Universidade de Aveiro
2022

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Aplicação do conceito de biorrefinaria na valorização de *blooms* cianobacterianos

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Fernando J. M. Gonçalves, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e co-orientação científica da Doutora Sónia P. M. Ventura, Professora Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Joana L. Pereira, Investigadora do Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio (SFRH/BD/123850/2016 e PTDC/BTA-BTA/30914/2017).

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Agradecimentos

Gostaria de agradecer aos meus orientadores, Prof. Doutor Fernando Gonçalves, Professora Sónia Ventura e Doutora Joana Pereira, por toda a disponibilidade ao longo destes últimos anos. Por todo o conhecimento, pela oportunidade de realizar este trabalho, e pela possibilidade de colaborar com várias pessoas diferentes. O vosso apoio e a vossa boa disposição constante foram muito importantes. Nunca me cortam as asas, e sinto que não poderia ter estado melhor acompanhada.

Em segundo lugar, agradeço a toda a equipa envolvida neste projeto, em especial à Telma Veloso, Margarida Martins, Andreia Fernandes, João Romão, Doutora Tânia Dias, Doutora Iola Duarte e Doutora Helena Oliveira, por toda a ajuda indispensável ao longo deste trabalho. Agradeço também ao Professor João Serôdio, Doutora Silja Frankenbach, Doutor Mario Torres-Acosta, Doutora Helena Passos e Doutora Clara Sousa por terem aceitado fazer parte deste trabalho por tudo o que me foram ensinando.

Agradeço ainda aos meus colegas, muitos deles amigos, que foram companheiros incríveis nesta jornada. As nossas belas horas de almoço são momentos que vou sempre recordar. Um especial agradecimento aos mais próximos, Joana, Telma, Libânia, Guilherme e Cuco. À Joana, o meu pilar de laboratório, que foi sempre uma boa amiga e um grande apoio, e à Telma, que foi a melhor amiga e companheira de trabalho que poderia ter pedido.

Por fim, agradeço aos meus pais e ao meu irmão, que sempre me apoiaram e que ajudaram a encarar estes anos com mais leveza. Ao Quitério por todo o apoio, carinho e por todas as conversas que tivemos, e onde tanto aprendi.

palavras-chave

Cianobactérias, biorrefinaria, produção de pigmentos, C-ficocianina, aplicações biotecnológicas, Marte

Resumo

Num contexto de alterações climáticas, verifica-se um aumento no desenvolvimento de *blooms* cianobacterianos, com consequências negativas para a qualidade da água. Ao mesmo tempo, assiste-se a um crescimento acentuado da população, o que exige a exploração de novos recursos. Neste contexto, as cianobactérias podem ser vistas como um problema, mas também como parte da solução. É nesta dicotomia que se foca esta Tese, utilizando o conceito de biorrefinaria para explorar diferentes vias de valorização desta biomassa. Apesar dos principais problemas associados ao desenvolvimento de *blooms*, potenciados pelas alterações climáticas e deficientemente mitigados pelas atuais estratégias de gestão ambiental, estes apresentam um potencial de aplicação biotecnológica relevante. Este contexto foi revisto e conduziu à elaboração de um modelo conceptual para a valorização de *blooms* cianobacterianos, que compreende (i) a remoção do *bloom*, (ii) a sua caracterização, (iii) a valorização da biomassa removida e (iv) a aplicação do retorno resultante da valorização em gestão e proteção ambiental.

Seguindo esta filosofia e trabalhando sob o conceito de biorrefinaria, foram estabelecidos os objetivos da componente experimental, que se centram na análise de algumas das características fisiológicas das cianobactérias, na caracterização de compostos potencialmente bioativos com origem nesta biomassa, bem como nas possíveis aplicações biotecnológicas das cianobactérias. Relativamente às características fisiológicas, foram analisadas as curvas de crescimento e os padrões de produção de pigmentos (clorofila *a* e C-ficocianina) de três espécies (*Arthrospira platensis*, *Anabaena cylindrica* e *Nostoc muscorum*). Todas foram capazes de crescer durante mais de 100 dias, sem renovação ou suplementação do meio de cultura, sendo que a produção de pigmentos não foi constante durante o período de crescimento e os padrões de produção mostraram-se diferentes de espécie para espécie. De entre os compostos possíveis de extrair das cianobactérias, os pigmentos são os que apresentam um maior valor de mercado. Dentro desta classe, o pigmento azul, C-ficocianina, é amplamente assumido como bioativo, por vezes sem dados empíricos de suporte. Extratos de C-ficocianina não purificados e purificados, extraídos de *Anabaena cylindrica*, foram caracterizados, considerando a sua atividade antioxidante, antimicrobiana, antitumoral e biocompatibilidade. Apesar dos resultados terem sido pouco expressivos, permitiram concluir que a atividade biológica atribuída *ad-hoc* à C-ficocianina poderá dever-se ao efeito sinérgico entre os vários constituintes do extrato e não apenas à presença do pigmento, dependendo ainda da espécie/estirpe usada para a extração.

As cianobactérias podem também produzir inúmeros outros compostos de interesse biotecnológico, o que foi demonstrado através do estudo do metaboloma de *Nostoc muscorum*, ao longo do seu crescimento, utilizando Ressonância Magnética Nuclear ^{1}H: açúcares, oligossacarídeos, lípidos (glicolipídeos, ómega 3 e ómega 6), aminoácidos tipo micosporina, péptidos e pigmentos. A produção destes compostos pôde ser associada a momentos específicos de crescimento de *Nostoc muscorum*. Por último, pelas suas múltiplas aplicações biotecnológicas e pelo facto de estas produzirem oxigénio, foi avaliada a possibilidade de utilização de cianobactérias na exploração espacial, mais precisamente na colonização de Marte. Foi avaliada a capacidade de crescimento de três cianobactérias (*Arthrospira platensis*, *Anabaena cylindrica* e *Nostoc muscorum*) e da microalga *Chlorella vulgaris*, utilizando apenas recursos que podem ser encontrados em Marte (água e regolito). As espécies diazotróficas foram as que mostraram um melhor desempenho. Para além do crescimento, foi também avaliada a possibilidade de utilização destas culturas como biofertilizantes, uma solução que se mostrou preliminarmente promissora.

Este trabalho demonstra o potencial para o desenvolvimento de novas estratégias integradas que permitam assegurar a conservação dos ecossistemas aquáticos através da exploração das aplicações biotecnológicas das cianobactérias.

keywords

Cyanobacteria, biorefinery, pigments, C-phycocyanin, biotechnological applications, Mars

abstract

Climate change increases the likelihood of cyanobacterial blooms formation, with negative impacts on water quality. Concomitantly, a steadily growing population demands the exploitation of new resources as feedstocks. In this context, recognition of cyanobacteria as a nuisance couples with their highlight as potential contributors to solutions. This Thesis focused on this dichotomy, using the biorefinery concept to explore different routes of valorization of cyanobacteria. Although the problems related to cyanobacterial blooms, positively linked to climate change, are insufficiently mitigated by the currently available management strategies, cyanobacteria present a relevant potential for many biotechnological applications. This context was reviewed and lead to the development of a conceptual model for the valorization of cyanobacterial blooms considering (i) the physical removal of the biomass, (ii) its characterization, (iii) its transformation into high market value compounds, and (iv) the investment of the return of this valorization into environmental management and protection.

Following this philosophy and using the biorefinery concept as an umbrella, several experimental goals were established that focused on relevant physiological features, on the characterization of cyanobacterial bioactive products, and on the pursue for biotechnological applications. Regarding its physiological features, the growth kinetics and pigment production patterns (i.e. chlorophyll *a* and C-phycocyanin) from three cyanobacterial species (*Arthrospira platensis*, *Anabaena cylindrica*, and *Nostoc muscorum*) were assessed. All species were proven to be able to live for more than 100 days, without medium supplementation, and the pigment production patterns showed fluctuations during the growth period, while pigment production patterns did neither follow monotonic trends through time nor was similar among different species. Indeed, pigments are among the most valuable cyanobacterial products, and among these the blue pigment C-phycocyanin has been touted as having attractive biological activity, yet often without supportive empirical data. Raw and purified C-phycocyanin extracts from *Anabaena cylindrica* were produced and characterized regarding antioxidant, antimicrobial, antitumor activity and biocompatibility. Mild or unrecognizable results were achieved with these extracts, but important conclusions followed this work, namely that the argued biological activities of C-phycocyanin can be due to synergic effects with components other than C-phycocyanin in extracts, or that C-phycocyanin biological activities are species/strain-specific.

Besides pigments, there are a plethora of cyanobacterial products of biotechnological interest, as demonstrated through the study of the metabolome of *Nostoc muscorum* during its long-term growth, using of ^{1}H Nuclear Magnetic Resonance: sugars and oligosaccharides, lipids (e.g. glycolipids, ω-3 and ω-6 fatty acids), mycosporin-like amino acids, peptides, and pigments. Moreover, the production of these compounds could be associated with specific moments of *Nostoc muscorum* growth. Finally, due to their many applications and oxygen production, the possibility of using cyanobacteria in Space exploration, namely on Mars colonization was addressed. The ability of three cyanobacteria (*Arthrospira platensis*, *Anabaena cylindrica*, and *Nostoc muscorum*) and the microalgae *Chlorella vulgaris* to grow using only resources found on Mars (i.e. water and martian regolith) were assessed, with diazotrophic species confirmedly performing better. In addition, the possibility of using the grown cultures as biofertilization agents was evaluated and this solution was preliminarily shown to be promising.

This work opens avenues to the development of integrative strategies that could meet conservation goals targeting aquatic ecosystems through the exploitation of the cyanobacteria biotechnological potential considering different applications.

Publications resulting from or linked to the work developed in this Thesis

Published papers

S. F. H. Correia; A. R. Bastos; M. Martins; I. P. E. Macário; T. Veloso; J. L. Pereira; J. A. P. Coutinho; S. P. M. Ventura; P. S. André; R. A. S. Ferreira (2022) Bio-based solar energy harvesting for onsite mobile optical temperature sensing in smart cities. *Advance Science*, 10.1002/advs.202104801

I. P. E. Macário; S. P. Ventura; F. J. M. Gonçalves; M. Torres-Acosta; J. L. Pereira (2021) The "bright side" of cyanobacteria: revising the nuisance potential and prospecting innovative biotechnology-based solutions to integrate water management programs. *ACS Sustainable Chemistry & Engineering* 9 (21), 7182-7197. doi.org/10.1021/acssuschemeng.1c00458

I. P. E. Macário; T. Veloso; A. P. M. Fernandes; M. Martins; T. R. Dias; H. Oliveira; F. J. M. Gonçalves; S. P. M. Ventura; J. L. Pereira (2021) Blue is not enough: Biological activity of C-phycocyanin extracts from *Anabaena cylindrica. Journal of Chemical Technology and Biotechnology.* doi.org/10.1002/jctb.7001

T. E. Sintra; S. S. Bagagem, F. G. Ahsaie; A. P. M. Fernandes; M. Martins; I. P. E. Macário; J. L. Pereira; F. J. M. Gonçalves; G. Pazuki; J. A. P. Coutinho; S. P. M. Ventura (2021) Sequential recovery of C-phycocyanin and chlorophylls from *Anabaena cylindrica. Separation and Purification Technology*, 255, 117538. doi.org/10.1016/j.seppur.2020.117538

Submitted papers

I. P. E. Macário; T. Veloso; A. P. M. Fernandes; M. Martins; S. Frankenbach; J. Serôdio; F. J. M. Gonçalves; S. P. M. Ventura; J. L. Pereira. Are Cyanobacteria a nearly immortal sources of high market value compounds? *Submitted to Algae Research*

