*, 2011; Niedermeyer *et al.*, 2014; Zanchett and Oliveira-Filho, 2013). Therefore, also in this case decreasing losses of cyanotoxins during the harvesting process of cyanobacteria are desired so that the production cost of these compounds is reduced and their commercialization becomes more feasible.

The aim of this study was to compare the efficiency on the removal of *M. aeruginosa* of four different harvesting methodologies – the utilization of well-known flocculantion agents (i.e. aluminum chloride ($AlCl_3$), iron chloride ($FeCl_3$), and chitosan (Ch)) and

pH-induced flocculation. An innovative approach based on the use of iron oxide magnetic microparticles (IOMMs) was also evaluated. The influence of zeta potential (ZP) was assessed and an optimization of all methods was performed.

5.3 Materials and methods

5.3.1 Microorganism culture and cyanobacterial suspension preparation

Microcystis aeruginosa LEGE 91094, a unicellular cyanobacterium, was kindly provided by the Interdisciplinary Centre of Marine and Environmental Research collection (CIIMAR - Porto, Portugal) and maintained in Z8 medium (Kotai, 1972) at 20 °C and pH 8.7 under 10 $\mu mol_{photons}.m^{-2}.s^{-1}$ of light intensity using a 12:12-hour L:D cycle. Cultures were aerated (0.38 vvm) and renewed every month. Cyanobacterial cells were washed with distilled water (8000 rpm, 15 min) before the preparation of each assay in order to ensure the same initial culture conditions. Biomass concentration was then fixed at 0.5 $g.L^{-1}$.

5.3.2 pH-induced flocculation optimization

Flocculation induced by pH was tested for values ranging from 1 to 12 and the experiments were conducted in duplicate for 24 h using glass test tubes containing 42 mL of culture. The pH of the assays was adjusted utilizing NaOH (0.5 and 1 $mol.L^{-1}$) and HCl (1 and 2 $mol.L^{-1}$). Samples were drawn at the intermediate point between the surface of the culture and the bottom of the test tube.

5.3.3 Comparison of harvesting methods

After selecting the pH responsible for the most efficient flocculation, a comparison was performed using three different flocculant agents: $AlCl_3$, $FeCl_3$, and chitosan (Ch). These techniques were carried out using the optimal concentrations reported in Sun *et al.* (2012), Chow *et al.* (1998), and Pei *et al.* (2014), respectively. The assays were followed during 24 h and done in duplicate as described in Section 5.3.2.

5.3.4 Optimization of harvesting methods using ZP as an indicative tool

Li *et al.* (2015) have carried out a study where a specific optimal interval of values of ZP (OIVZP) between -6.7 and -20.7 mV was found to improve *HE* during chitosan

modified soil induced flocculation. Therefore, we decided to assess the influence of this parameter on harvesting by varying either the amount of $AlCl_3$, $FeCl_3$, and Ch applied on treatment or the pH of the culture.

5.3.5 Synthesis of IOMMs

The iron oxide magnetic microparticles (IOMMs) were prepared following a similar protocol as described in Prochazkova *et al.* (2013a). Briefly, 1 g of $FeSO_4.7H_2O$ was dissolved in 100 mL of distilled water and then the pH of the solution was adjusted to 11-12 using NaOH 1 $mol.L^{-1}$. The addition of NaOH was done drop by drop under continuous stirring conditions. Using distilled water, the solution was then made up to 200 mL and subject to 800 W for 10 min in a regular kitchen microwave oven device. Upon reaching room temperature, IOMMs were washed 3 times with distilled water (4000 rpm, 5 min). Particles size was characterized by Prochazkova *et al.* (2013a), ranging from 0.15 to 20 µm.

5.3.6 IOMMs methodology optimization

The separation of cells using IOMMs was carried out in glass test tubes containing 42 mL of culture by means of external magnetic fields (cylindrical NdFeB magnets, 25 × 10 mm, Neomag, Czech Republic). The optimization process of this technique was divided into two stages: i) optimization of IOMMs:cells concentration (g/g) ratio (0:1, 0.2:1, 0.4:1, 0.6:1, 0.8:1, and 1:1), and ii) finding the optimal pH to perform magnetic separation (pH values tested were 2, 4, 7, 10, and 12). All the assays were done in triplicate.

5.3.7 Analytical methods

HE was determined by measuring the optical density of the culture at 750 nm in a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., USA) according to Eq. 4.1:

$$HE\% = \frac{OD_0 - OD_t}{OD_0} \times 100\ \% \qquad \text{(Eq. 4.1)}$$

where OD_0 refers to the optical density (750 nm) measured at the beginning of the process and OD_t is the optical density (750 nm) after a certain period of time (t, h).

All the experiments were analysed by flow cytometry (EC800 Flow Cytometer Analyser, Sony Biotechnology Inc., USA) to ensure that no damage was inflicted to cells during harvesting methods and no significant changes in terms of size, membrane complexity, and viability were observed (data not shown). The same conclusions were drawn by evaluating the absorbance of supernatant (at 254 nm) with a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., USA) in order to determine the amount of intracellular organic matter released (data not shown). The supernatant was collected after harvesting methods were applied by centrifuging the cultures at 12000 rpm for 5 min.

ZP was also determined in triplicate for all tests performed using a Zetasizer Nano-ZS (Malvern Instruments Ltd., UK).

5.4 Results and discussion

5.4.1 Optimization of pH-induced flocculation

pH-induced flocculation assays were carried out in order to study the harvesting efficiency of *M. aeruginosa* (Figure 5.1).

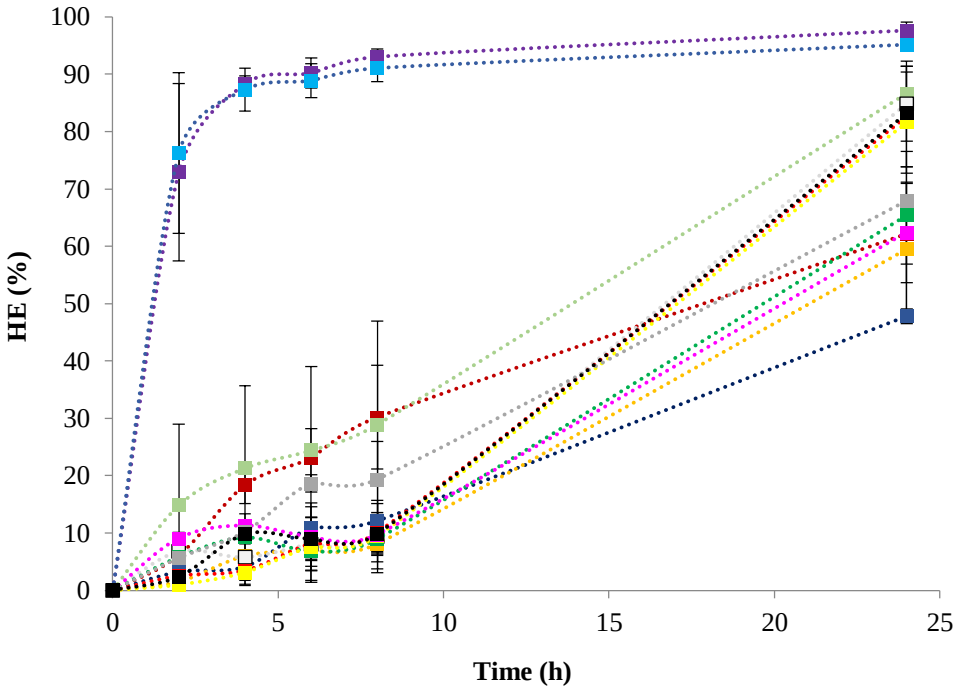

Figure 5.1 – Assessment of *HE* on pH-induced flocculation experiments (n=2) (Control (■), pH 1 (■), pH 2 (■), pH 3 (■), pH 4 (■), pH 5 (■), pH 6 (□), pH 7 (■), pH 8 (■), pH 9 (■), pH 10 (■), pH 11 (■), pH 12 (■)). Error bars correspond to minimum and maximum values.

According to Figure 5.1, the highest *HE*s were obtained by adjusting pH values to 3 and 4, resulting in a sedimentation of more than 90 % of *M. aeruginosa* cells after 8 h of treatment. This behaviour is in accordance with findings reported by Liu *et al.* (2013) for three different freshwater microalgae: *Chlorococcum nivale*, *C. ellipsoideum* and *Scenedesmus* sp.. Although the optimal pH interval suggested by these authors is in agreement with our results, other studies presented completely different results. This is the case of Wu *et al.* (2012) who have tested flocculation induced by pH in three freshwater microalgae (among which were *Chlorococcum* sp. and *Scenedesmus* sp.) as well as two marine species. Alkaline environments, especially above pH 9, were frequently considered the most suitable conditions to reach the most efficient flocculation processes (Wu *et al.*, 2012). The variations found in these works indicate that the success of pH-induced flocculation methods is strongly dependent on the strain studied.

As mentioned previously in this section, changes in pH of the environment entail a series of modifications, being ZP one of the properties significantly affected by those changes. Figure 5.2 presents the differences observed for ZP of *M. aeruginosa* when pH of the medium ranges from 1 to 12.

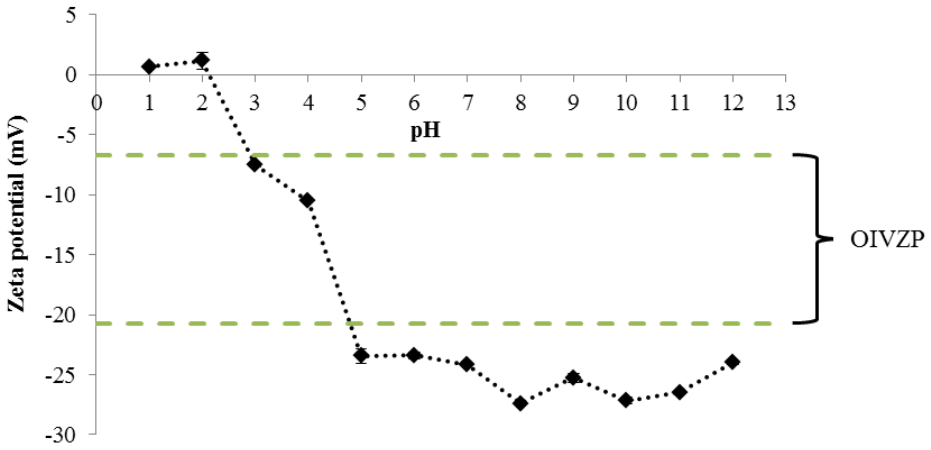

Figure 5.2 – Variation of ZP of *M. aeruginosa* at different pH values (*n*=3). Green dashed lines (–) represent the optimal interval of values for the zeta potential (OIVZP) for flocculation, according to Li *et al.* (2015). Error bars correspond to the standard deviation of the average value determined for triplicates.

Through Figure 5.2 it is possible to conclude that both assays where highest *HE*s were observed, carried out at pH 3 and 4, presented ZP values comprised in the OIVZP. pH 4 was selected as optimal value to induce *M. aeruginosa* flocculation.