I. P. E. Macário; T. Veloso; J. Romão; F. J. M. Gonçalves; J. L. Pereira; I. F. Duarte; S. P. M. Ventura. Metabolic composition of the cyanobacterium *Nostoc muscorum* as a function of culture time: a ^{1}H NMR metabolomics study. *Submitted to Algae Research*

I. P. E. Macário; T. Veloso; S. Frankenbach; J. Serôdio; H. Passos; C. Sousa; F. J. M. Gonçalves; S. P. M. Ventura; J. L. Pereira. Cyanobacteria as candidate to support Mars colonization: growth and biofertilization evaluation using Mars regolith. *Submitted to Frontiers in Microbiology*

S. F. H. Correia; A. R. Bastos; M. Martins; I. P. E. Macário; T. Veloso; J. L. Pereira; L. D. Carlos; J. A. P. Coutinho; S. P. M. Ventura; P. S. André; R. A. S. Ferreira. Self-powered sustainable temperature sensor using solar energy and nature-based molecules for smart windows. *Submitted to Fuel*

L. Fu; A. M. P. Botas; T. R. Dias; I. P. E. Macário; J. L. Pereira; J. A. P. Coutinho; S. P. M. Ventura; R. A. S. Ferreira. Entrapment of natural luminescent pigments into solid matrices retaining the optical properties/long-term viabilities. *Submitted to MDPI*

Oral communications

I. P. E. Macário; T. Veloso; A. P. M. Fernandes; S. P. M. Ventura; F. J. M. Gonçalves; J. L. Pereira. The "bright side" of cyanobacteria – a water management perspective. Limnologia, 26-29 October 2020 (online event)

Poster communications

I. P. E. Macário; T. Veloso; J. Romão; F. J. M. Gonçalves; J. L. Pereira; I. F. Duarte; S. P. M. Ventura. Discovering the cyanobacterium *Nostoc* sp. composition through NMR metabolomics. Jornadas CICECO, 6-7 October 2021 (online event)

I. P. E. Macário; S. P. M. Ventura; F. J. M. Gonçalves; M. Torres-Acosta; J. L. Pereira; Cyanobacteria – the link between biotechnology and water management. Jornadas CICECO, 6-7 October 2021 (online event)

I. P. E. Macário; S. P. M. Ventura; J. L. Pereira; F. J. M. Gonçalves. Cyanobacteria. #SETACkissUK, 15 September 2021 (online event)

I. P. E. Macário; T. Veloso; A. P. M. Fernandes; M. Martins; J. L. Pereira; H. Oliveira; F. J. M. Gonçalves; S. P. M. Ventura. Phycocyanin from Cyanobacteria: production, extraction, and biological activity. Jornadas CICECO, 19-20 November 2020 (online event)

I. P. E. Macário; T. Veloso; A. P. M. Fernandes; M. Martins; S. P. M. Ventura; H. Oliveira; F. J. M. Gonçalves; J. L. Pereira. The silver lining of Cyanobacteria – the case of phycocyanin. Encontro Ciência 20, 3-4 November 2020, Lisbon, Portugal

I. P. E. Macário; S. P. M. Ventura; J. L. Pereira; F. J. M. Gonçalves. The Dark and the Bright side of Cyanobacteria. #SETACkissUK, 16 September 2020 (online event)

M. Martins; A. P. M. Fernandes; I. P. E. Macário; J. L. Pereira; F. J. M. Gonçalves; J. A. P. Coutinho; S. P. M. Ventura. Sequential extraction of phycocyanin and chlorophyll from *Anabaena cylindrica*. Biopartitioning & Purification Conference, November 2019, São Paulo, Brazil

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CHAPTER I

General introduction

In a scenario of climate change, water degradation is pointed out as one major consequent problem and the promotion of good water quality is entailed in the United Nations Sustainable Development Goals, as well as in official management strategies such as that regulated under the European Union Water Framework Directive. In this context, the necessity of exploring integrative novel strategies to efficiently deal with old problems urges. Cyanobacteria blooms are a remarkable example of a well-known and old problem, expected to become potentiated by climate changes events, representing a major threat to water quality that already affects a large part of the world population (Janse et al. 2004; Paerl and Huisman 2009; O'Neil et al. 2012; Paerl and Paul 2012; Taranu et al. 2012; Visser et al. 2016; Hofer 2018).

Cyanobacteria are the oldest known oxygen-producing organisms, thriving on Earth for approximately 2.4 billion years. These organisms, belonging to the Division Cyanophyta, are close to Gram-negative bacteria but are able to perform photosynthesis (Flores and Herrero 2014). Cyanobacteria present a characteristic bluish-green colour due to the pigment phycocyanin that gave them the name cyanobacteria or, in some particular arenas, blue green algae. These organisms can occur singly or grouped in colonies and can increase to such large densities (blooms) that colour the water, ultimately forming a clearly visible thick scum (WHO 2003). Several environmental factors contribute to cyanobacterial growth, being the most important ones, light intensity, CO_2 concentration, nutrient availability (especially nitrogen, phosphorus, iron, and molybdenum), hydrologic characteristics of the water body, and aquatic ecosystem structure and function (Merel et al. 2013). Interactions between environmental factors and climate change events play a major role promoting the development of cyanobacterial blooms (Visser et al. 2016). Cyanobacteria and eukaryotic algae may respond differently to climate change manifestations, with former often outcompeting the latter, and changes in typical phytoplankton community composition are expected to occur locally as a consequence (Visser et al. 2016), as well as a global geographical expansion of cyanobacteria (Paerl and Huisman 2009).

Several studies show that, with increased temperature, cyanobacterial growth rates can reach their optimum and continue at high levels in the long term, even at temperatures higher than 25 ºC (Paerl and Paul 2012). Indeed, optimum temperatures for cyanobacterial growth range within 27-37 ºC, while optimal ranges are shorter for green algae (27-32 ºC), and lower (17-27 ºC and 17-22 ºC) for dinoflagellates and diatoms (Paerl 2014). Also, the

warming of superficial waters intensifies vertical stratification, and many cyanobacteria species are able to explore stratified conditions, because as long as there is low turbulence, and thus weak mixing, cyanobacteria can control their buoyancy by exploring their gas vesicles (Paerl and Paul 2012). Since most of cyanobacterial species decrease in abundance in the winter, the expected increase in temperature may affect the annual life cycle of these organisms promoting the extension of their growth season (Visser et al. 2016). Also, the toxin composition of cyanobacterial blooms may be affected, with higher temperatures potentially increasing the ratio between toxic and non-toxic strains. Still, not all species will behave in the same way in this context. For instance, in the case of *Microcystis*, Davis et al. (2009) verified that toxic strains will benefit with an increase in temperature, which is also true for saxitoxin production from *Aphanizomenon* sp. (Dias et al. 2002). However, this tendency was not verified for saxitoxins production by *Cylindrospermopsis raciborskii* (Castro et al. 2004). UV stress also reflects in a competitive advantage of cyanobacteria comparing to other phytoplankton groups since they contain photoprotective pigments and can more prominently increase the production of antioxidant enzymes (Carreto and Carignan 2011).

Extensive summer droughts, sea level rising, and increased use of freshwater for industrial proposes and for drinking water supply, have been leading to increased salinization worldwide (Paerl 2014). Increased salinization of freshwaters can favour some cyanobacterial species that are salt tolerant (Paerl and Paul 2012). Drought conditions leads to important changes in the water physical and chemical characteristics as well as in the composition of the aquatic communities (Wiedner et al. 2007). In these conditions, groups such as Cyanobacteria are favoured (Smith et al. 2008) due to increase temperature of water and enhanced stratification (Mosley 2015). The development of pathogenic bacteria (e.g. *Aeromonas, Vibrio, Actinobacter* and *Pseudomonas*) can also occur favoured by cyanobacterial blooms, threatening further human health if affected waters are used as drinking water resources or for recreation purposes (Berg et al. 2009).

Algal blooms can impair the ecosystem structure and functioning by adversely affecting the biota, with potential negative impacts in human health. These types of blooms are commonly known as harmful algal blooms (HABs). The greatest public health concerns related to HABs apply when cyanobacteria are dominant and this type of blooms are hereinafter referred to as CyanoHABs (Backer 2002). This specific type of bloom has been

increasing at a high pace since the 1960s, and has been spreading worldwide. Cyanobacteria dominance is linked to eutrophication, and human activity is the primary driver of accelerated eutrophication. Therefore, CyanoHABs occurrence is clearly related to human activities and generally promoted during the warmer and dryer periods of the year (Carmichael 2008).

Cyanobacteria can cause not only ecological problems, but also economic constrains and public health problems (de Figueiredo et al. 2004). From an ecological perspective, cyanobacterial blooms lead to the reduction of specific biodiversity and to habitat deterioration due to the entailed increase in turbidity and reduction of light levels in the subsurface. This reduction of light penetration in the water column leads to an overall decrease of photosynthesis, with the consequent reduction of oxygen released to the water, which can be lethal to some fish and invertebrate species. Cyanobacteria can also cause changes in the organoleptic parameters of the water. They can produce substances that confer bad taste and odour to water (e.g. geosmin and 2-methylisoborneol; Izaguirre et al. 1982), and more stringently they can produce toxins that, if released in water (after cell lysis in the majority of species, or during the growth phase in the case of e.g. *Cylindrospermopsis raciborskii*), are noxious to a great variety of organisms (Metcalf and Codd 2012). Consequentially, socio-economic problems eventually arise due to the deterioration of water quality. The use of water from contaminated reservoirs requires expensive and additional treatment processes needed to remove cyanotoxins (Rapala et al. 2006). Regarding recreational activities, water quality degradation *via* cyanobacteria contamination may also have great economic impacts, mainly through reduction of local tourism preferences (WHO 2021), but ultimately due to direct contact with hazardous substances.

The health problems associated to cyanobacteria are related to their ability to produce toxins (Metcalf and Codd 2012), commonly referred to as cyanotoxins. These toxins represent a potential hazard for waters used for human and animal drinking-water supplies, for aquaculture, agriculture, and recreation. Moreover, there are also health risks associated with the ingestion of cyanotoxins, in the form of food supplements (Roy-Lachapelle et al. 2017), or in the form of crops irrigated with contaminated water (Miller and Russell 2017). Toxic cyanobacteria are found worldwide in both inland and coastal waters. Still, it should be noted that not all of cyanobacterial blooms are toxic since the production of toxins is associated to specific genes that may or may not be present (Kurmayer and Christiansen

2009), and even if these genes are present, their expression is dependent on environmental conditions (Merel et al. 2013).

Cyanotoxins can be classified according to their mode of action and target organs in neurotoxins, hepatotoxins, cytotoxins, irritants and gastrointestinal toxins. The most common neurotoxins include anatoxin-a, homoanatoxin-a or saxitoxins and the guanidin methyl phosphate ester. Hepatotoxins, the most frequent and dangerous cyanotoxins, include nodularins and microcystins. Cytotoxins include cylindrospermopsin, and amongst the toxins with irritant and gastrointestinal effects are aplysiatoxin, debromoaplysiatoxin and lyngbyatoxin (which are tumour promoters and cause skin irritation), as well as lipopolysaccharide endotoxins (LPS) that may enhance inflammatory and gastrointestinal incidents (Metcalf and Codd 2012). While some cyanotoxins (e.g. saxitoxins and lipopolysaccharide endotoxins) are not exclusively produced by cyanobacteria, others such as microcystins and anatoxins, are only produced by these organisms (Metcalf and Codd 2012).

Most of these cyanotoxins have been found in a wide array of genera and some species may produce more than one toxin. In freshwaters, the most widespread cyanobacterial toxins are microcystins and neurotoxins, and some species can contain several types of toxins simultaneously (WHO 2021). Neurotoxins (e.g. anatoxin-a, anatoxin-a (S) and saxitoxin) can be lethal at high doses by causing asphyxia through paralysis of respiratory muscles, although they differ in their mode of action (WHO 2003). Although acute exposure is well-known, the chronic effects of some neurotoxins are still under study (Metcalf et al. 2021). On the contrary, chronic exposure to microcystins in drinking water is already well-known and can promote liver cancer (Shi et al. 2021). Microcystins are among the most common cyanotoxins and more than 200 isomers are already reported, being microcystin-LR the most common worldwide (Shi et al. 2021). Although commonly considered an hepatotoxin, this description is no longer sufficient to fully appraise its mode-of-action in animals because it can accumulate in multiple organs and tissues of mammals and fish, such as heart, liver, gonads, lungs, brain and kidney, with consequent tissue and cell damage (Metcalf and Codd 2012). In Caruaru (Brazil), numerous deaths occurred when 136 dialysis patients were exposed to microcystins through water used for dialysis (Pouria et al. 1998). A similar case occurred in Portugal, in 1993, during a dry summer, when a cyanobacterial bloom occurred at Monte Novo and Divor reservoirs (Évora), used as drinking water supply (Oliveira 1995).

Cylindrospermopsin is also a hepatotoxin, first discovered after a poisoning incident on Palm Island (Queensland, Australia) in 1979, when 148 people, mainly children, were hospitalized with hepatoenteritis due to *Cylindrospermopsis raciborskii* contamination of a drinking water reservoir (Griffiths and Saker 2003). This toxin behaves as a general cytotoxin that blocks protein synthesis, potentially causing kidney and liver failure, ultimately widespread organ damage (Chorus et al. 2000). Besides the acute symptoms, cylindrospermopsin is also considered genotoxic and potentially carcinogenic (Messineo et al. 2010).

Cyanotoxins can reflect in both acute and chronic effects. Some low-level short-term exposures can lead to health effects from which full recovery should occur (e.g. dermal exposure which may lead to skin irritations and allergic reactions), but long-term exposure, even at low levels, may result in damage to target organs, thus promoting long-lasting effects in exposed organisms, including humans (Funari and Testai 2008). Most reported cases of human injury by cyanotoxins involved exposure through drinking water. Also, people swimming in contaminated lakes have experienced abdominal pain, nausea, vomiting, diarrhoea, sore throat, dry cough, headache and elevated liver enzymes in the serum, as well as high fever symptoms, dizziness, fatigue, skin and eye irritations, allergic dermatitis, swollen lips, conjunctivitis, earaches and a hay fever-like syndrome (WHO 2003).

Allergic or irritant dermal reactions may arise from exposure through dermal contact and can vary in severity. Several freshwater Cyanobacteria *taxa* (*Anabaena, Aphanizomenon, Nodularia, Oscillatoria* and *Gloeotrichia*) have been reported to cause these reactions after recreational exposure. Bathing suits, particularly wet suits, tend to intensify such effects by accumulating cyanobacterial material, and enhancing disruption and the release of the cell content (WHO 2003).

The natural growth progress of cyanobacteria, especially when forming noxious blooms poses several problems as detailed above. However, the production of these organisms in controlled industrial settings reflects important benefits. This is because these organisms have the potential to be efficiently used in several applications in fields such as agriculture, aquaculture, bioremediation, bioenergy and the sourcing of valuable compounds (Pandey et al. 2013; Kamal and Ahmad 2014). However, amongst more than 10 000 species of cyanobacteria, only a few have been partly characterized and even a lower number of species is currently used for exploitation by the dedicated industry (Raheem et al. 2018). Considering the number of species that still remain to explore, the knowledge about the

biotechnological potential of these organisms should be recognised as very limited. Moreover, the market for cyanobacterial products has been increasing, and nowadays it reached a relevant value, with bioenergy, nutrition and cosmetics being the main areas of related industrial investment (https://www.gminsights.com, assessed on December 7th 2021).

Increased consciousness about the harmful effects of synthetic or chemical products led consumers and industry to search for natural alternatives. Indeed, the search for natural compounds with biological activity is an active and growing area of research that has been mostly focused on bacterial, and fungal microorganisms (*Actinomycetes* and *Hyphomycetes*). However, research has more recently opening perspectives towards consideration of microalgae and cyanobacteria (Singh et al. 2005). The biological activity of cyanobacteria metabolites is of significant relevance to various applications and it is possible to extract from them a wide range of bioactive compounds with high commercial value (e.g. polyunsaturated fatty acids, amino acids and pigments, with antibacterial, antifungal, antiviral and antitumor activity, just to mention a few) (Eriksen 2008; Sekar and Chandramohan 2008). Pigments are among the extractable products with a higher market value. Different pigments with different colours can be found in cyanobacteria, such as chlorophylls, carotenoids, and phycobiliproteins, being the last two those with the highest commercial value (Koller et al. 2014). Also, beside its appealing colour featuring its primary potential as natural dyes in food and in cosmetic products (Spolaore et al. 2006), other sectors may also benefit from these products. Fluorescence properties, antitumor activity, antioxidant properties, and anti-inflammatory potential, are some of the attractive features of carotenoids and phycobiliproteins (Saini et al. 2018; Pagels et al. 2021).

The applications of cyanobacteria are not just focused on the extraction of valuable compounds. The biomass as a whole can also be used with a positive contribution to relevant problems like food scarcity, climate change mitigation, and bioenergy. An important quality that favours its application is that the culturing of these organisms does not need the use of fertile soil, oppositely to common crops. By itself, this has a huge impact considering the continuous growth of the humankind and the scarcity of arable soils for food production (FAO 2011). Indeed, since the human population of our planet is projected to reach *ca.* 9.7 billion by 2050 (DESA 2015), new solutions to overcome or prevent food scarcity are an urgency. A common application for cyanobacteria is their use as supplements in the food

and in aquaculture feed markets. Cyanobacteria are a non-conventional source of protein, healthy lipids, minerals, antioxidants, and vitamins (Singh et al. 2005), with several species being considered as edible and consumed for a reasonable time (e.g. *Arthrospira, Nostoc,* and *Anabaena*) (Pathak et al. 2018). One of the well-known examples is *Arthrospira platensis* that is industrially cultivated in outdoor ponds or bioreactors, and commercially available in several forms (flakes, powder, capsules or pills). This species is an important source of proteins, thiamine, riboflavin, beta-carotene, vitamin E and vitamin B12 (Abed et al. 2009). Therefore, and owing to its nutritional profile, there are already projects running in some African countries aiming to produce *Arthrospira* to fight malnutrition (e.g. the project JustSpirulina, a collaboration between Rwanda, United Nation's Food and Agriculture Organization (FAO) and the non-governmental organization YaLa Africa). The studies concerning the incorporation of cyanobacteria in aquaculture are fewer, but some positive effects have been demonstrated in the growing rates of Tilapia fish (Fadl et al. 2020). Another role of cyanobacteria that can contribute for a sustainable food production is their use as biofertilizers. Indeed, "Green Revolution" practices have the goal of increasing agriculture production while reducing the risk of chemical-based fertilizers on human health and in the environment. Exploring cyanobacteria to improve crop production and soil fertility can be tagged as a 'green technology' in this context (Chittora et al. 2020) given that these organisms are important bio-geo-chemical players in the fixation of atmospheric CO_2 and N_2 (Grzesik and Kalaji 2017). A successful example is the association *Azolla-Anabaena,* for N_2 fixation and nutrient enrichment in rice paddy fields (Hove and Lejeune 2002).

Due to their photosynthetic capacity, these organisms are natural CO_2 mitigation agents. Indeed, cyanobacteria along with microalgae are very efficient in capturing CO_2. A remarkable example of the interplay between profitable applications of these organisms and CO_2 mitigation is a recently designed bioreactor (Eos Bioreactor) of a relatively small size (90 x 90 x 210 cm) that can use the carbon retrieved from the atmosphere to produce biofuels. It is expected that *Chlorella vulgaris* cultured in this bioreactor could be 400 times more efficient capturing CO_2 than trees taking up the same footprint (https://newatlas.com/environment/algae-fueled-bioreactor-carbon-sequestration/ assessed on November 16th 2021).

Cyanobacteria can also act as bioremediation agents, able to remove several heavy metals, such as cadmium, lead, zinc, iron, copper and manganese (Zeng et al. 2012; Fawzy

and Mohamed 2017), crude oil (El-Sheekh and Hamouda 2014), and complex organic compounds such as herbicides (Ibrahim et al. 2014), from aquatic matrices. However, some studies showed that cyanobacteria are not the single responsible for the degradation of these compounds, but the consortia between cyanobacteria and aerobic organotrophic bacteria (e.g. Sánchez et al. 2005). In these consortia, aerobic bacteria directly degrade the compounds while cyanobacteria provide them oxygen and fixed nitrogen, which is often limited in different environments (Abed et al. 2009).

The energy sector represents another opportunity for cyanobacteria, and a wide range of biofuels can be produced using this feedstock (i.e. biohydrogen, biomethane, bioethanol and biodiesel) (Raheem et al. 2018). The problems associated to the use of fossil fuels are well known and the use of renewable resources and biofuels is pointed out as one major solution. However, biofuels produced using terrestrial plants, such as soybean, sugarcane and corn, compete for fertile soil with food production (Scharlemann and Laurence 2008). Therefore, the development of third-generation biofuel feedstocks based on cyanobacteria is advantageous, especially given their rapid growth rates, high CO_2 fixation capacity, high yields in applied lipid extraction, the possibility of growing in facilities installed in non-arable lands, and the possibility of implementing a non-seasonal production dynamics (Raheem et al. 2018). In addition to biofuels production, electricity can also be generated directly using cyanobacteria in systems such as Microbial Fuel Cells (Zhao et al. 2012).

Another hot topic regarding cyanobacteria applications that constitutes also a major challenge faced recently by the humankind is Space exploration. Many years ago, the visionary Carl Sagan suggested the use of these organisms in the planet Venus, to render it more Earth-like (Sagan 1961). Nowadays, the use of cyanobacteria in Space exploration continues to be an option with great potential. Long-duration travels and future inhabitation in space will require almost zero dependency on Earth supplies. Therefore, one of the essential steps to ensure independency is to develop a controlled ecological life-support system that can recycle the atmosphere (producing oxygen and capturing CO_2), purify water, and generate food for human consumption. The regeneration of resources could be achieved through a combination of biological and physicochemical process (Mitchell 1994). The use of photosynthetic organisms such as cyanobacteria in these systems is now under study (e.g. incorporation of *Arthrospira* in the project MELiSSA - Micro-Ecological Life Support System Alternative; https://www.melissafoundation.org, assessed on December 4th 2021).

Cyanobacteria are indeed remarkable oxygen producers, food suppliers, a source of bioenergy and biofertilizers. Their ecophysiological features as detailed above also promote these organisms as possible supporters for Mars colonization (Arai 2009; Verseux et al. 2016).

In order to achieve a better and more sustainable World, the United Nations promote the Sustainable Development Goals (SDG) to address the most significant global challenges of the humankind, including poverty, inequality, climate change, environmental degradation, peace and justice. Cyanobacteria exploitation can be part of solutions developed to tackle some of the UN SDG (Figure I-1). Among the 17 SDG established under the 2030 Agenda, the possible use of nutritional rich cyanobacteria as food supplements and in aquaculture, as well as their use as biofertilizers to improve crop cultures, is in line with SDG 2 (Zero Hunger). Also, the extraction of specific metabolites with application in the health and pharmaceutical sector meets SDG 3 (Good Health and Wellbeing). The bioremediation abilities could help to contribute to insure cleaner water, in line with SDG 6 (Clean Water and Sanitation). For the SDG 7 (Affordable and Clean Energy), cyanobacteria can contribute as feedstock for biofuels and electricity production. Finally, their ability to absorb the atmospheric CO_2 is in line with SDG 13 (Climate Action).