5.4.2 Comparison of *HE*

After the optimization of pH-induced flocculation process for *M. aeruginosa* (pH = 4), and using the optimal concentrations defined in the bibliography (Sun *et al.*, 2012; Chow *et al.*, 1998; Pei *et al.*, 2014) for $AlCl_3$, $FeCl_3$ and Ch – 15, 30, and 7.31 $mg.L^{-1}$, respectively (cf. Section 5.3.3) –, it was possible to compare all methods with respect to *HE* (Figure 5.3).

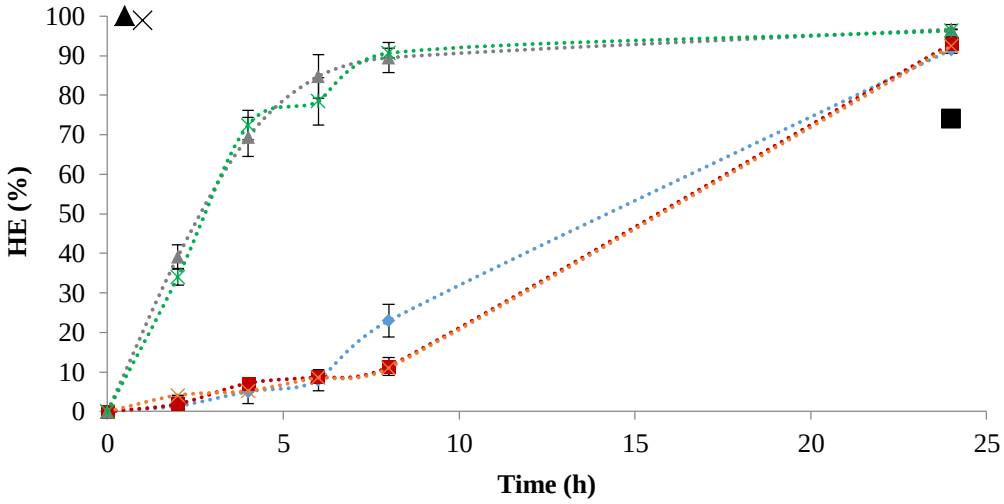

Figure 5.3 – Comparison of *HE* of flocculation of *M. aeruginosa* induced by pH 4 (*), $FeCl_3$ (■), $AlCl_3$ (▲), and Ch (×) and control (♦) on a 24-h treatment (n=2). The *HE*s obtained in Sun *et al.* (2012), Chow *et al.* (1998), and Pei *et al.* (2014), from which the optimal dosage of $AlCl_3$ (▲), $FeCl_3$ (■), and Ch (×) were retrieved, respectively, are also presented here. Error bars correspond to minimum and maximum values.

According to Figure 5.3, $AlCl_3$ and pH 4 presented the best *HE*s being considerably higher than those achieved by using the other two well-known flocculant agents ($FeCl_3$ and Ch), which have shown a behaviour similar to control tests. Taking into account the effect of ZP on the *HE* and the performance of $AlCl_3$ on sedimentation of *M. aeruginosa*, it was expected that ZP of cyanobacteria treated with this compound would be in the OIVZP. However, this was not verified (Table 5.1) and thus other parameters besides ZP could have had influence in the harvesting process. Metal precipitates formation during the process, which results in the dragging of cyanobacteria to the bottom, is one of the possibly occurring events described in literature (Wu *et al.*, 2012) that might have concurred to the results presented here.

Table 5.1 – ZP values of the assays performed to compare the *HE* of all methods tested (*n*=3)

Assay	Control	$AlCl_3$	pH 4	Ch	$FeCl_3$
ZP (mV)	-27.5 ± 0.3	7.2 ± 0.1	-17.7 ± 0.4	15.5 ± 0.4	18.5 ± 0.2

The rather low efficiency of cells removal determined for $FeCl_3$ and Ch tests in the first hours of the process, besides the high value of ZP, can also be associated with some other factors such as the biomass concentration and strain used, the sampling point, or the agitation applied. Comparing the work of Pei *et al.* (2014) with the results obtained here for Ch, it is possible to see that in that work some tests reached approximately 90 % of *HE* just after 1 h of treatment, which is significantly higher than the values obtained in our experiments. However, the biomass concentration was roughly 10 times lower and an optimization of the agitation time and speed was performed, while in our study no agitation was used. Additionally, the sampling point defined by Pei *et al.* (2014) was just 1 cm below the surface of the culture, which is very close to the surface relatively to ours, thus yielding in higher values of efficiency.

5.4.3 *HE* optimization – evaluation of ZP's impact

Due to the significant differences observed for *HE*s found in literature and those obtained in our flocculant agents' assays (Figure 5.3), together with the fact that none of the corresponding ZP values are in the OIVZP (see Table 5.1), led us to study the role of ZP on the harvesting of *M. aeruginosa*. For that purpose, several concentrations of the flocculant agents were selected in a way that it would be possible to assess the effect over the *HE* whether the ZP values applied were within, above, or below the OIVZP.

5.4.3.1 $AlCl_3$ experiments

Besides the assay performed initially with a concentration of 15 $mg.L^{-1}$, three other concentrations of $AlCl_3$ were tested: 1.88, 3.75, and 7.5 $mg.L^{-1}$ (Figure 5.4).

Figure 5.4 shows that intermediate concentrations are more effective for harvesting of cyanobacteria, especially those around 3.75 $mg.L^{-1}$. Regarding the initial concentration tested (15 $mg.L^{-1}$), *HE* has improved reasonably at the beginning of the process; approximately 14 and 9 % after 2 h and 4 h of treatment, respectively.

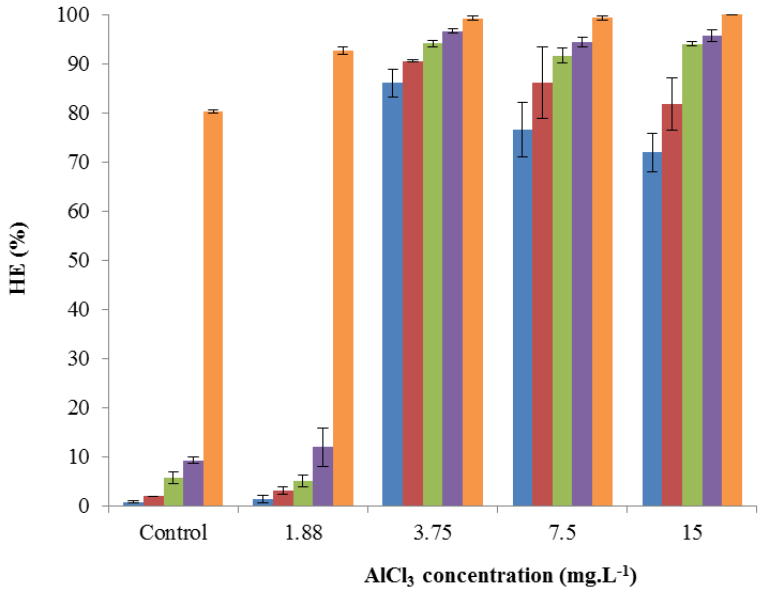

Figure 5.4 – *HE*s obtained using different concentrations of $AlCl_3$ after 2 (■), 4 (■), 6 (■), 8 (■), and 24 h (■) (*n*=2). Error bars correspond to minimum and maximum values.

Considering the relationship between *HE* and ZP, it is possible to say that the optimal condition to promote flocculation of *M. aeruginosa* using $AlCl_3$ was within the range of the OIVZP (Table 5.2).

Table 5.2 – ZP values of the assays performed to compare the *HE* using different concentrations of $AlCl_3$ (*n*=3)

$AlCl_3$ (mg.L^{-1})	**Control**	**1.88**	**3.75**	**7.5**	**15**
ZP (mV)	-27.5 ± 0.6	-26.8 ± 0.2	-14.4 ± 0.1	3.3 ± 0.2	10.3 ± 0.3

5.4.3.2 $FeCl_3$ experiments

The optimization of flocculation caused by the addition of $FeCl_3$ was also carried out reducing the concentration of this agent employed initially. For that purpose, assays containing 30, 7.5, 3.75 and 1.88 mg.L^{-1} of $FeCl_3$ were conducted during 24 h (Figure 5.5).

Similarly to what has been verified for $AlCl_3$, the application of 3.75 mg.L^{-1} revealed to be the most suitable concentration of $FeCl_3$ to remove *M. aeruginosa*. The improvement of the *HE* comparing to the results obtained with 30 mg.L^{-1} of flocculant agent was noteworthy, as it increased between 85 and 88 % in the first 8 h.

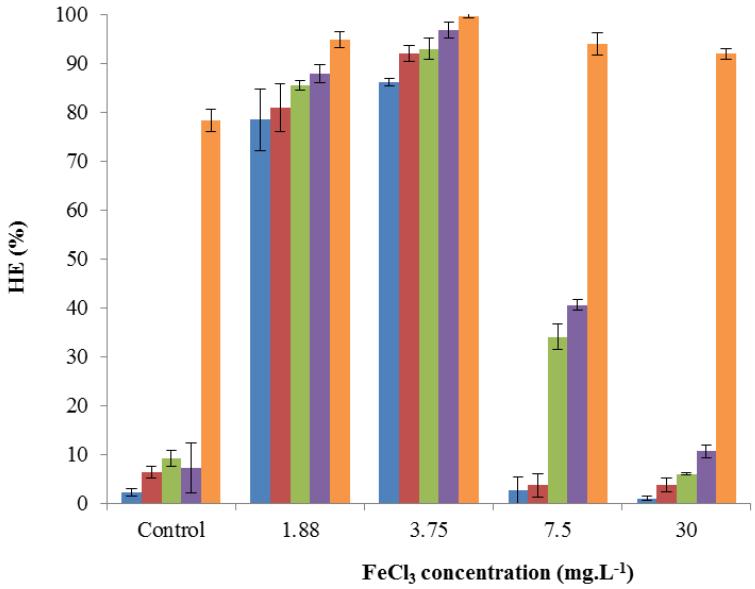

Figure 5.5 – Comparison of *HE*s using different concentrations of $FeCl_3$ after 2 (■), 4 (■), 6 (■), 8 (■), and 24 h (■) (*n*=2). Error bars correspond to minimum and maximum values.

With respect to ZP, and analysing Table 5.3, these results followed the same trend exhibited previously, where values included in the range of OIVZP were associated with the highest *HE*.

Table 5.3 – ZP values of the assays performed to compare the *HE* using different concentrations of $FeCl_3$ (*n*=3)

$FeCl_3$ (mg.L^{-1})	**Control**	**1.88**	**3.75**	**7.5**	**30**
ZP (mV)	-27.2 ± 0.4	-22.4 ± 0.3	-7.9 ± 1.2	11.7 ± 0.1	20.7 ± 0.4

5.4.3.3 Ch experiments

Maintaining the same rationale of the previous sections for the reduction of ZP, in the assays using Ch it was decided to reduce concentrations – 0.25, 0.5, and 2 mg.L^{-1} – and compare their removal efficiency with those attained by applying 7.31 mg.L^{-1}, the starting point of this experiment (Figure 5.6).

Although the lowest concentrations of Ch (0.25 and 0.5 mg.L^{-1}) showed similar behaviour 2 h after starting the harvesting process, the fact is that the use of 0.5 mg.L^{-1} revealed to be a better approach reaching efficiencies above 93 % at 6 h. Once again,

the increase of the *HE* regarding the initial assay (7.31 mg.L^{-1}) within the first 8 h was remarkable, varying between 65 and 76 %.