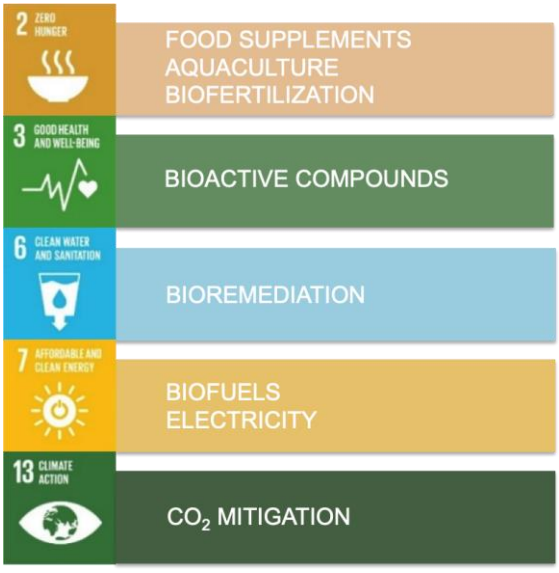

Figure I-1: Link between the UN SDG and applications of cyanobacteria, or cyanobacterial compounds.

In addition, the Green Deal is also one important framework at the European level towards achieving a more sustainable world. Among its goals is a more efficient use of resources by moving to a clean and circular economy. In this context, blue bioeconomy is a fundamental pillar to meet the EU environmental and climate objectives (European Commission 2021). Furthermore, the exploitation of cyanobacteria following a biorefinery concept is a meaningful strategy used to promote blue bioeconomy (the algae blue bioeconomy sector has been gaining increased attention; European Commission, 2021). Biorefinery is a concept that can be defined as "the sustainable processing of biomass into a spectrum of marketable products and energy" (IEA 2008). It explores the idea of multiple valorization routes using the same biomass (IEA 2008) and allowed a transition into a bio-based economy, using renewable sources and circular economy philosophy (Veríssimo et al. 2021). Considering the many applications and exploitable products of cyanobacteria, as described above, this is a concept that could be applied to the valorization of these organisms.

I-1. Objectives and structure of the Thesis

The main goal of this Thesis is the exploration of the biorefinery concept in the valorization of cyanobacteria. This concept was applied herein to cyanobacteria to explore which could be the future valorization of natural blooms and cultured strains, allying environmental management (of the old problem posed by cyanobacteria blooms) to biotechnological exploitation. Also, following the inner logic of the biorefinery concept, different studies focused on specific routes of valorization of specific products or applications.

This Thesis is structured in seven chapters. The first and seventh chapters correspond to a brief introduction and to the final remarks of the Thesis, respectively. The other five correspond to individual research papers, published or submitted to peer-reviewed journals. A summary of the major aims of the studies reported in each of these chapters is described as follows, and depicted in Figure I-2.

Chapter II contains a review that complements the Introduction of this Thesis. Therein, the conceptual possibility of retrieve value from the removal of natural blooms is discussed, by reasoning on the "bright side" of cyanobacteria. It additionally develops and

proposes a model for promoting efficient environmental management of natural blooms through linking with major biotechnology approaches.

Chapter III contains the work developed to better understand the differential exploitability possibilities of several cyanobacteria species. Indeed, the study was triggered from the acknowledgment that different species would produce different compounds at different rates depending on the culturing conditions and on their growth dynamics, thus any exploitation investment should stem from previous evidence on these aspects rather than on convenience. This first experimental work aimed specifically to compare the growth dynamics and the pigment production patterns of three species. The cyanobacterium most commonly selected for exploitation *Arthrospira platensis* and two alternative species, *Anabaena cylindrica* and *Nostoc muscorum*, were studied, and their production of chlorophyll *a* and C-phycocyanin was monitored during the growth period. While the major aim was to understand if species other than *Arthrospira platens*is could be a valuable source of pigments, different methods (spectrophotometry and fluorimetry) were explored to understand whether there is an appropriate proxy for biomass increase and pigment production that can be used for monitoring purposes in industrial exploitation settings.

Since phycocyanin is one of pigments with higher market value, Chapter IV aimed at assessing the biological activity of cyanobacteria extracts rich in C-phycocyanin. The cyanobacterium *Anabaena cylindrica* was used as a model for extraction and extract biological activity assessment (antioxidant, antimicrobial, biocompatibility, and antitumor against human cell lines). Raw extracts were compared with purified extracts to understand if the desired biological activity was achievable using less purified (easier and cheaper to produce) extracts, and extracts lyophilisation was also addressed.

Chapter V was driven by the search for interesting compounds other than pigments that could be valorised from cyanobacteria feedstocks. Here the goal was to explore the metabolic profile of the cyanobacterium *Nostoc muscorum* through high resolution ^{1}H NMR. The long-term culturing and the periodic samples to metabolic analysis allowed an insight on how the metabolic profile evolved during the growth, providing important insights on the dependency of yields on the harvesting schedules established for up-scaled cyanobacteria cultures.

In Chapter VI the goal was to evaluate the possible role of cyanobacteria in Space exploration, namely supporting Mars colonization. Considering the necessity of becoming less dependent of Earth supplies, this study focused on the ability of several species of cyanobacteria and microalgae (*Anabaena cylindrica, Nostoc muscorum, Arthrospira platensis,* and *Chlorella vulgaris*), to grow using only resources existing in Mars (i.e. water and Martian regolith). In addition, the capacity of these cultures as biofertilizers was also evaluated using the macrophyte *Lemna minor* as a vegetable model.

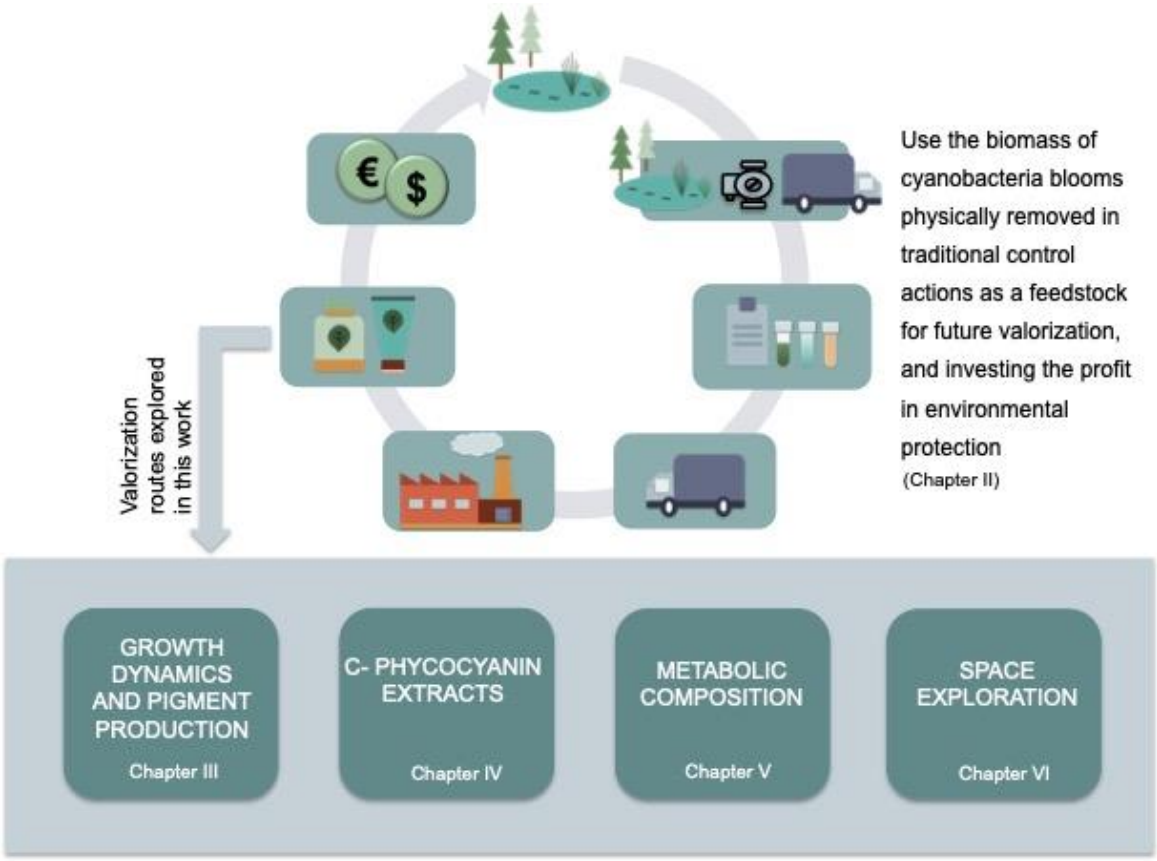

Figure I-2: Schematic representation of the work developed within this Thesis.

The final outcome of this thesis is a multidisciplinary approach focusing on cyanobacteria. Therefore, it connects many fields of expertise through collaborative work within the fields of environmental management, ecophysiology, toxicology, metabolomics, economics and biotechnology. In this way, problems associated with these organisms were addressed as a background, but this Thesis essentially focuses on possible exploitation routes applicable to cyanobacteria, through the exploration of different features such as growth

kinetics, pigment production patterns, C-phycocyanin extracts characterization, metabolic profile evaluation, and finally, the possibility of using these organisms in Space exploration.

References

Abed RMM, Dobretsov S, Sudesh K (2009) Applications of cyanobacteria in biotechnology. J Appl Microbiol 106:1–12. doi: 10.1111/j.1365-2672.2008.03918.x

Arai M (2009) Cyanobacteria for Space agriculture on Mars. Biol Sci Sp 23:203–210. doi: 10.2187/bss.23.203

Backer LC (2002) Cyanobacterial harmful algal blooms (CyanoHABs): Developing a public health response. Lake Reserv Manag 18:20–31. doi: 10.1080/07438140209353926

Berg KA, Lyra C, Sivonen K, et al. (2009) High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms. ISME J 3:314–325. doi: 10.1038/ismej.2008.110

Carmichael WW (2008) A world overview — One-hundred-twenty-seven years of research on toxic cyanobacteria — Where do we go from here? In: Hudnell HK (ed) Cyanobacterial Harmful Algal Bloom. State Sci. Res. Needs. Advances in Experimental Medicine and Biology, pp 105–125

Carreto JI, Carignan MO (2011) Mycosporine-like amino acids: Relevant secondary metabolites. Chemical and ecological aspects. Mar Drugs 9:387–446. doi: 10.3390/md9030387

Castro D, Vera D, Lagos N, et al. (2004) The effect of temperature on growth and production of paralytic shellfish poisoning toxins by the cyanobacterium *Cylindrospermopsis raciborskii* C10. Toxicon 44:483–489. doi: 10.1016/j.toxicon.2004.06.005

Chittora D, Meena M, Barupal T, Swapnil P (2020) Cyanobacteria as a source of biofertilizers for sustainable agriculture. Biochem Biophys Reports 22:100737. doi: 10.1016/j.bbrep.2020.100737

Chorus I, Falconer IR, Salas HJ, Bartram J (2000) Health risks caused by freshwater

cyanobacteria in recreational waters. J Toxicol Environ Heal Part BCritical Rev 3:323–347. doi: 10.1080/109374000436364

Davis TW, Berry DL, Boyer GL, Gobler CJ (2009) The effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms. Harmful Algae 8:715–725. doi: 10.1016/j.hal.2009.02.004