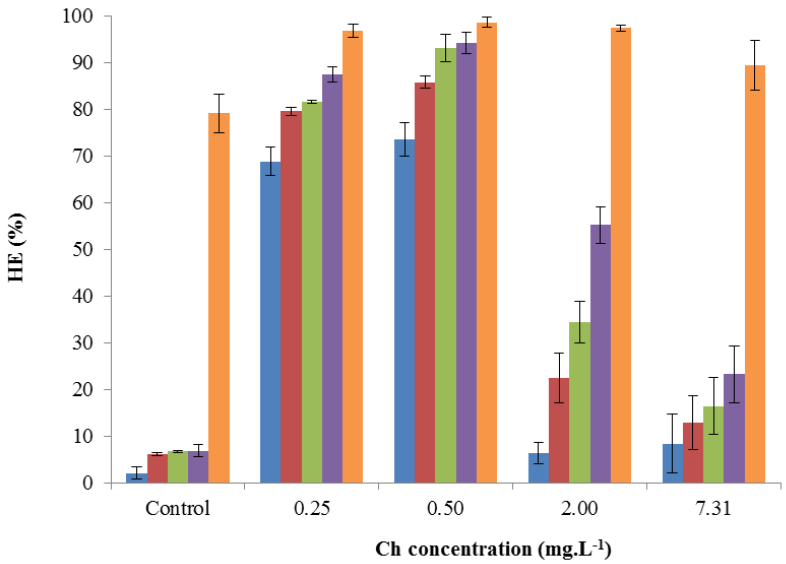

Figure 5.6 – *HE*s of the assays performed with different concentrations of Ch after 2 (■), 4 (■), 6 (■), 8 (■), and 24 h (■) (*n*=2). Error bars correspond to minimum and maximum values.

The results presented in Table 5.4 confirmed that *HE* of flocculation of *M. aeruginosa* using Ch is strongly dependent on ZP values, which means that these two parameters appear to be deeply related, regardless the flocculation derives from a pH change or the use of flocculant agents.

Table 5.4 – ZP values of the assays performed to compare the *HE* using different concentrations of Ch (*n*=3)

Ch (mg.L^{-1})	**Control**	**0.25**	**0.5**	**2**	**7.31**
ZP (mV)	-28.4 ± 2.2	-21.5 ± 0.3	-7.7 ± 0.1	4.6 ± 0.3	14.4 ± 0.3

5.4.3.4 Comparison of optimal conditions

To finalize the comparison of the *HE* obtained for the four different methods, one last experiment was performed (Figure 5.7) with the optimal conditions selected (cf. Sections 5.4.1, 5.4.3.1, 5.4.3.2, and 5.4.3.3), ensuring that ZP of the cultures would be comprised between -6.7 and -20.7 mV.

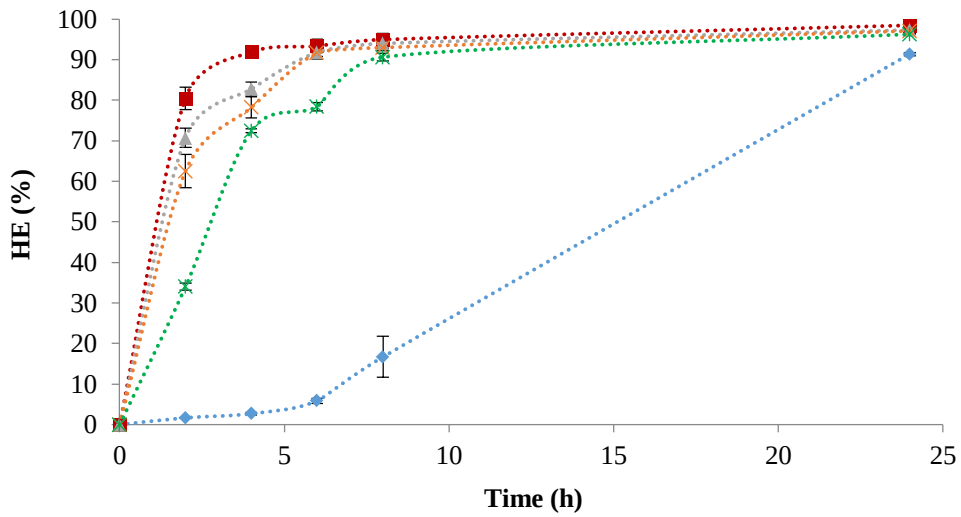

Figure 5.7 – Comparison of *HE* under optimal conditions of flocculation of *M. aeruginosa* induced by pH 4 (*), $FeCl_3$ (3.75 $mg.L^{-1}$) (■), $AlCl_3$ (3.75 $mg.L^{-1}$) (▲), and Ch (0.5 $mg.L^{-1}$) (×) and the control (♦) (*n*=2). Error bars correspond to minimum and maximum values.

With no exception, the modification of ZP of cells enabled to reach more than 91 % of *HE* in all procedures tested within the first 8 h (Figure 5.7), being $FeCl_3$ the most effective treatment since it has attained sedimentation rates of around 92 % in 4 h. The benefits of using ZP to favour the harvesting of cells seems to be of utmost importance, especially if comparing these results with those obtained at the beginning of the study (Figure 5.3), namely in the case of $FeCl_3$ and Ch.

As presented in Figure 5.7, Gonzalez-Torres *et al.* (2014) have previously proved that the application of $FeCl_3$ is more suitable for removal of *M. aeruginosa* than $AlCl_3$. Regarding Ch, Chen *et al.* (2014) have gathered relevant information about the harvesting of an extended group of microalgae making use of this coagulant. Although most of the harvesting efficiencies range from 80 to 95 %, the fact is that chitosan dosage frequently exceeds 10 $mg.L^{-1}$. Although Ch is seen as a very promising agent to replace metal-based flocculants such as $AlCl_3$ and $FeCl_3$, it also entails serious cell membrane damaging risks, which is unacceptable in the case of *M. aeruginosa* due to the release of intracellular toxins (i.e. MC). According to Mucci *et al.* (2017), negligible changes were observed in the normal behaviour and structure of *M. aeruginosa* when subjected to low concentrations of Ch, such as 0.5 $g.L^{-1}$. In contrast, dosages above 1 $mg.L^{-1}$ have demonstrated to significantly affect these cyanobacteria. In this work we succeeded to show that it is possible to have excellent *HE* results with low

concentrations of Ch if the conditions under which flocculation is performed are such that the adequate value of ZP is met.

5.4.4 *HE* optimization of IOMMs technique

The magnetic separation of *M. aeruginosa* using IOMMs was not compared with the flocculantion harvesting tests performed in previous sections since the process time is extremely short, as it is shown throughout Sections 5.4.4.1 and 5.4.4.2.

5.4.4.1 *HE* variation with IOMMs:cells concentration ratio

The influence of IOMMs:cells ratio on *HE* of the process is shown by Figure 5.8.

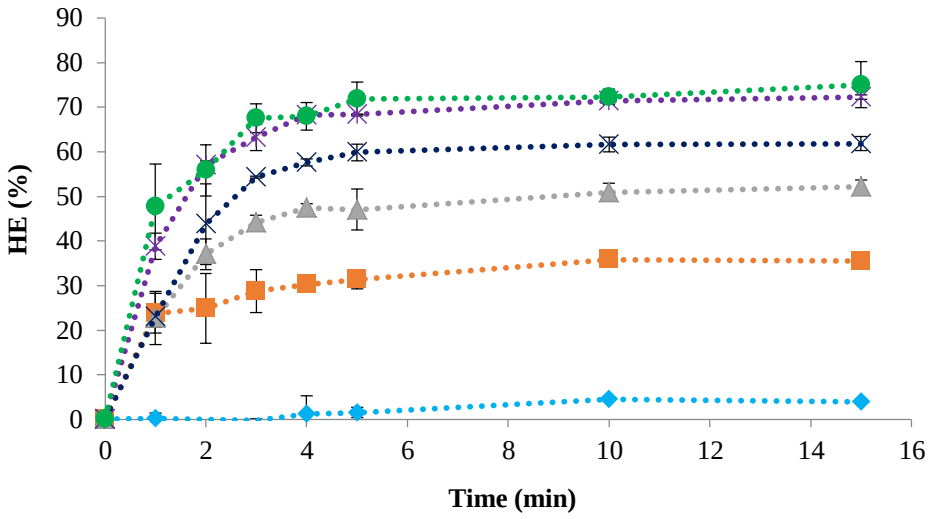

Figure 5.8 – Variation of *HE* of *M. aeruginosa* using different IOMMs:cells (g/g) ratios: control (♦), 0.2:1 (■),0.4:1 (▲), 0.6:1 (×), 0.8:1 (*), and 1:1 (●) (*n*=3). Error bars correspond to the standard deviation of the average value determined for triplicates.

These results allow us to infer that *HE* is very dependent on IOMMs:cells ratio using lower concentrations of magnetic particles once a continuous increase was observed until higher ratios (0.8:1 and 1:1) are attained. Interestingly, through Figure 5.8 it is possible to see that cells removal generally remain stable from 5 min onwards in all the experiments. The same behaviour – an increase of *HE* from lower to higher concentrations reaching then a stabilization – was also reported in other works using magnetic particles to remove *Chlorella* sp. (Liu *et al.*, 2016; Prochazkova *et al.*, 2013a; Prochazkova *et al.*, 2013b; Seo *et al.*, 2015; Wang *et al.*, 2016).

Figure 5.8 clearly indicates that the highest ratios are the best for *M. aeruginosa* harvesting, presenting *HE*s above 70 % within 15 min of treatment. This in agreement with Prochazkova *et al.* (2013a), where ratios between 0.4:1 and 1.6:1 revealed the greatest HEs utilizing the same IOMMs of our study on *C. vulgaris*. Despite of the concordant results regarding the most effective ratios, differences can be found both in *HE* and time of the process since those authors collected 95 % of the culture in 2 min. These inconcistencies might derive from the different microorganisms used and the optimization of growth medium pH, which was previously done by Prochazkova *et al.* (2013a) and applied in their cultures. Additionally, different growth medium composition – in our case cyanobacteria were washed and resuspended in distilled water – have a great impact on *HE* due to the presence of numerous ions that can change electrostatic interactions between cells and IOMMs (Wang *et al.*, 2015b).

5.4.4.2 Environmental pH assessment to improve *HE*

To proceed with the optimization of harvesting by means of IOMMs, namely pH optimization, and considering the results of Section 5.4.4.1, we decided to select 0.8:1 as the ratio to apply and to evaluate the *HE* just within the first 5 min. The variation of *HE* with pH can be observed through Figure 5.9.

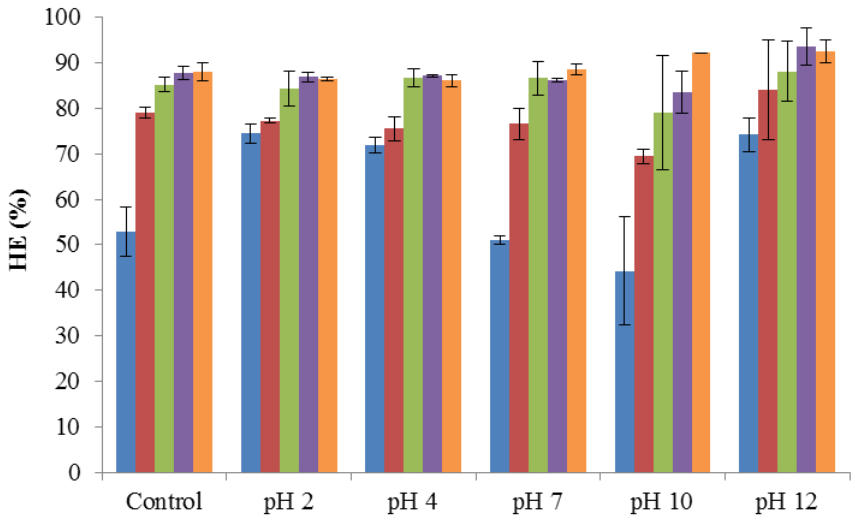

Figure 5.9 – Variation of *HE* using different pH values and a IOMMs:cells ratio of 0.8:1 (g/g) after 1 (■), 2 (■), 3 (■), 4 (■), and 5 (■) min of treatment (*n*=3). Error bars correspond to the standard deviation of the average value determined for triplicates.

According to these data, pH 12 was the most effictive condition resulting in a *HE* of aproximately 93.6 % after just 4 min of treatment. This result is considerably better than

those presented in Section 5.4.4.1 (Figure 5.8) and is closer to the values and period of time obtained by Prochazkova *et al.* (2013a), as described in the previous section as well. Regarding the other pH values, only pH 10 has shown significant differences comparing to control (*HE* around 88 %), reaching the maximum sedimentation of *M. aeruginosa*, around 92.2 %, after 5 min.

Although these are good results, the behaviour presented in Figure 5.9 was not expected. Considering the ZP of both IOMMs and cyanobacteria when exposed to the different pH values (Figure 5.10), pH 4 should have been the most successful assay since the biggest difference between ZPs of cells and magnetic particles is at this point. That was, for instance, the justification found by Prochazkova *et al.* (2013a) to explain their highest efficiency at pH 4, where a biggest gap between ZPs of IOMMs and cells would lead to stronger electrostatic interactions and, consequently, greater sedimentation rates. Furthermore, the same authors indicated that the attachment of IOMMs to *C. vulgaris* was favoured by pH values below the isoelectric point of magnetic particles, at pH 6.2 (Figure 5.10), which is in contrast with our results (Figure 5.9).

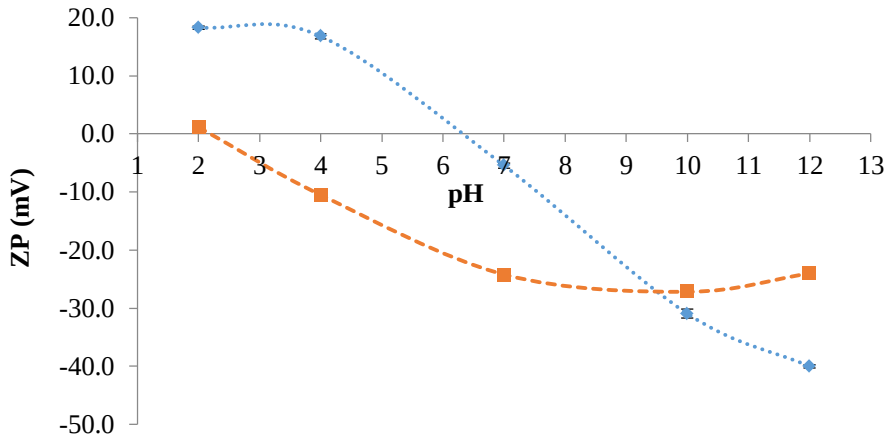

Figure 5.10 – ZP measurements of IOMMs (♦) and *M. aeruginosa* cells (■) at different pH values. Error bars correspond to the standard deviation of the average value determined for triplicates.

Although other research works support the thesis of harvesting using magnetic particles being enhanced under acidic environments and especially at pH 4 (Lin *et al.*, 2015; Liu *et al.*, 2016; Prochazkova *et al.* (2013b); Wang *et al.*, 2016), likewise our case several studies have obtained higher *HE*s applying alkaline conditions (Hu *et al.*, 2013; Zhao *et al.*, 2015), including at pH 12 (Cerff *et al.*, 2012). Besides the variability of

species/strains and media composition used, as reported in Section 5.4.4.1, the differences presented by all those experiments can be associated with the numerous magnetic particles tested and the influence of pH on the ionization degree of cells and particles, which makes that distinct pH values might be optimal for different microorganisms even on processes applying the same magnetic particles (Wang *et al.*, 2015b). Similarly to Hu *et al.* (2013), the divergence of the optimal pH values reported by Prochazkova *et al.* (2013a) when compared to ours could also rely on the increased diameter of IOMMs:cells aggregates promoted by alkali environments, thus forming some kind of net that have dragged a great number of cyanobacteria quickly to the bottom.

5.5 Conclusions

The evaluation of pH-induced flocculation showed that the use of environments with pH values of 3 and 4 results in the highest *HE*, reaching more than 90 % of cells removal within the first 8 h of the process.

In spite of the interference of other mechanisms, ZP has demonstrated to directly affect the *HE* of cyanobacteria. Generally, values within the OIVZP have proven to be the most suitable to be used in all the methods performed in this study: flocculation induced by pH, $FeCl_3$, $AlCl_3$, and Ch. The pursuit of higher *HE* by changing the ZP, allowed not only to improve significantly the settling rates attained utilizing the same flocculation agents (improvements ranging from 9 to 88 % within the first 8 h), but also to decrease the dosage used for all of them.

Although the four harvesting methodologies have attained efficiencies higher than 91 % after 8 h, the best one revealed to be the use of $FeCl_3$, reaching 92 % within the first 4 h of the process.

The study of harvesting of *M. aeruginosa* with IOMMs have shown that IOMMs:cells (g/g) ratios below 0.8:1 were not very efficient, presenting, in general, *HE*s not exceeding 60 %. On the other hand, a similar behaviour was observed for 0.8:1 and 1:1 ratios, which have attained more than 70 % of culture's sedimentation after 15 min. Regarding the pH value of the medium, the highest *HE* was approximately 93.6 %, obtained after 4 min at pH 12. Apart from pH 10, which have resulted in a *HE* around

92.2 %, no other significant differences were found by varying the pH when compared to control.

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Chapter 6

Optimization and evaluation of different cell's disruption techniques in *Microcystis aeruginosa*

Most of the information described in this Chapter was adapted from:

Geada, **P**., Loureiro, L., Escobar, L., Jewkes, A., Teixeira, J.A., Vasconcelos, V., Vicente, A.A., Fernandes, B., *Microcystis aeruginosa* disruption/permeabilization methodologies study and comparison in order to obtain high yields of microcystin release. Submitted to Bioresource Technology.

6.1 Abstract

The extraction of the wide range of useful bioactive compounds displayed by cyanobacteria is still a major bottleneck at industrial scale. In addition to the high costs, extraction efficiencies are also commonly low, with cell disruption efficiencies playing a significant role in intracellular compounds' extraction. In order to increase the chances of an extended use of MC in several biotechnological fields, we decided to optimize five different disruption techniques: bead milling, microwave, freeze-thaw cycles (FTC), high-speed homogenization (HSH), and sonication.

For each of the methods tested, the conditions that maximized the intracellular organic matter release (*IOMR*) were: ***i)*** 20 % of beads and treatment time of 7 min (bead milling); ***ii)*** 800 W for 1.5 min (microwave); ***iii)*** three 12-h freezing cycles at -20 ˚C (FTC); ***iv)*** 15000 rpm for 7 min (HSH); and ***v)*** 40 kHz for 10 min (sonication). Sonication and FTC followed by sonication revealed to be the most effective methodologies to ensure a maximum *IOMR* and, consequently, MC availability for being extracted. The decrease of cells' viability was however more evident in FTC, FTC followed by sonication, and microwave where only 0.3, 0.05 and 0.9 % of the initial cells, respectively, maintained their viability after being treated. On the other hand, sonication and bead milling reduced the viability of the original culture to 5 and 15.5 %, respectively, while HSH did not show any significant differences compared to control.

6.2 Introduction

Because of the high production and downstream processing costs, the number of microalgal- and cyanobacterial-based biotechnological products launched in the market is still low when compared to its broad potential. A significant market shift is though expected especially due to recent advances found in PBR engineering, systems biology, genetic engineering, and biorefining (Wijffels and Barbosa, 2010).

As shown in Section 2, cyanobacteria produce important secondary metabolites (including vitamins, toxins, enzymes and pigments) and consequently represent a tremendous source of potential high added-value compounds to apply in food, feed, pharmaceutical, chemical, and biofuels sectors (Geada *et al.*, 2017; Thajuddin and Subramanian, 2005). *M. aeruginosa*, for instance, is pointed by numerous studies as source of a great range of bioproducts or even as an added-value itself by integrating

this species in some industrial processes (Ashokkumar *et al.*, 2014; Chen *et al.*, 2005; Philippis and Vicenzini, 1998; Rós *et al.*, 2012; Singh and Singh, 2014). In spite of the arising interest originated by recent findings, most of these compounds (including MC) are intracellular and cell rupture techniques are required, commonly representing a major bottleneck at industrial scale (Geada *et al.*, 2017).

Generally, disruption processes can be divided into two distinct groups: mechanical (e.g. microwave, bead milling, ultrasonication) and non-mechanical (e.g. chemical, enzymatic) methods (Günerken *et al.*, 2015). In spite of the variety of effective techniques to apply, there is a need for mild, inexpensive, and low-energy consumption methodologies to meet the exploitation of more unstable cyanobacterial metabolites. The conventional disruption methods usually utilize chemicals or very high pressure optimized conditions to obtain a given product, but might result in severe damage for many other products (Vanthoor-Koopmans *et al.*, 2013). The use of mild-processing techniques – such as pulsed electric fields, enzymes, ultrasonication – might represent an appropriate alternative to overcome this major bottleneck (Günerken *et al.*, 2015; Vanthoor-Koopmans *et al.*, 2013). Although several studies have already been published about the disruption of *M. aeruginosa* cells, there is a lack of a thorough comparison between the disruption efficiency of the methods and their impact on cells and products of interest (Ma *et al.*, 2012; Pestana *et al.*, 2014; Silva-Stenico *et al.*, 2009; Wu *et al.*, 2012). Additionally, harsh conditions are frequently applied in downstream processes since complete removal of both cells and secondary metabolites, namely MCs, is the main goal – especially when it concerns to wastewater treatment plants. However, bearing in mind the potential applications of MC, the purpose of this work is to achieve very high disruption efficiencies without compromising the stability of the cyanotoxin (i.e. operating under mild and effective conditions). In this sense, we propose to optimize a series of disruption methods – bead milling, freeze-thaw cycles (FTC), microwave, sonication, and high-speed homogenization (HSH) – and compare them in terms of disruption efficiency and MC and intracellular organic matter release (*IOMR*).

6.3 Materials and methods

6.3.1 Microorganism and culture conditions

Cyanobacterium *M. aeruginosa* LEGE 91094 utilized in this study was kindly provided by the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR - Porto, Portugal) collection and maintained in Z8 medium (Kotai, 1972) under 10 $\mu mol_{photons}.m^{-2}.s^{-1}$ using a 12:12-hour light-dark cycle at room temperature. Stock cultures were renewed on a monthly basis.