DESA (2015) World Population Prospects: The 2015 Revision, Key Findings and Advance Tables. Working Paper No ESA/P/WP. 241. New York

Dias E, Pereira P, Franca S, et al. (2002) Production of paralytic shellfish toxins by *Aphanizomenon* sp. LMECYA 31 (Cyanobacteria). J Phycol 38:705–712. doi: https://doi.org/10.1046/j.1529-8817.2002.01146.x

El-Sheekh MM, Hamouda RA (2014) Biodegradation of crude oil by some cyanobacteria under heterotrophic conditions. Desalin Water Treat 52:1448–1454. doi: 10.1080/19443994.2013.794008

Eriksen NT (2008) Production of phycocyanin - a pigment with applications in biology, biotechnology, foods and medicine. Appl Microbiol Biotechnol 80:1–14. doi: 10.1007/s00253-008-1542-y

European Commission (2021) The Blue Economy Report 2021. doi: 10.2771/8217

Fadl SE, Elsadany AY, El-Shenawy AM, et al. (2020) Efficacy of cyanobacterium *Anabaena* sp. as a feed supplement on productive performance and immune status in cultured Nile tilapia. Aquac Reports 17:100406. doi: 10.1016/j.aqrep.2020.100406

FAO (2011) The state of the world's land and water resources for food and agriculture (SOLAW) – Managing systems at risk. Rome and Earthscan, London

Fawzy MA, Mohamed AKSH (2017) Bioremediation of heavy metals from municipal sewage by cyanobacteria and its effects on growth and some metabolites of *Beta vulgaris*. J Plant Nutr 40:2550–2561. doi: 10.1080/01904167.2017.1380822

de Figueiredo DR, Azeiteiro UM, Esteves S. M, et al. (2004) Microcystin-producing blooms - a serious global public health issue. Ecotoxicol Environ Saf 59:151–163. doi: 10.1016/j.ecoenv.2004.04.006

Flores E, Herrero A (2014) The cell biology of cyanobacteria. Caister Academic Press,

Norfolk, UK

Funari E, Testai E (2008) Human health risk assessment related to cyanotoxins exposure. Crit Rev Toxicol 38:97–125. doi: 10.1080/10408440701749454

Griffiths DJ, Saker ML (2003) The Palm Island mystery disease 20 years on: A review of research on the cyanotoxin cylindrospermopsin. Env Toxicol 18:78–93. doi: 10.1002/tox.10103

Grzesik M, Kalaji HM (2017) Effectiveness of cyanobacteria and green algae in enhancing the photosynthetic performance and growth of willow (*Salix viminalis* L.) plants under limited synthetic fertilizers application. Photosynthetica 55:510–521. doi: 10.1007/s11099-017-0716-1

Hofer U (2018) Climate change boosts cyanobacteria. Nat Rev Microbiol 16:122–123. doi: 10.1038/nrmicro.2018.15

Hove CV, Lejeune A (2002) The *Azolla*: *Anabaena* Symbiosis. Biol Environ Proc R Irish Acad 102B:23–26. doi: http://www.jstor.org/stable/20500136

Ibrahim WM, Karam MA, El-Shahat RM, Adway AA (2014) Biodegradation and utilization of organophosphorus pesticide Malathion by cyanobacteria. Biomed Res Int 2014:392682. doi: 10.1155/2014/392682

IEA (2008) IEA bioenergy Task 42 on biorefineries: co-production of fuels, chemicals, power and materials from biomass. In: Minutes of the third Task meeting. 25–26 March 2007.

Izaguirre G, Hwang CJ, Krasner SW, McGuire MJ (1982) Geosmin and 2-methylisoborneol from cyanobacteria in three water supply systems. Appl Environ Microbiol 43:708–714. doi: 10.1128/aem.43.3.708-714.1982

Janse I, Kardinaal WE, Meima M, et al. (2004) Toxic and nontoxic *Microcystis* colonies in natural populations can be differentiated on the basis of rRNA gene internal transcribed spacer diversity. Appl Environ Microbiol 70:3979–3987. doi: 10.1128/AEM.70.7.3979-3987.2004

Kamal A, Ahmad IZ (2014) Cyanobacteria “the blue green algae” and its novel applications: A brief review. Int J Innov Apllied Stud 7:251–261.

Koller M, Muhr A, Braunegg G (2014) Microalgae as versatile cellular factories for valued products. Algal Res 6:52–63. doi: 10.1016/j.algal.2014.09.002

Kurmayer R, Christiansen G (2009) The genetic basis of toxin production in Cyanobacteria. Freshw Rev 2:31–50. doi: 10.1608/FRJ-2.1.2

Merel S, Walker D, Chicana R, et al. (2013) State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environ Int 59:303–327. doi: 10.1016/j.envint.2013.06.013

Messineo V, Melchiorre S, Di Corcia A, et al. (2010) Seasonal succession of *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* blooms with cylindrospermopsin occurrence in the volcanic Lake Albano, Central Italy. Environ Toxicol 25:18–27. doi: 10.1002/tox.20469

Metcalf JS, Codd GA (2012) Cyanotoxins. In: Whitton BA (ed) Ecol. Cyanobacteria II - Their Divers. Sp. Time. Springer Science + Business Media B.V., pp 651–675

Metcalf JS, Tischbein M, Cox PA, Stommel EW (2021) Cyanotoxins and the nervous system. Toxins (Basel) 13:1–19. doi: 10.3390/toxins13090660

Miller A, Russell C (2017) Food crops irrigated with cyanobacteria-contaminated water: an emerging public health issue in Canada. Environ Heal Rev 60:58–63. doi: 10.5864/d2017-021

Mitchell CA (1994) Bioregenerative life-support systems. Am J Clin Nutr 60:820–824.

Mosley LM (2015) Drought impacts on the water quality of freshwater systems; review and integration. Earth-Science Rev 140:203–214. doi: 10.1016/j.earscirev.2014.11.010

O'Neil JM, Davis TW, Burford MA, Gobler CJ (2012) The rise of harmful cyanobacteria blooms: the potential roles of eutrophication and climate change. Harmful Algae 14:313–334. doi: 10.1016/j.hal.2011.10.027

Oliveira FA (1995) Effects of cyanobacteria on drinking water and human health: An epidemiological study in Évora, Portugal. Proc Int Symp Assess Manag Heal Risks from Drink Water Contam Approaches Appl 101–110.

Paerl H (2014) Mitigating harmful cyanobacterial blooms in a human- and climatically-impacted world. Life 4:988–1012. doi: 10.3390/life4040988

Paerl HW, Huisman J (2009) Climate change: a catalyst for global expansion of harmful cyanobacterial blooms. Environ Microbiol Rep 1:27–37. doi: 10.1111/j.1758-2229.2008.00004.x

Paerl HW, Paul VJ (2012) Climate change: Links to global expansion of harmful cyanobacteria. Water Res 46:1349–1363. doi: 10.1016/j.watres.2011.08.002

Pagels F, Vasconcelos V, Guedes AC (2021) Carotenoids from cyanobacteria: Biotechnological potential and optimization strategies. Biomolecules. doi: 10.3390/biom11050735

Pandey VD, Pandey A, Sharma V (2013) Biotechnological applications of cyanobacterial phycobiliproteins. Int J Curr Microbiol Appl Sci 2:89–97.

Pathak J, Rajneesh, Maurya PK, et al. (2018) Cyanobacterial farming for environment friendly sustainable agriculture practices: Innovations and perspectives. Front Environ Sci. doi: 10.3389/fenvs.2018.00007

Pouria S, de Andrade A, Barbosa J, et al. (1998) Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. Lancet 352:21–26. doi: 10.1016/s0140-6736(97)12285-1

Raheem A, Prinsen P, Vuppaladadiyam AK, et al. (2018) A review on sustainable microalgae based biofuel and bioenergy production: Recent developments. J Clean Prod 181:42–59. doi: 10.1016/j.jclepro.2018.01.125

Rapala J, Niemela M, Berg KA, et al. (2006) Removal of cyanobacteria, cyanotoxins, heterotrophic bacteria and endotoxins at an operating surface water treatment plant. Water Sci Technol 54:23–28.

Roy-Lachapelle A, Solliec M, Bouchard MF, Sauvé S (2017) Detection of cyanotoxins in algae dietary supplements. Toxins (Basel) 9:1–17. doi: 10.3390/toxins9030076

Sagan C (1961) The Planet Venus. Science (80) 133:849–858.

Saini DK, Pabbi S, Shukla P (2018) Cyanobacterial pigments: Perspectives and biotechnological approaches. Food Chem Toxicol 120:616–624. doi: 10.1016/j.fct.2018.08.002

Sánchez O, Diestra E, Esteve I, Mas J (2005) Molecular characterization of an oil-degrading

cyanobacterial consortium. Microb Ecol 50:580–588. doi: 10.1007/s00248-005-5061-4

Scharlemann LJPW, Laurence WF (2008) How Green Are Biofuels? Science (80-) 319:43–44. doi: DOI: 10.1126/science.1153103

Sekar S, Chandramohan M (2008) Phycobiliproteins as a commodity: Trends in applied research, patents and commercialization. J Appl Phycol 20:113–136. doi: 10.1007/s10811-007-9188-1

Shi L, Du X, Liu H, et al. (2021) Update on the adverse effects of microcystins on the liver. Environ Res 195:110890. doi: 10.1016/j.envres.2021.110890

Singh S, Kate BN, Banecjee UC (2005) Bioactive compounds from cyanobacteria and microalgae: An overview. Crit Rev Biotechnol 25:73–95. doi: 10.1080/07388550500248498

Smith MJ, Shaw GR, Eaglesham GK, et al. (2008) Elucidating the factors influencing the biodegradation of cylindrospermopsin in drinking water sources. Environ Toxicol 23:413–421. doi: 10.1002/tox.20356

Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006) Commercial applications of microalgae. J Biosci Bioeng 101:87–96. doi: 10.1263/jbb.101.87

Taranu Z, Zurawell R, Pick F, Gregory-Eaves I (2012) Predicting cyanobacterial dynamics in the face of global change: the importance of scale and environmental context. Glob Chang Biol 18:3477–3490. doi: 10.1111/gcb.12015

Veríssimo NV, Mussagy CU, Oshiro AA, et al. (2021) From green to blue economy: Marine biorefineries for a sustainable ocean-based economy. Green Chem.

Verseux C, Baqué M, Lehto K, et al. (2016) Sustainable life support on Mars - The potential roles of cyanobacteria. Int J Astrobiol 15:65–92. doi: 10.1017/S147355041500021X

Visser PM, Verspagen JMH, Sandrini G, et al. (2016) How rising CO_2 and global warming may stimulate harmful cyanobacterial blooms. Harmful Algae 54:145–159. doi: 10.1016/j.hal.2015.12.006

WHO (2003) Algae and cyanobacteria in fresh water. Guidel. safe Recreat. water Environ. World Health Organization, Geneva, Switzerland, pp 136–158

WHO (2021) Toxic cyanobacteria in water, 2nd ed. doi: https://doi.org/10.1201/9781003081449

Wiedner C, Rucker J, Bruggemann R, Nixdorf B (2007) Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. Oecologia 152:473–484. doi: 10.1007/s00442-007-0683-5

Zeng J, Zhao DY, Ji YB, Wu QL (2012) Comparison of heavy metal accumulation by a bloom-forming cyanobacterium, *Microcystis aeruginosa*. Chinese Sci Bull 57:3790–3797. doi: 10.1007/s11434-012-5337-2

Zhao J, Li X-F, Ren Y-P, et al. (2012) Electricity generation from Taihu Lake cyanobacteria by sediment microbial fuel cells. J Chem Technol Biotechnol 87:1567–1573. doi: 10.1002/jctb.3794

CHAPTER II

The "bright side" of cyanobacteria: revising the nuisance potential and prospecting innovative biotechnology-based solutions to integrate water management programs

I. P. E. Macário; S. P. Ventura; F. J. M. Gonçalves; M. Torres-Acosta; J. L. Pereira (2021) The "bright side" of cyanobacteria: revising the nuisance potential and prospecting innovative biotechnology-based solutions to integrate water management programs. *ACS Sustainable Chemistry & Engineering*. 9 (21), 7182-7197. doi.org/10.1021/acssuschemeng.1c00458

Contributions: I. P. E. M. wrote the manuscript; M. T. A. performed the economical analysis; F. J. M. G., S. P. V., and J. L. P. supervised

Abstract: Global warming and the anthropogenic degradation of water quality are pointed out as main causes of the worldwide increase in frequency, severity, and duration of harmful algal blooms (HABs). Cyanobacteria, major constituents of HABs, can cause ecological, economic, and human health problems, configuring a “dark side” requiring management attention. Their growth can be potentiated by climate change consequences, highlighting further the urgency of improving HABs management strategies to ensure water quality. An innovative perspective for cyanobacteria management is the exploitation of their “bright side”. Several exploitable products produced by cyanobacteria (e.g. bioactive pigments, lipids, proteins) present high market value. Thus, this work provides a critical perspective on how HABs management may be connected with biotechnology in the future. We propose the use of the biomass of cyanobacteria blooms physically removed in traditional control actions (much needed to ensure environmental and even human health safety) as a feedstock for future valorization, thus allying profit to water quality management, in a win-win relationship between economics and environmental sustainability. Such a proposal was validated with an economic analysis, which evidenced a relevant potential for a positive Return (hence rendering profit likely to occur), both considering only the delivery of harvested biomass to production units and the full valorization route from harvesting to the selling of the extracted/purified product using phycocyanin as a model.

Keywords: cyanobacteria blooms, water management, biomass exploitation, biotechnology, economic impact

II-1. Introduction

The warming of the climate system is unequivocal. Due to the changing in precipitation patterns or the melting of snow and ice, hydrologic systems suffer alterations in their common cycles, with consequences in water quantity and quality (IPCC 2014a). Under a global change scenario, water scarcity in many regions will became (or already is) a reality, and contamination of freshwater sources will continue to be an issue (e.g. 80% of the world’s population already suffers serious threats on water security; IPCC 2014b). One of the threats to water degradation is the proliferation of phytoplankton groups such as cyanobacteria. Along with the anthropogenic eutrophication and increasing CO_2 levels in the atmosphere, climate change manifestations, namely rising temperatures and altered hydrologic patterns, are strong drivers of the increased frequency, intensity and duration of cyanobacterial blooms (Paerl and Huisman 2009; Paerl and Paul 2012; Visser et al. 2016b). Thus, water management programs need to increasingly consider these organisms to assure human health and ecosystems safety. On the other hand, cyanobacteria have the biochemical potential to be commercially exploited in agriculture, aquaculture, bioremediation, biofuels, nutraceutical and pharmaceutical products (Eriksen 2008; Sekar and Chandramohan 2008; Pandey et al. 2013; Kamal and Ahmad 2014). This imposes the key question on whether exploiting cyanobacteria blooms can be a sustainable strategy supporting the management of affected waterbodies. Here, we document the suitability of exploring the nuisance biomass removed in environmental control actions and the economic income that can offset management costs. We first provide key definitions for the understanding of the field, regarding the ecophysiological advantages of cyanobacteria and how climate change promotes them. Then, the critical problem of bloom formation and cyanotoxins production, configuring the “dark side” of cyanobacteria, and (linked) management actions are discussed, these crossed with the “bright side” represented by their biotechnological valorization. Moreover, an exercise will be presented considering the potential economic impact of working the “bright side” of cyanobacteria through biotechnology approaches, aiming to allow a deeper understanding of the positive consequences of promoting the ecosystems conservation through their biotechnological valorization. Overall, this review aims to be the trigger for innovative approaches to deal with the cyanobacteria-driven environmental and health issues, interconnecting the fields of water quality, water management, and biotechnology.

II-2. The nuisance potential of cyanobacterial harmful algal blooms (CyanoHABs)

Cyanobacteria are gram-negative bacteria performing photosynthesis (Domain Bacteria: Phylum Cyanobacteria) that can occur singly or grouped in colonies, often forming blooms. A complex interaction of environmental factors linked to global climate change has been contributing to cyanobacterial growth and leading to the outcompeting of eukaryotic algae (Visser et al. 2016b) (Figure II-1). Higher optimal temperature ranges for growth (Paerl 2014), buoyancy capacity allowing to benefit (better than non-bouyant microalgae) from vertical stratification caused by the water surface warming (Paerl and Paul 2012), higher production and accumulation of photoprotective pigments and antioxidant amino acids supporting protection against increased UV irradiation (Carreto and Carignan 2011), and even a better efficiency using increased CO_2 levels in the atmosphere as a source of carbon (Visser et al. 2016b), feature the capacities of cyanobacteria in this context (Figure II-1). Even under drought conditions, cyanobacteria can be favoured (Smith et al. 2008) due to increased water residence (Paerl 2014), as well as with the increase in salinization of freshwaters (Paerl and Paul 2012). Finally, diazotrophic cyanobacteria (e.g. *Anabaena*, and *Cylindrospermopsis*) can fix atmospheric nitrogen in heterocysts to grow successfully under nitrogen scarcity (Moisander et al. 2012).

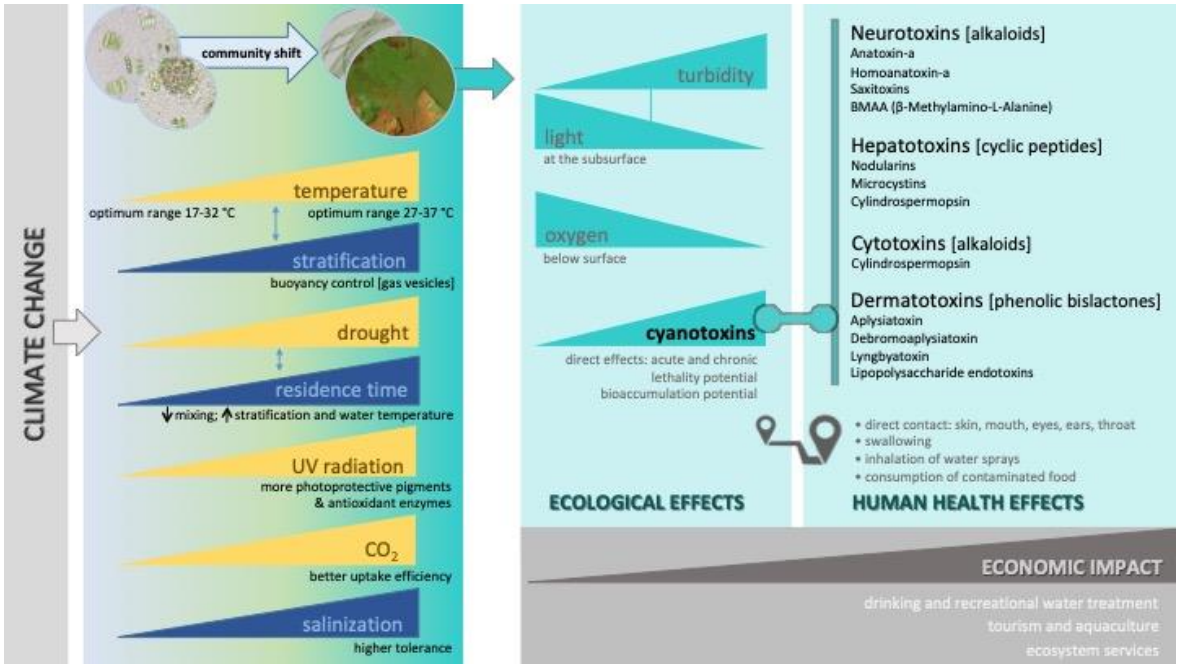

Figure II-1. Synthesis on the driving role of climate change towards cyanobacteria dominance over other phytoplankton groups, with emphasis on the consequent noxious effects in ecosystem and human health, as well as negative socio-economic impacts resulting from nuisance management demands.

Harmful algal blooms (HABs) is the generalist name given to algal blooms that affect adversely the environment, plants or animals’ health. This is a worldwide nuisance and the greatest public health impacts related to HABs are linked to CyanoHABs, considering drinking water supply and recreational waters (Backer 2002). As a worldwide problem, it affects some of the largest and resourceful water bodies (e.g. Lake Vitoria, Africa; Lakes Erie and Michigan, US-Canada; Lake Okeechobee and Lake Ponchartrain, USA; Lake Taihu, China; Lake Biwa, Japan; Baltic Sea, Northern Europe; Caspian Sea, West Asia; Chesapeake Bay, USA; North Carolina’s Albermarle-Pamlico Sound, USA; Florida Bay, USA; Swan River Estuary, Australia; Patos Lagoon, Brazil) (Paerl 2014). A worldwide overview on reports of cyanotoxins showed its presence in 14 countries (76 freshwater systems) in Africa, 15 countries (131 freshwater systems) in Asia, 106 freshwater ecosystems in Australia and New Zealand, 25 countries (273 freshwater systems) in Europe, 4 countries (204 freshwater systems) in North and Central America, and 6 countries (79 freshwater systems) in South America (Svirčev et al. 2019). This analysis was made based on the published literature, meaning that the problem can be largely underestimated as

blooms can remain largely unreported at this level. From an ecological perspective, CyanoHABs lead to the reduction of biodiversity, habitat deterioration or even production of toxins (de Figueiredo et al. 2004) as detailed in Figure II-1. Consequently, socio-economic impairment occurs, especially regarding affected drinking water reservoirs and recreational waters (WHO 2003), seed on the treatment demands (Westrick et al. 2010) (Figure II-1). While information on HABs prevalence and management costs is particularly difficult to find for example regarding Europe, data for USA can be found in scientific and official reports (Sanseverino et al. 2016). Costs associated to monitoring programs can be more easily assessed than the overall costs of blooms (these must account to the ecological and economic impacts of blooms, which are difficult to comprehensively measure), and are dependent on the monitoring strategy applied and also the area of the affected lake or reservoir (Hamilton et al. 2013). In large lakes like Taihu (2537 km^2) and Chaohu (775 km^2) in China, severe events of cyanoHABs imposed the closure of water treatment plants and caused losses of more than RMB 100 million in each lake (Hamilton et al. 2013). The costs associated to additional water treatment and to the service relocation to other water sources where safe use is ensured are also remarkable. These costs can range within $12 and $56 millions for dealing with a problem in a town of 100 000 people, in the USA (Naidenko et al. 2012). More data for USA can be found (e.g. Sanseverino et al. 2016), with Bingham and collaborators (2015) estimating $43 millions/year of losses for recreation and tourism, $18 million for property values and $4 millions for treatment of drinking water following cyanotoxin contamination in Ohio. An interesting study by Smith et al. (2019) in Canada demonstrated the long-term impacts of blooms. These authors pointed out an equivalent annual cost equal to $272 millions (2015 rates) over a period of 30 years, in a business-as-usual scenario. Commercial fisheries affected by problems of eutrophication could generate losses of £ 29-118 000 annually in the United Kingdom (Pretty et al. 2003). For a more complete compilation of economic data regarding the losses in sectors such human health, commercial fisheries, tourism and recreation, monitoring and management, we refer to the report on the topic to the European Commission by Sanseverino and co-workers (2016). Still, not many studies focus systematically on the effects of cyanoHABs regarding human health impairment due to the difficulty of establishing consequence links.