6.3.2 Disruption techniques

Five different disruption methods were performed and compared: i) bead milling; ii) microwave; iii) freeze-thaw cycles (FTC); iv) high-speed homogenization (HSH); and v) sonication. Tests were made in triplicate using 25 mL of a culture of *M. aeruginosa* presenting a biomass concentration of approximately 0.5 $g.L^{-1}$.

6.3.2.1 Bead milling

Bead milling assays were carried out using glass beads of small diameter (149-250 µm) and a vortex mixer Clifton Cyclone CM-1 (Nickel Electro Ltd, UK). Several bead ratios (between 0 and 80 % of the culture volume) and treatment time intervals (1-10 min) were tested.

6.3.2.2 Microwave

Cell disruption caused by microwave treatment was conducted in a Speedy 21 L microwave oven (Taurus, Spain) varying the time (0.5-2 min) and the power (Defrost-800 W). To avoid overheating and intracellular content release caused by temperature (above 60 ˚C – data not shown), short-period cycles were applied for each power used (four 30-s cycles for defrost; eight 15-s cycles for the remaining power intensities) and samples were kept in ice between cycles until room temperature (roughly 20 ˚C) was reached.

6.3.2.3 Freeze-thaw cycles (FTC)

The effect of FTC was tested at two different temperatures (-20 and -70 ˚C). The number of cycles to apply was also assessed. Since defrosting process using hot water led to release of intracellular material (data not shown), it was done placing tubes containing frozen samples on a vessel with tap water.

6.3.2.4 High-speed homogenization (HSH)

A T-25 digital Ultra-Turrax® (IKA®, Germany) was utilized to perform cell disruption experiments under HSH conditions. The influence of three distinct agitation speeds (10000, 15000 and 20000 rpm) and treatment time (1-15 min) was determined.

6.3.2.5 Sonication

Sonication was performed using an ultrasonic processor VCX 500 (Sonics & Materials, Inc., USA) and subjecting cells to different frequencies (20 and 40 kHz) during certain periods of time (1-15 min). Cycles applied consisted of 3 s of treatment on and 9 s off. The culture was continually kept in ice to prevent overheating.

6.3.3 Cell disruption efficiency analysis

The cell disruption methods were compared using three different techniques: i) flow cytometry; ii) *IOMR*; and iii) toxin quantification.

6.3.3.1 Flow cytometry

Flow cytometry analysis was performed using an EC800™ flow cytometer analyzer (Sony Biotechnology Inc., USA). A total of 50000 events was collected for each triplicate of the samples in order to construct confidence intervals of cell concentrations and consistent fluorescence results. Red fluorescence signals, which are associated with the total chlorophyll content, were collected by a 665 nm long-pass filter, FL3. The number of events and fluorescence signals were evaluated through the EC800 1.3.6 analysis software (Sony Biotech) and the Flowing software 2.5.1. Cell disruption efficiency results obtained by flow cytometry were compared using the methods described in Günerken *et al.* (2017). Briefly, the direct cell counting data was one of the methods used to evaluate the disruption efficiency; the other method consisted in splitting the histograms of FL3-Peak-Lin (radius of spherical cells) and FL3-Lin (area) into quadrants, corresponding the upper right quadrant to the healthy population of microalgae.

6.3.3.2 *IOMR* measurement

After disruption treatment, samples of 500 µL were taken and then centrifuged at 12000 rpm for 10 min. The supernatant was collected and its absorbance was measured at a wavelength of 254 nm in a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., USA). This procedure allowed detecting the *IOMR* from the cells at different conditions (Henderson *et al.*, 2008). The extracellular organic matter present in the original sample (before starting the disruption process) was also determined applying the same method. To facilitate the interpretation of results, an *IOMR* factor was calculated for each sample tested according to the following equation:

$$IOMR\ factor = (Abs_t - Abs_0) / Abs_0 \qquad \text{(Equation 6.1)}$$

where Abs_t refers to the absorbance measured at 254 nm after disruption treatment during a certain period of time (t, min) and Abs_0 is the absorbance measured at 254 nm of the original sample (before treatment).

6.3.3.3 Toxin quantification

MC quantification was performed using the Microcystins-ADDA ELISA Kit (Abraxis, Inc., Pennsylvania, USA), as described in Section 3.3.3.

6.3.3.4 Statistical analysis

The experimental data referring to the release of MC (Section 6.4.2.3) were analysed through one-way ANOVA followed by a *post hoc* Tukey test using Statistica 10.0.228.8 software (Statsoft Inc., USA).

6.4 Results and discussion

6.4.1 Cell disruption techniques optimization

The comparison of all tested techniques was divided into two major steps: optimization of each disruption method mainly considering the *IOMR* during the process (this section) and further comparison of optimal conditions of each strategy by measuring the disruption efficiency and organic matter and toxin release (Section 6.4.2).

6.4.1.1 Bead milling

The first stage of bead milling process characterization was developed by comparing the effect of several beads relative percentages (between 0 and 80 % of the culture volume) at a fixed treatment time of 5 min (Figure 6.1).

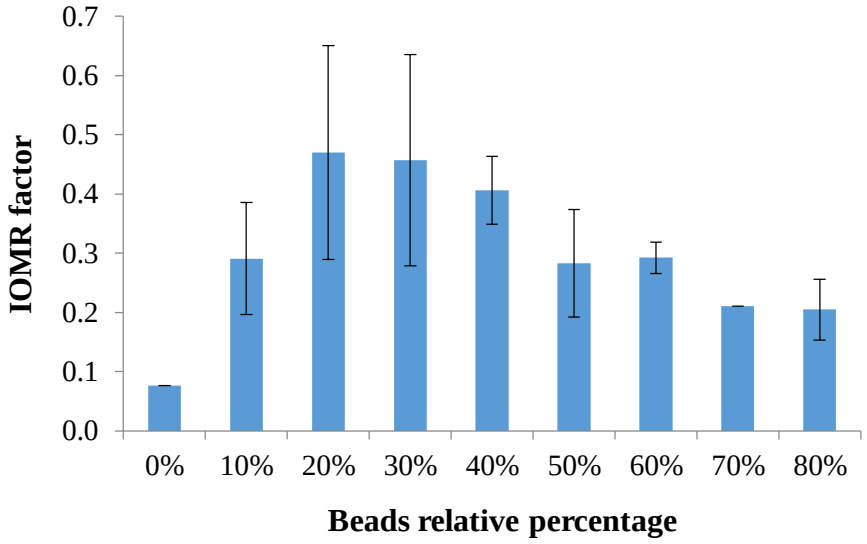

Figure 6.1 – Cell disruption efficiency assessment (based on *IOMR* variation) utilizing different glass beads relative percentages on bead milling process carried out for 5 min. Error bars represent the standard deviation for three experiments.

As shown in Figure 6.1, the use of a relative percentage of glass beads around 20 % was found to cause the highest release of IOM and thus to be the most efficient ratio causing cell disruption/permeabilization. Taking into account this information, it was decided to fix this percentage of beads on bead milling process for the following evaluation tests on the influence of treatment time (between 1 and 10 min) – Figure 6.2. The filling ratio employed here as being the optimal condition is considerably lower when compared to microalgae processing information sources stating that beads' volume should be kept between 50 and 90 %, typically being 80-85 % (Richmond, 2004). Our results also showed a different behaviour in terms of the disruption efficiency with bead load variation, since some authors observed higher cell disintegration yields when increasing ratios are applied (Doucha and Lívanský, 2008; Montalescot *et al.*, 2015). However, these differences might be explained by the use of different beads (in terms of size and composition), cell concentration, and microorganisms, which affect significantly the process effectiveness.

The influence of treatment time on cell disruption using the bead milling technique was evaluated and the highest *IOMR* was detected after 7 min (Figure 6.2). Generally, increasing cell disintegration is expected with treatment time extension until it reaches a

maximum owing to complete disruption of microorganisms, which seems to be the case. The optimal duration for liberation of *IOM* from *M. aeruginosa* applying bead milling technique – 7 min – is in agreement with other studies performed using distinct microalgae where a period between 4 and 9 min was considered the best treatment time for cell disruption and, consequently, high-value metabolites release (Byreddy *et al.*, 2016; Postma *et al.*, 2015).

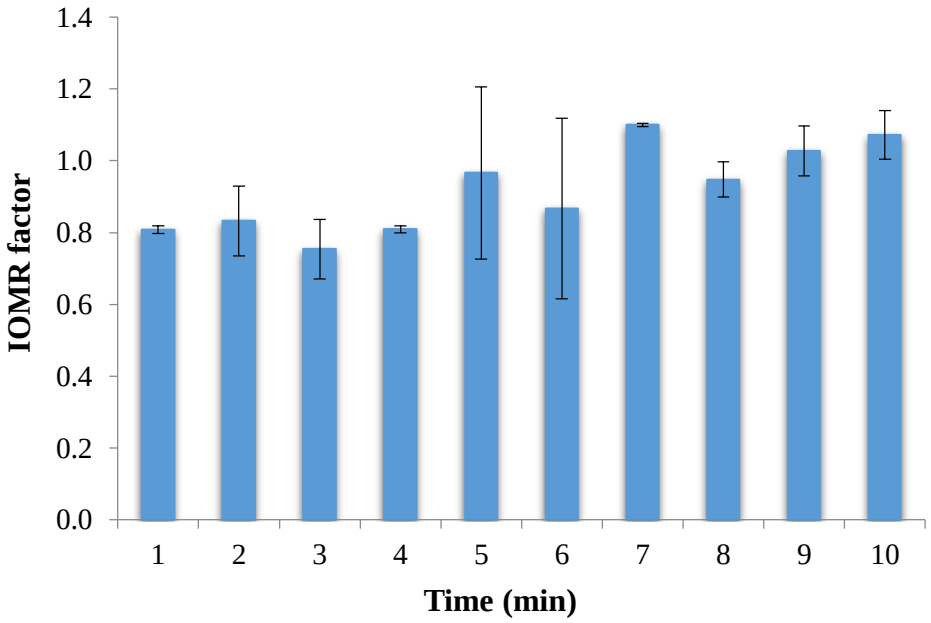

Figure 6.2 –Treatment time effect on cell disruption efficiency (according to *IOMR* factor) for bead milling process. Error bars represent the standard deviation for three experiments.

6.4.1.2 Microwave

Microwave effectiveness on cell disruption was assessed by testing four different power intensities (defrost, 400, 600, and 800 W) following the *IOMR* during 2 min (Figure 6.3).

Generally, 800 W power intensity treatments revealed higher efficiency for all time intervals measured, resulting in higher *IOMR* of *M. aeruginosa* cells. As shown in Figure 6.3, subjecting cyanobacteria to a 1.5 min treatment using this power intensity was considered the optimal condition to apply microwave as a disruption/permeabilization technique. Comparing to other studies, high disruption (approximately 94 %) and oil extraction yields were obtained in tests performed at 80-95 ˚C for periods of time ranging from 20 to 30 min (Balasubramanian *et al.*, 2011; McMillan *et al.*, 2013). However, our goal was to assess the efficiency of microwaves

only and avoid possible interferences of temperature and solvents that would possibly increase *IOMR* effectiveness. Additionally, metabolites' denaturation is frequently observed as a consequence of increasing temperature, which should be prevented as well. Despite of the significant effect of long-lasting microwave treatments, Balasubramanian *et al.* (2011) also found that there is no direct relationship between treatment time and extraction efficiency, since extraction yields of some algae oils were higher for shorter periods of time and then decreased when extended treatment times were applied. Thus, optimal extraction conditions are deeply dependent on the type of metabolite targeted.