II-2.1. Cyanotoxins

The production of cyanotoxins is associated to specific genes that may or may not be present (Kurmayer and Christiansen 2009), and even if present, expression occurs depending on environmental conditions (Merel et al. 2013). Still, toxic blooms may occur frequently with potential health risks associated to direct contact, bioaccumulation of cyanotoxins through the food chain and/or through the inhalation of water particles due to crop-spray irrigation, and haemodialysis (WHO 2021). Cyanotoxins can act as neurotoxins (including neurodegenerative agents), hepatotoxins, cytotoxins, and dermatotoxins (Figure II-1). Occurrence of cyanotoxins and its association with cyanobacterial *taxa* can be pictured, as previously reviewed (Metcalf and Codd 2012; Sanseverino et al. 2016; WHO 2021), but expectable concentrations are difficult to ascertain due the scarcity of studies addressing the time and space variability of blooms’ toxin content, although this is critical information to conduct proper risk assessment and protective water management (WHO 2003).

Despite sub-lethal doses allow full recovery and no effects of chronic exposure are usually observed, neurotoxins can be lethal at high doses by causing asphyxia through paralysis of respiratory muscles (WHO 2003). Besides direct action as hepatotoxins, microcystins accumulate in mammals and fish, with consequent tissue and cell damage (Metcalf and Codd 2012). Microcystin toxicity is cumulative, and it can be lethal in vertebrates depending on the dose by liver necrosis, within hours to a few days following exposure (NHMRC 2008). Meanwhile, chronic exposure of humans to low microcystin levels in drinking water can promote cancer (de Figueiredo et al. 2004). In Caruaru (Brazil), numerous deaths occurred by exposure to microcystins in water used for the dialysis (Pouria et al. 1998). This is the most common toxin found worldwide (Srivastava et al. 2013), but cylindrospermopsin is also a concern. It was first discovered after a poisoning incident on Palm Island (Australia) in 1979, when 148 persons were hospitalized with hepatoenteritis due to contamination of a drinking water reservoir (Griffiths and Saker 2003). This toxin acts by blocking protein synthesis, potentially causing kidney and liver failure (Chorus et al. 2000). Besides acutely hepatotoxic, cylindrospermopsin is genotoxic and potentially carcinogenic (Messineo et al. 2010).

While cyanotoxins can entry in the human body *via* direct contact, swallowing or inhalation during recreational or occupational exposure to contaminated water, less common exposure routes are through renal dialysis, irrigation water used in crops and possible uptake

into the food chain and dietary supplements (WHO 1999).

II-3. Brief notes on the regulatory appraisal of cyanoHABs

The nuisance potential of cyanoHABs was ignored in socio-political arenas worldwide until the 1990s, when evidence of environmental and human health impairment by cyanobacteria and cyanotoxins became exposed. The United States Environmental Protection Agency (USEPA) included freshwater cyanobacteria and their toxins in the first Candidate Contaminant List in 1998, and since then, regulatory agencies worldwide started considering cyanotoxins as potential bioterrorism agents (OPCW 2005). In the European Union (EU), the main piece of legislation concerning water quality management is the Water Framework Directive (WFD - 2000/60/EC), establishing the concept of ecological water quality, while the specific Bathing Water Directive (BWD - 2006/7/EC) regulates the quality of recreational waters. Phytoplankton is a defined indicator of the ecological quality of water by the WFD; although this includes Cyanobacteria, no guidelines specifically targeting cyanotoxins are given. Regarding the BWD, HAB-causing organisms are neither assessed in practice nor given a relevant role in the qualitative classification ruling management actions, although cyanobacteria are specifically mentioned as part of the bathing water profile. Currently, a new Directive is under preparation, which will certainly follow the recommendations by the World Health Organization (WHO) on cyanobacteria. These recommendations include on-site public awareness on the risks in susceptible areas following on dedicated monitoring of cyanobacteria biovolume, chlorophyll-*a,* phycocyanin, water transparency or cyanotoxin concentration (WHO 2018). An important example of supportive WHO guidelines are those for safety levels in drinking and recreational waters recently published (WHO 2021) building up from the previous equivalent (WHO 1999). This document suggests several guideline values for several cyanotoxins. The guidelines are based on animal studies, except for saxitoxins due to the rapid onset of highly specific symptoms caused by the consumption of contaminated seafood. The provisional lifetime drinking-water guideline values are around 1 $\mu g.L^{-1}$ for microcystin-LR and 0.7 $\mu g.L^{-1}$ for cylindrospermopsin, while the provisional short-term drinking water guideline values are close to 12 $\mu g.L^{-1}$ for microcystin-LR, 3 $\mu g.L^{-1}$ for cylindrospermopsin and 30 $\mu g.L^{-1}$ for anatoxin-a, with an acute drinking water guideline value given also for saxitoxins (3 $\mu g.L^{-1}$). The provisional recreational water guideline

values are of 24 µg.L^{-1} for microcystin-LR, 6 µg.L^{-1} for cylindrospermopsin and 60 µg.L^{-1} for anatoxin-a, while there is a recreational water guideline value of 30 µg.L^{-1} for saxitoxins. Besides providing these guidelines, the WHO also suggests an Alert Level Framework, with specific guidelines and steps for both drinking and recreational waters. As each level is met, the WHO suggests specific management measures ranging from the triggering of closer surveillance to the use an alternative water supply or an effective water treatment (in the case of drinking water), as well as bathing restriction (in the case of recreational waters) (WHO 2021). In parallel to the WHO protocol, several countries established their guidelines focusing on cyanotoxin concentration reviewed by Chorus et al. (WHO 2021). Recently, the European Union released a new Directive on the quality of water for human consumption (EU Directive 2020/2184) that should be transposed to the national legislation of the Member States by 12 January 2023, and microcystin-LR was added to the list of compounds of mandatory monitoring by 12 January 2026 (Part B of Annex 1). The same guideline as used by the WHO (1 µg.L^{-1} for microcystin-LR) is proposed as a safety benchmark, but this toxin only needs to be measured when there is a risk of blooms occurrence in the water sources.

II-4. Current management strategies targeting cyanoHABs

Risk management programs are dependent on the political, social and economic context, as well as on the scientific data available in each region (WHO 1999). Despite the inexistence of a “rule of thumb” to deal with cyanobacteria, Alert Level frameworks are a common management instrument, applicable in drinking water facilities (WHO 1999) and water bodies used recreationally (e.g. Macário et al. 2017). Management can be done by taking preventive measures, to reduce nutrient inputs in the water, or control measures, to remove cyanobacteria or their toxins from affected waters.

II-4.1. Common preventive measures targeting cyanoHABs

The application of preventive measures targeting cyanoHABs requires the identification and limitation of human- or animal-driven contaminants’ input into waterbodies. As management at the source level is expensive and time-consuming (Jančula and MarŠálek 2011), and given that natural feedback mechanisms in aquatic ecosystems stem from the benthic compartment (Merel et al. 2013), there is a delay (that can be of several

years) between implementation and the significant dropping of nutrient concentrations below levels expected to sustain an algal bloom (Merel et al. 2013). Alternative prevention measures have been deemed effective in the short-term, which can be of physical, chemical or biological nature (Table II-1) as follows.

The disruption of natural water stratification is meant to favour the access to light of non-buoyant green algae and diatoms (Visser et al. 2016a). Bubble plume aerators and mechanical mixers are the most common equipment for artificial de-stratification, its efficiency being dependent on the interplay of several operational conditions (Newcombe 2009; Visser et al. 2016a). Such systems were already used successfully to depress cyanobacteria, favouring diatoms and chlorophytes in the Bleiloch Reservoir, Germany (Becker et al. 2006), but validation is still scarce to assume their broad efficiency (Chen et al. 2018) and evidence actually exists that the technique can promote rather than control some cyanobacteria species (Nürnberg et al. 2003). Despite the increasing interest and use of these techniques, the information is still scarce to assume their efficiency as a tool applicable to different types of waterbodies and to different types of phytoplankton blooms (Chen et al. 2018). These methods are based mostly in responses of different phytoplankton groups at a laboratory scale and not through experiments using model ecosystems (Lofton et al. 2019). The costs associated to water destratification are variable according to the situation and size of the reservoir, the reference for large systems being within the hundred thousand dollars range (Steffensen 2008). For example, after a damaging bloom of *Planktothrix rubescens* with high microcystin concentrations in Lake Boerhringen (0.735 km^2), in Germany, an artificial destratification device was installed with associated costs of EU 80 000 plus EU 3000 annually for installation, coupled with EU 10 000 annually for monitoring (Hamilton et al. 2013). Water-level fluctuations can be applied to disrupt water stratification and managing water retention times every few days for lake-restoration, by controlling the magnitude and timing of the discharge. Reducing retention time can successfully reduce cyanobacteria provided that dilution rates are higher than cyanobacteria growth rates (WHO 2021). This technique can be applied in reservoirs, rivers (Bormans and Webster 1997), and lakes (Coops and Hosper 2002) where it is possible to control the magnitude and timing of the discharge. The removal of sediments to limit the release of nutrients to the water column is another option, but very labour intensive, typically with short-term implications in water quality and questionable ecosystem quality improvement

(Paerl et al. 2016). Draglines, dry mechanical removal, or hydraulic dredging are techniques available for sediment removal, but this is not a frequently used prevention strategy towards cyanoHABs; indeed, the last known (i.e. reported in accessible literature) action of this type was undertaken long time ago for the restoration of Lake Springfield, Illinois, USA (Buckler et al. 1988). In this case, the unit cost was $ 3 *per* cubic yard to dredge the sediment from the lake. Among the additional costs, $ 46 000 annually were spent for the treatment of the high turbidity levels at the city filtration plant, caused by the continued sediment transport. Hypolimnetic oxygenation decreases the release of nutrients from the sediments without disrupting the stratification of the water body, but it is expensive and requires deep understanding of hydro- and nutrient dynamics, which is often difficult to achieve (Newcombe 2009). For the purpose, airlift pumps, side stream oxygenation and direct oxygen injection can be used (Beutel and Horne 1999). Phosphorus (phosphate) precipitation (Cairns et al. 2013; Nakamura 2019; Nie et al. 2019) followed with treating the sediment by capping with different agents (Newcombe 2009) can also apply to chemically prevent cyanoHABs, while inefficacy has been noted in some systems (Paerl et al. 2016).

II-4.2. Common control measures targeting cyanoHABs

Chemical control approaches can be very efficient in the rapid deterioration of existing cyanoHABs (e.g. Matthijs et al. 2012), most commonly by the application of coagulants and algaecides (Table II-1). Coagulants, such as aluminium and ferric salts (Ho et al. 2012) deposit cyanobacteria cells preventing access to light, the deposits being then removed mechanically. However, they can in parallel disrupt cell walls and membranes, with the consequent release of cyanotoxins, and residues can easily exceed water quality standards. In this way, the use of coagulants should be coupled with methods for the removal of the deposited mass from the water body, hence preventing the release of toxins to the water column (Newcombe 2009) and eliminating the threat posed by the burdening of the system with potential pollutants.

New coagulants are being developed with better performance regarding cell lysis (Wang et al. 2018) and improved environmental safety (Ahmad et al. 2011; Wang et al. 2013), although with some recognised constraints. Synthetic algaecides (House and Burch 2002; Jančula and MarŠálek 2011; Matthijs et al. 2012) are applied to cause cell lysis, being mostly used in early stages of a bloom to minimize toxin release (Newcombe 2009).