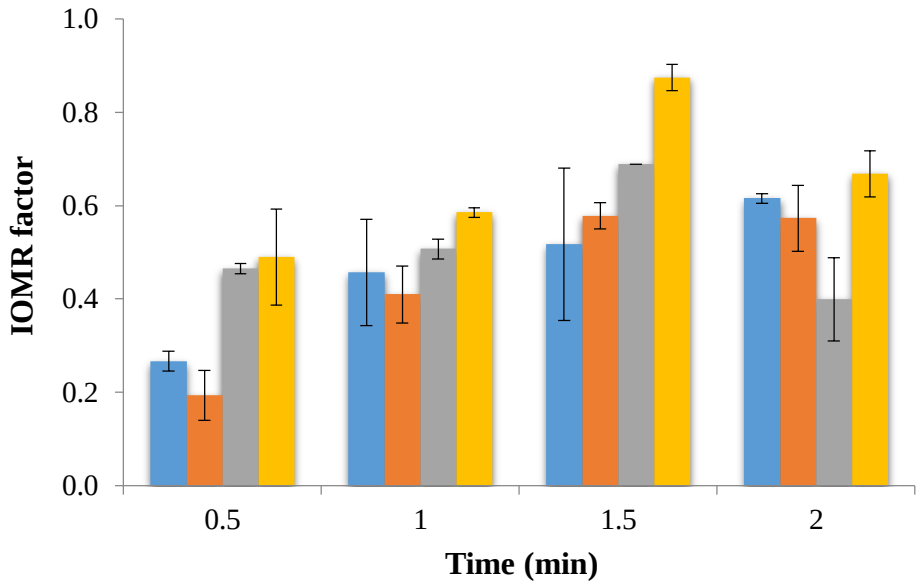

Figure 6.3 – Impact of treatment time and microwave power intensity (defrost (■), 400 W (■), 600 W (■), and 800 W (■)) on cell disruption efficiency according to the *IOMR*. Error bars represent the standard deviation for three experiments.

6.4.1.3 Freeze-thaw cycles (FTC)

Freeze-thaw methodology showed higher *IOMR* from *M. aeruginosa* cells using three 12 h freezing cycles at -20 ˚C (Figure 6.4). This is in agreement with the common use of this cell disruption technique since many ELISA quantification kits, for instance, have instructions to use three freeze-thaw cycles as a means to cause rupture of the cells. This method takes advantage of ice crystals formation inside cells to damage cell wall and release intracellular compounds. The duration of freezing cycles can however vary significantly, being in some cases as short as 2 h (Pestana *et al.*, 2014).

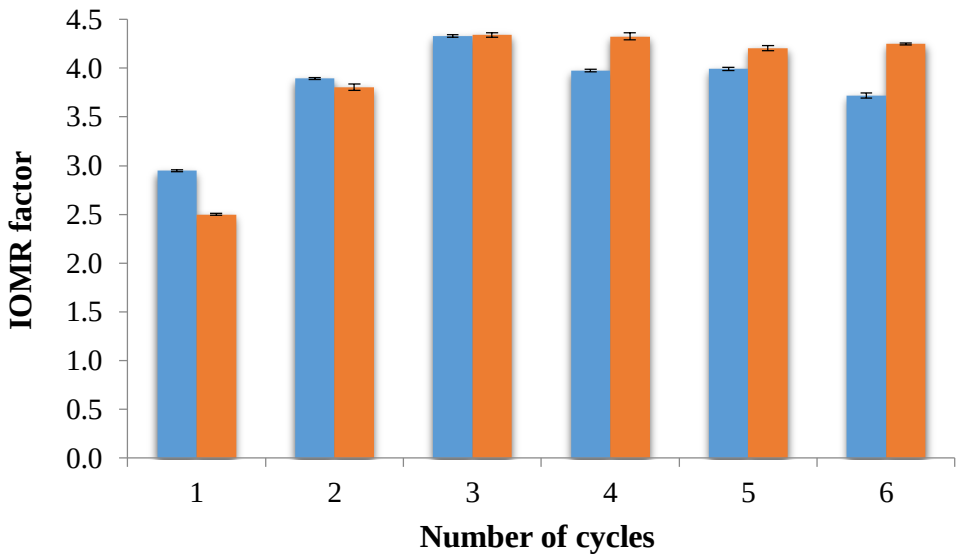

Figure 6.4 – *IOMR* efficiency utilizing a variable number of FTC and two different freezing temperatures (-20 ˚C (■) and -70 ˚C (■)). Error bars represent the standard deviation for three experiments.

6.4.1.4 High-speed homogenization (HSH)

HSH tests were carried out up to 10 min under 10000, 15000, and 20000 rpm. As shown in Figure 6.5, the use of an agitation velocity of 15000 rpm frequently reveals higher extraction rates of IOM throughout the disruption treatment. According to the results obtained, the release of intracellular compounds applying this speed is maintained constant from 7 min onwards. Consequently, this might be considered the most suitable time to operate when *M. aeruginosa* cells disruption is intended via HSH.

This mechanical method is frequently applied to extract compounds of interest from a number of microorganisms – as the case of microalgae (Günerken *et al.*, 2015). Although the process may, in some cases, be as short as 1 min (or even less) (Guedes *et al.*, 2013; Wang and Wang, 2012) due to the combination of hydrodynamic cavitation and solvents, rotation speed is commonly set in the range 10000 to 14000 rpm, which is close to the value achieved in this study. When solvents are not part of the extraction process, treatment duration can, however, be longer (6 min) and approximate to our optimum (Sun *et al.*, 2016). Nevertheless, the agitation rate applied by Sun *et al.* (2016) was considerably higher than 15000 rpm.

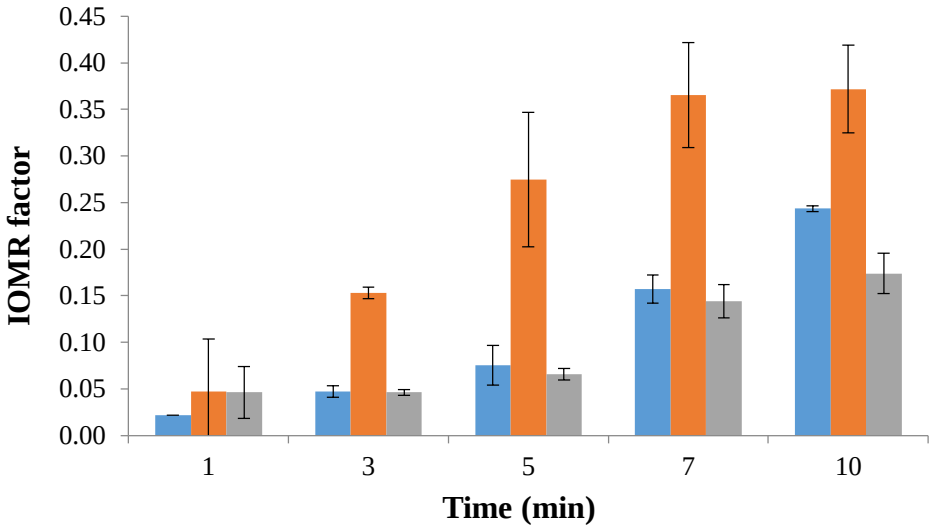

Figure 6.5 – Evaluation of *IOMR* of *M. aeruginosa* cells using a HSH at distinct velocities (10000 (■), 15000 (■), and 20000 (■) rpm) and different treatment times. Error bars represent the standard deviation for three experiments.

6.4.1.5 Sonication

By comparing both frequencies tested, it is possible to infer that sonication performed at 40 kHz always presented better disruption/permeabilization yields, regardless of the treatment time (Figure 6.6). Additionally, these data also showed that maximum *IOMR* was attained after 10 min.

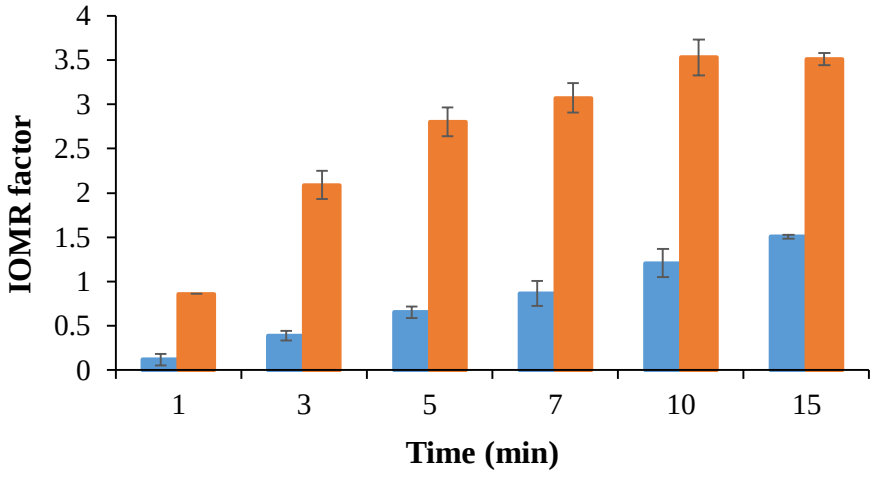

Figure 6.6 – Influence of sonication operating time and frequency (20 (■) and 40 (■) kHz) on *M. aeruginosa* cells *IOMR*. Error bars represent the standard deviation for three experiments.

Despite of being widely used for the disruption of microalgae and subsequent extraction of added-value products (Günerken *et al.*, 2015; Richmond and Hu, 2013),

(ultra)sonication process optimization is deeply associated with the microorganism under study (Wang and Yuan, 2015), the metabolite(s) of interest to be recovered and whether solvents are part of the process or not (Parniakov *et al.*, 2015). In the specific case of *M. aeruginosa*, the optimal treatment time presented in Figure 6.6 is in agreement with the procedure implemented by other authors such as Pestana *et al.* (2014), although the frequency applied in that study is just 50 Hz. Regarding the other parameter studied, Wu *et al.* (2012) compared the inactivation of *M. aeruginosa* using low frequency (20 kHz) and high frequency (580 and 1146 kHz) sonication concluding that lower frequencies are more effective than higher ones.

6.4.2 Comparison between the different cell disruption techniques

6.4.2.1 Damage of cells

The optimal conditions of each disruption method were selected and a new experiment was performed where all techniques were compared. Since sonication and FTC were found to promote the highest rates of *IOMR* individually, an additional test was performed using FTC as pre-treatment of sonication. The number of absolute counts and, among these, the corresponding number of cells that remained viable after applying the disruption methods, are presented in Figure 6.7.

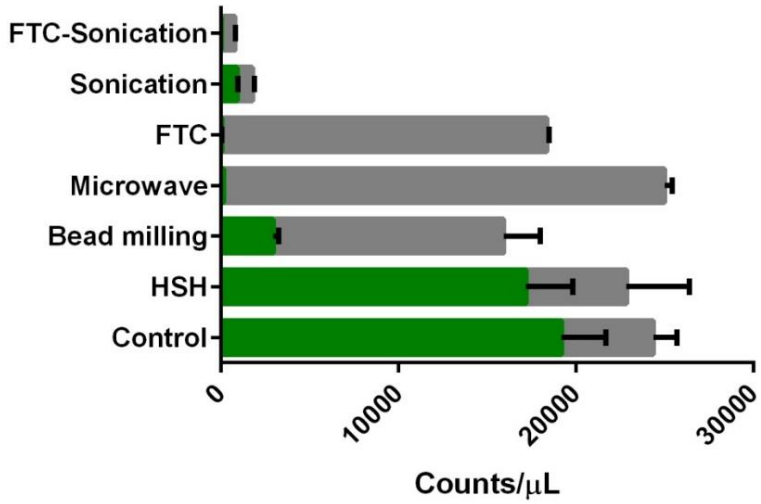

Figure 6.7 – Quantification of the absolute counts (■) and surviving *M. aeruginosa* cells counts (■) by flow cytometry after applying the different disruption methods. Error bars represent the standard deviation for three experiments.

According to these results it is possible verify that, after the treatments, most of the absolute counts obtained do not correspond to the region of *M. aeruginosa* cells. The

only exception seems to be the HSH since it presents a similar number to the control, indicating that almost all the cells weren't affected by this technique. The sonication and the FTC followed by sonication processes appear to be highly destructive and to reduce the debris of cells in such a way that a great part of them is not even accounted by the cytometer (Figure 6.7). Despite of the effectiveness shown by sonication, approximately 5 % of the initial culture survived to this method. Differently, the number of cells that survive to FTC followed by sonication methodology can be neglected once it represents just 0.05 % of the original amount of biomass. As opposed to sonication, bead milling, and particularly microwave and FTC methods, present a high number of absolute counts, but the number of viable cells found in the culture is significantly lower (Figure 6.7). Although FTC and microwave methods seem not to be as aggressive to cells as sonication (the number of absolute counts is considerably greater), higher impact on cells' viability was obtained (99.7 and 99.1 %, respectively, of the initial sample was damaged and did not keep viable). Regarding bead milling, the number of viable cells found in culture after treatment was around 15.5 %.

These results seem not to be in agreement with some other studies since sonication is commonly pointed as an inefficient disruption method (Heo *et al.*, 2017; McMillan *et al.*, 2013). For instance, the difference between the disruption efficiency obtained for sonication and microwave was reported as high as 27.2 % by McMillan *et al.* (2013), which is clearly not the case in our results. However, this disparity may rely on the species-dependency of disruption effectiveness once most of the studies use *C. vulgaris* (Heo *et al.*, 2017; McMillan *et al.*, 2013), commonly considered a more robust and difficult-to-break microorganism.

By evaluating the fluorescence of the cells that were observed in treated samples (Figure 6.7), we were able to get interesting information about the effect of each disruption technique over *M. aeruginosa* (Figure 6.8).

Through these data, a more linear profile of fluorescence is observed for sonication and HSH, suggesting that for these treatments most of the cells kept a cellular structure which is more similar to that of cells in control samples. This means that sonication and HSH provide more uniform treatment conditions in the whole volume of the samples to which they are applied. However, the majority of the absolute counts detected after HSH (Figure 6.7) is placed in the upper right quadrant, while in the case of sonication only about half of them are in that quadrant (Figure 6.8). This means that from the

number of absolute counts obtained after treatment, almost all of them are cells that survive to HSH and keep their viability, whereas only circa 52 % have shown the same behaviour under sonication.

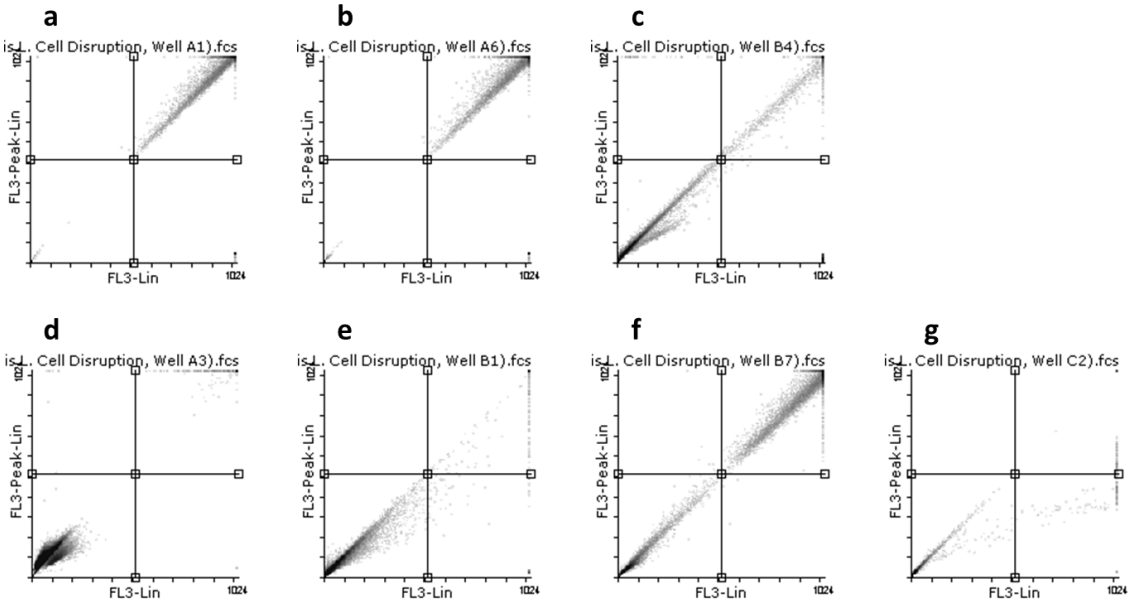

Figure 6.8 – Fluorescence profile of *M. aeruginosa* cells present in solution after applying the optimal disruption conditions selected for the different techniques tested. a) control; b) HSH; c) bead milling; d) microwave; e) FTC; f) sonication; and g) FTC followed by sonication.

However, the number of absolute counts after sonication is considerably lower than HSH, as shown in Figure 6.7. Although the profiles exhibited by cells treated with FTC and bead milling generally show a linear fluorescence profile, it is possible to see some counts displaced and scattered throughout the lower left quadrant. The impact on cells' viability is though distinct in these two methods. The 15.5 % of cells that survived to bead milling represent approximately 32 % of the absolute counts, whilst the use of FTC left less than 0.4 % with viability. Consequently, several counts are observed in the upper right quadrant of the bead milling and very few appear there in the case of FTC. In spite of being slightly less efficient than FTC, microwaves (0.9 % of the cells survived to the method, Figure 6.7) do not present any trend on fluorescence profile, which is associated with great variability in terms of cells' size and shape. The dispersity observed in the lower left quadrant of FTC, bead milling and especially microwave (Figure 6.8), indicates a random effect over cells and it makes sense considering that the processing involving these three methodologies is not as constant and uniform as in the case of sonication and HSH. Heo *et al.* (2017) have verified the same behaviour applying microwave irradiation and sonication to *C. vulgaris*.

According to those authors, cells' membranes were severely damaged when microwave was utilized, whereas sonication was responsible for cracking or torning the cells. Furthermore, it was also concluded that microwave irradiation treatment was not able to disrupt cells evenly (Heo *et al.*, 2017). Regarding the case of FTC followed by sonication, it is possible to see a conjugation of both individual profiles of each treatment; however, the predominant effect seems to be induced by sonication (Figure 6.8).

6.4.2.2 IOMR

Besides the damage inflicted to *M. aeruginosa* by the optimized technologies, another parameter was also evaluated – the *IOMR* (Figure 6.9).

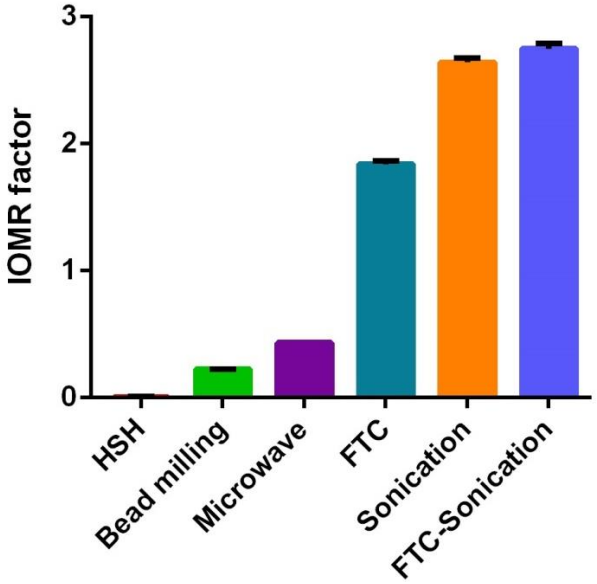

Figure 6.9 – Evaluation of *IOMR* of *M. aeruginosa* cells under the optimized conditions of each disruption method. Error bars represent the standard deviation for three experiments.

Figure 6.9 shows that the rate of organic matter liberated by cells was considerably promoted by sonication, being the FTC responsible just for a slight increment when applied as pre-treatment. The *IOMR* caused by microwave irradiation was surprisingly low in spite of being one of the most effective techniques with regards to cells' viability reduction (Figure 6.7). The HSH method, in turn, had no effect on *IOMR* (Figure 6.9), which was already expected since it did not show to induce any significant changes in cells' structure (Figure 6.7 and Figure 6.8). Despite of the greater *IOMR* derived from sonication treatment, and as discussed in the previous section, this methodology is

frequently taken as unsuitable for extraction purposes. As example, Lee *et al.* (2010) determined that the extraction of lipids involving three different microalgae (*C. vulgaris*, *Botrycoccus* sp., and *Scenedesmus* sp.) is more efficient using microwave irradiation, followed by bead-beating and sonication, respectively. The same conclusions were drawn by Heo *et al.* (2017) when comparing the extraction of lipids from *C. vulgaris* using microwave and sonication, being the efficiencies 82.87 and 69.56 %, respectively. Differently, Pan *et al.* (2002) have found similar extraction yields of bioactive compounds from a plant by applying microwave irradiation and sonication. In addition to the utilization of different organisms, the variability observed between our results and the other studies might be explained by the use of different disruption conditions of the tested methods and the application of solvents to enhance the extraction of certain compounds.

6.4.2.3 Toxin release

As displayed in Figure 6.10, the release of MC from *M. aeruginosa* cells was also evaluated for each optimized disruption process.

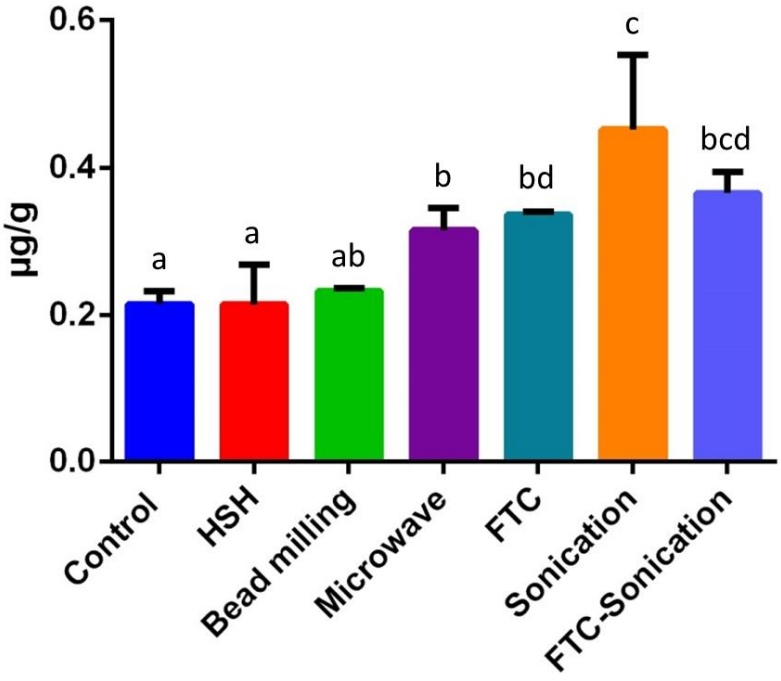

Figure 6.10 – MC release assessment of *M. aeruginosa* cells under the optimized conditions of each disruption method. Error bars represent the standard deviation for three experiments. According to Tukey's test, the methods presenting the same letter do not differ significantly ($\alpha = 0.05$).

Despite of the lower impact on cells' viability when compared to microwave, FTC, and the combination with FTC (Figure 6.7), sonication was, together with the combination with FTC, the most successful methodology with respect to toxin release, similarly to what was observed for the *IOMR* (Figure 6.9). On the contrary, HSH and bead milling, that generally presented none and intermediate impact on *M. aeruginosa* cells (cf. Sections 6.4.2.1 and 6.4.2.2), respectively, do not seem to be interesting methods to conduct MC's extration processes, once no significant changes were determined when compared to control. However, bead milling also demonstrated to be statitiscally identical to microwave, FTC, and sonication followed by FTC, which allows us to conclude that this method is not very efficient on MC release but, at the same time, it isn't as inefficient as HSH. This distinction is clearly supported by the results obtained for cells' viability (Figure 6.7) and *IOMR* (Figure 6.9) where the impact of HSH is neglectable, while bead milling has shown to affect *M. aeruginosa* in both parameters. Regarding microwave and FTC, these techniques were not able to reach a toxin release significantly different from that observed with FTC followed by sonication method. The similarities between these three methodologies could be expected taking into account the impact of all of them over cells (Figure 6.7); however, it is interesting to see that microwave irradiation and FTC did not present comparable yields of *IOMR* (Figure 6.9) and MC release (Figure 6.10) to sonication, that revealed lower reduction on cells' viability. Even more surprising is the fact that FTC is pointed by the manufacturers' instructions of most of the toxin quantification ELISA kits (including the one used throughout this thesis) as the disruption method to apply. Figure 6.10 clearly shows that FTC is not the best option to promote the extraction of MC and, when applied as pre-treatment of sonication, no increment was noted.

These results are not in agreement with Silva-Stenico *et al.* (2009), once microwave and sonication approaches revealed similar toxin release yields. Additionally, the amount of MC obtained by these authors corresponds to concentrations 1000 times higher than ours, which is very surprising. The hypotheses for such huge differences might be the use of distinct strains, which have certainly different toxin production capacities and probably different resistance to disruption methods, or the use of different treatment conditions. For instance, the microwave irradiation process described by Silva-Stenico *et al.* (2009) was applied for a longer period of time (15 min) and the solution was boiled, being thus more aggressive to cells than ours (1.5 min of treatment divided into

15 s cycles and temperatures always below 60 °C throughout the whole process). Furthermore, all the samples undergone disruption processing twice since the treatments were repeated and re-extraction was performed, which might have led to this exponential release of MC. Additionally, the cells used were previously lyophilized and that might have weakened the cells' membranes before applying the disruption methods, contributing to higher amounts of toxin extracted.

6.5 Conclusions

Each disruption technique was optimized through the assessment of *IOMR* and the most suitable conditions were defined as follows: i) 20 % of beads and treatment time of 7 min (bead milling); ii) 800 W for 1.5 min (microwave); iii) three 12 h freezing cycles at -20 ˚C (FTC); iv) 15000 rpm for 7 min (HSH); and v) 40 kHz for 10 min (sonication). Among the optimized methodologies, HSH revealed to be ineffective to extract the toxin or any other intracellular matter, which is a consequence of the absence of structural alterations inflicted to cells. In contrast, FTC followed by sonication, FTC, and microwave irradiation had a great impact over *M. aeruginosa* cells and all of them presented less than 1 % of viable cells after treatment application. However, cell damage was not accompanied by the values of *IOMR* and MC extraction in the case of FTC and microwave. Sonication, which was not as effective in reducing cells' viability – 5 % of the initial biomass concentration maintained its viability –, has shown to be the best methodology to apply both for *IOMR* and toxin extraction, together with FTC followed by sonication. Generally, bead milling has demonstrated a low to intermediate effect on MC extraction and *IOMR* but was able to reduce the cells' viability in 84.5 %.

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Chapter 7

General conclusions and future work

7.1 General conclusions

In order to enhance the growth of *M. aeruginosa* and to improve microcystin (MC) productivity, as well as increase the cost-effectiveness of the whole process, a series of studies was conducted to characterize: i) the effect of environmental factors; ii) different growth strategies; and iii) efficient downstream processes.

A great impact of the combined effect of light intensity, CO_2 concentration, temperature, and pH was observed on both *M. aeruginosa* growth-related parameters and MC production. Results indicate that light intensities below 80 $\mu mol_{photons}.m^{-2}.s^{-1}$ and low CO_2 concentrations (< 1% v/v) represent a suitable environment to reach high toxin production. On the other hand, the combination of high light intensities and CO_2 concentrations (155 $\mu mol_{photons}.m^{-2}.s^{-1}$ 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.

Flat panel (FP) was found to be the best cultivation system to grow *M. aeruginosa*, presenting the highest biomass concentration (1.274 ± 0.013 $g.L^{-1}$), productivity (0.067 ± 0.002 $g.L^{-1}.d^{-1}$), and specific growth rate (0.440 ± 0.009 d^{-1}). Although the X_{max} obtained in bubble column (BC) (1.134 ± 0.125 $g.L^{-1}$) was close to the FP, the time needed to reach this value was the double. Despite of the very low cell concentration obtained in the split cylinder airlift photobioreactor (0.643 ± 0.098 $g.L^{-1}$), the fact is that the P_{max} attained (0.047 ± 0.003 $g.L^{-1}.d^{-1}$) was the same as the BC (0.048 ± 0.001 $g.L^{-1}.d^{-1}$).

In the two-phase growth approach, Stage 1 has shown very high growth rates when compared to Stage 2, being P_{max} and μ_{max} four- and twelve- to thirteen-fold greater, respectively. On the other hand, the accumulation of toxin in the Stage 2 was 66.7 % higher than the control, while it did not exceed 42 % of increment in Stage 1. This behaviour was expected since the environmental conditions applied in Stage 1 were meant to enhance the growth of cyanobacteria, while those used in Stage 2 intended to boost MC production.

Most of the filtrates and extracts tested have shown to affect the growth of toxin-producer *M. aeruginosa*. Nevertheless, the use of 20 % of extract of non-toxic *M.*

aeruginosa was, among the selected assays, the only condition enhancing the growth with an increment of 17 %. This increase was though not followed by an increment on the production of MC, which has maintained invariable. The greatest inhibition was determined using 20 % of filtrate of *S. obliquus* and 20 % of extract of *C. vulgaris*, which have caused cyanobacterial growth reductions of around 13 and 12 %, respectively. The highest concentrations of MC were detected in cultures utilizing 20 % of filtrate and 10 % of extract of *C. emersonii* where increases of approximately 49.3 and 57.5 % were attained, respectively. Surprisingly, these were the conditions that presented the lowest impacts on growth.

pH-induced flocculation was found a promising harvesting technique for *M. aeruginosa* particularly using environments with pH values of 3 and 4, which have presented more than 90 % of cells' removal within the first 8 h of the process. Generally, the most suitable conditions to be applied in all the methods performed in this study (flocculation induced by pH, $FeCl_3$, $AlCl_3$, and Ch) comprised zeta potential (ZP) values in a specific interval, the optimal interval of values of ZP (OIVZP). The optimization of ZP allowed not only to improve significantly the settling rates attained using the flocculation agents (improvements ranging from 9 to 88 % within the first 8 h), but also to decrease the dosage utilized for all of them. Among the harvesting methodologies tested, the best one revealed to be the use of 3.75 $mg.L^{-1}$ of $FeCl_3$, reaching 92 % within the first 4 h of the process.

Harvesting of *M. aeruginosa* with iron oxide magnetic microparticles (IOMMs) using higher IOMMs:cells (g/g) ratios, 0.8:1 and 1:1, revealed to be the best option, since more than 70 % of the culture settled after 15 min of treatment. With respect to the pH of the medium, the highest harvesting efficiency was approximately 93.6 %, obtained after 4 min at pH 12.

The optimization of disruption techniques allowed setting the following conditions for each one of them: i) 20 % of beads and treatment time of 7 min (bead milling); ii) 800 W for 1.5 min (microwave); iii) three 12 h freezing cycles at -20 ˚C (freeze-thaw cycles, FTC); iv) 15000 rpm for 7 min (high-speed homogenization, HSH); and v) 40 kHz for 10 min (sonication). Among these, HSH revealed to be ineffective to extract the toxin or any other intracellular matter, which is a consequence of the absence of structural alterations inflicted to cells. In contrast, FTC followed by sonication, FTC, and microwave irradiation had a great impact over *M. aeruginosa* cells and all of them

presented less than 1 % of viable cells after treatment. However, the damage observed in cells was not accompanied by the highest intracellular organic matter release (*IOMR*) and MC extraction in the case of FTC and microwave. Sonication, which was not as effective in reducing cells' viability – 5 % of the initial biomass concentration maintained its viability –, has shown to be the best methodology to apply both for *IOMR* and toxin release, together with FTC followed by sonication. Generally, bead milling has demonstrated a low to intermediate effect on MC extraction and *IOMR*, but was able to reduce the cells' viability in 84.5 %.

7.2 Future work

The results obtained throughout this PhD have shown that cost-effectiveness of MC's production process is possible. However, some additional aspects should be addressed to minimize the risks and increase the chances of success when operating at industrial scale.

It is suggested to validate the optimized conditions of growth of *M. aeruginosa* and production of MC in cultures carried out at larger scale. It would also be interesting to evaluate the growth of cyanobacteria in continuous mode and determine the impact over toxin production when compared to batch cultures. The assessment of different L:D cycles and light qualities should be considered as well, since these conditions are known to induce changes into the production of other secondary metabolites.

The introduction of electric technologies in downstream processing is gathering attention from research projects involving microalgae. Either for harvesting (e.g. electrofiltration) or for extraction (e.g. moderate electric fields, MEFs) purposes, there is a huge potential that might be applied to cyanobacteria too. Although we have tested the use of MEFs to release MC, the results obtained were not relevant enough to be discussed in this thesis, being thus necessary to proceed with more studies and alternative approaches.

Finally, a techno-economic analysis of the whole optimized process should be conducted to verify its feasibility at industrial scale.