Commonly used algaecides are chemicals based on metal elements such as aluminium, iron, copper, and calcium (Jančula and MarŠálek 2011). Their high and unspecific toxicity ranges are a problem (Jančula and MarŠálek 2011), and alternative promising bio-based substances (Lin et al. 2019) have been studied. Although bearing lower toxicity to other phytoplankton groups and higher biodegradability, these substances are typically less effective and have higher production costs. As for coagulants, parallel measures must be coupled to the use of algaecides, to safely deal with potentially released cell metabolites. Ideally, after a treatment with an algaecide, the treated waterbody should be isolated for a period of time in order to allow the degradation of the toxins and odours (Newcombe 2009). Additionally, it is worth remarking that, although chemicals like coagulants or algaecides can reduce the proliferation of cyanobacteria via decreasing the nutrient concentration or growth inhibition, these generally have poor specificity and can have negative impacts in non-target species sharing requirements or uptake/exposure routes, thus causing secondary pollution of the treated aquatic systems (Jančula and MarŠálek 2011; Huh and Ahn 2017). Also, due to biotic or abiotic degradation processes, many of these treatments should be applied repeatedly, which renders this strategy very costly (Paerl et al. 2016). For example, treating reservoirs ranging from 1300 to 26 000 ML can cost $ 20 000 to $ 50 000 in South Australia. Estimations suggest $ 1 million cost *per* year for the use of algaecides and the correspondent disposal of the algaecide contaminated water sludge (Steffensen 2008).

Biological strategies emerged as alternatives to chemical dosage (Table II-1). These are based on biomanipulation techniques over the trophic structure of the system. Top-down strategies (e.g. favouring grazing by reducing zooplanktivory) fail to control blooms of inedible species (e.g. filamentous morphotypes) (Gragnani et al. 1999). In cases where a bloom composed mainly by inedible species occurs (e.g. filamentous cyanobacteria), grazing by zooplankton will be focused on algae rather than cyanobacteria, ultimately leading to the increase of cyanobacteria due to the operated reduction of competition (Gragnani et al. 1999). Bottom-up control is based on the increase of competition for nutrients limiting the cyanobacterial growth e.g. by manipulating macrophyte density to promote competitive nutrient removal from water (Demeke 2016) and to favour the release of allelopathic compounds to cyanobacteria (Mohamed 2017).

Table II-1: Non-exhaustive compilation of current and proposed methods to prevent and control cyanoHABs. Physical (Phys.), chemical (Chem.) and biological (Biol.) methods are covered and examples of entailed agents are given. The operational constraints (or requirements) to successfully apply each method, as well as advantageous or disadvantageous aspects (relative among different alternatives within each type of method), were interpreted from the literature and are more limited for strategies that are still at very early development stages.

	Technique	Agents/Equipment	Operational constraints	Advantages	Disadvantages
			Prevention		
Phys.	Destratification	Bubble plume aerators Mechanical mixers	Depth; air flow rates or intensity/duration of treatment; degree of stratification	Do not use noxious chemicals	Results are dependent on the structure of the phytoplankton and even the cyanobacteria community
	Water level fluctuations	Outlets	Manipulation of high volumes of water	Successfully applied for lake restoration. Relevant nutrient decrease by dilution	Affects the whole hydrodynamics, and potentially the non-target biota
	Sediment removal	Draglines; dry mechanical removal; hydraulic dredging	Very labour intensive and expensive	Limits sediment sourcing of nutrients	Lack of specificity; may severely affect the benthos
Chem.	Hypolimnetic oxygenation	Airlift pumps Side stream oxygenation Direct oxygen injection	Expensive. Deep knowledge of hydrodynamics, nutrient release and external loads	Improves quality of habitats; limits sediment sourcing of nutrients and noxious compounds	Maintenance of thermal stratification, potentially benefiting cyanobacteria
	P precipitation coupled with sediment capping	Lime, $CaCO_3$, Ca_2O_4Si, $CaCl_2$ Insoluble iron compounds, zeolites, bauxite, clay, calcite, Phoslock™	Expensive Requires repeated treatment	Effectively reduces phosphate levels and prevents (re)mobilization to the water column	Potentially with severe effects on the benthic biota
			Control		
Chem.	Traditional coagulants	Aluminium salts Ferric salts	Expensive by the need of multiple treatment and removal of deposited residues	Fast action and effectiveness	Inherent cell lysis. Low specificity - impairs non-target species. Polluting residues. Ti xerogels are more expensive and not comprehensively known yet
	Innovative coagulants	Titanium xerogel		Improved efficacy and easier removal; targets also cyanotoxins	
		Clays, Chitosan, Tannins	Expensive by the need of multiple treatment	Lower environmental toxicity and better biodegradability	Relatively new, thus with inconsistent evidence on efficiency

	Algaecides	Metallic salts (e.g. $CuSO_4$) Photosensitizers (e.g. H_2O_2, phtalocyanines, TiO_2) Triazine herbicides	Used in early bloom stages. Expensive due to the need of multiple treatment. Post-treatment isolation required	Fast action and effectiveness	Possible cell lysis. Low specificity - impairs non-target species
	Biological chemicals	Polyphenols, nonanoic acids Sanguinarine	Dosing as plant extracts or as purified biochemicals	Less toxic in general and more easily biodegradable	Less efficient. Their modes of action are still not fully known
Biol.	Biomanipulation	Increase grazing by decreasing zooplanktivory	Depends on the prevalence of edible cyanobacteria species	Environmentally friendlier	Highly complex and with inconsistent efficiency evidence
		Macrophytes	Inefficient in eutrophic waters		
	Biological control agents	Cyanophages Fungi (parasitism)	--	High cyanobacteria specificity	Easy development of resistances (cyanophages and fungi) or are unspecific. Difficult to isolate and culture at large scale
		Bacteria (Extracellular metabolites) Protozoans (predation)		--	
Phys.	Biomass removal	Oil-spill skimmers Pumps	Application at late bloom stages (scum). Expensive	Does not use noxious chemicals	Requires further investment to treat removed biomass

Although some reports of success exist in the literature (e.g. Wang et al. 2012; Amorim et al. 2019), this strategy may have limited efficiency in eutrophic lakes or reservoirs with high phosphorus concentration (Demeke 2016). Regardless the typology, biological control methods are invariably complex since they use living organisms, but they also normally integrate different aspects: food web manipulation for the control of cyanoHABs can be based in one or more ecophysiological features of aquatic ecosystems, such as grazing, predation, parasitism or the release of metabolites (Demeke 2016). Other organisms like viruses (cyanophages), bacteria (through photosynthetic inhibition promoted by extracellular lytic substances; Lin et al. 2008), parasitic fungi and predatory protozoa, have been equated (Demeke 2016), but also bearing significant shortcomings (Cannon et al. 2008; Gerphagnon et al. 2017). Cyanophages are extremely difficult to isolate and/or culture, and cyanobacteria can easily develop resistance to them (Cannon et al. 2008) while there is generally a high specificity of the cyanophage to particular cyanobacterial strains (Waterbury and Valois 1993). Parasitism of cyanobacteria by fungi is also possible, but control strategies based on such biotic interaction are very difficult to upscale (Gerphagnon et al. 2017). Also, although there are reports on predation over cyanobacteria by several protozoans (Dryden and Wright 1987), the suitability of such a strategy can be questioned considering that many cyanobacterial species form colonies preventing that protozoans graze them (Demeke 2016).

Oil-spill skimmers and pumps were successfully applied for the physical control of cyanoHABs (Table II-1), especially at scum stages, combined or not with coagulation (Atkins et al. 2001; Kozacek 2014). A successful case where an oil-spill skimmer was used following coagulation with polyaluminium chloride was reported by Atkins et al. (2001), regarding a *Microcystis aeruginosa* bloom in the Swan River, Australia. A different system for physical control is the use of pumps to remove dense cyanobacterial blooms. This system was used in 2007 in Taihu Lake, China, in a bloom that left more than 2 million people with no potable water (Kozacek 2014), and vessels have been developed for the removal of cyanobacteria from water through filtration (e.g. ASIO vessel; www.asio.cs, assessed on April 1st 2021). A type of oil skimmer was also used in Southern Florida in 2018 after a severe bloom, with a $ 700 000 grant from the Florida Harmful Algal Bloom Management Grant Program being contracted to the company AECOM for solving the problem (Harpster 2018; Levi 2018). Still, examples of this type of physical removal as a cyanoHABs control

strategy are very scarce. A potential problem of such strategies is the retainment of non-target organisms along with the collected cyanobacteria, especially in the first stages of the bloom, which are not the stages where mechanical removal is typically equated. These control strategies are preferably applied in advanced stages of the bloom, when the cells accumulate mostly on the surface; under such circumstances, the risk of collecting non-target organisms dramatically decreases. Although physical removal may overcome the problem of cell lysis, this strategy does not seem to collect the preference of managing entities, possibly because of added costs of operation, i.e. those related to biomass removal and transport for landfilling or wastewater treatment plants (Atkins et al. 2001). Given the advantages of physical removal over other control treatments, which are inefficient regarding contamination with cyanotoxins and/or burden ecosystems with hazardous chemicals, the search for solutions to improve the economic sustainability of physical removal is a worthwhile research arena, potentially with important impacts on the successful management of cyanoHABs under a circular economy rationale. In practice, the economic income driven by the valorization of cyanobacteria biomass should easily cover the costs of physical control methods relying on the removal of cyanoHABs and eliminating or greatly reducing waste generation.

II-5. Cyanobacterial biomass valorization

Climate change will increase the pre-existent risks and create new risks in several environmental arenas, including those imposed by cyanoHABs. Thus, the adaptation or development of solutions contributing to reduce the risk vulnerability and exposure is needed (IPCC 2014b). The relevant drawbacks of current control strategies targeting cyanoHABs prevent guarantying the nullification of the threat posed by cyanotoxins or withstanding economic sustainability levels. However, the “dark side” of cyanobacteria can be compensated by exploring their “bright side”, integrating cyanoHABs management by physical removal with their biotechnological valorization. Firstly microalgae and, recently, cyanobacteria have been considered amongst the most promising feedstock for biofuels and biochemicals (Wijffels et al. 2013). However, only 10 % of the species were partially or totally characterized in this context and only a few are industrially cultured (Raheem et al. 2018), suggesting high losses regarding the exploitation of their biotechnological potential. Besides, cyanobacteria are biochemically rich in antibacterial, antifungal, antiviral, and

antitumor compounds, some like polyunsaturated fatty acids and phycobiliproteins already displaying high commercial value (Eriksen 2008; Sekar and Chandramohan 2008).

II-5.1. High market value compounds produced by cyanobacteria

Elucidating on the broadness of application possibilities, Burja et al. (2001) analysed 424 marine cyanobacteria compounds and found that around 40 % were lipopeptides, including compounds with cytotoxic, antitumor, antiviral, antibiotic, antimalarial, and antimycotic activity, as well as multi-drug resistance reversers, antifeedants, herbicides and immunosuppressive agents. Figure II-2 synthetises the most valuable cyanobacteria products, along with their bioactivity and application ranges. Carotenoids are light-harvesting pigments that are also protective for excessive solar radiation (Koller et al. 2014). These are powerful antioxidants and can be used as colorants, applied as food additives, in the cosmetics industry and in aquaculture (Olaizola 2000; Cardozo et al. 2007). Phycobiliproteins are water-soluble fluorescent pigment-protein complexes acting as secondary light-harvesting components in the photosynthesis (Pandey et al. 2013). The primary application of these molecules is as natural dyes, although with potential for the pharmaceutical sector (Spolaore et al. 2006) considering their bioactivity (Sekar and Chandramohan 2008). Chlorophylls, the primary photosynthetic pigments, have been used as food colorants (Hosikian et al. 2010) and more recently argued as cancer preventive agents (Díaz et al. 2003). Although their potential, pigments exploitation is still in its infancy due to low productivities and high recovery costs (Koller et al. 2014).

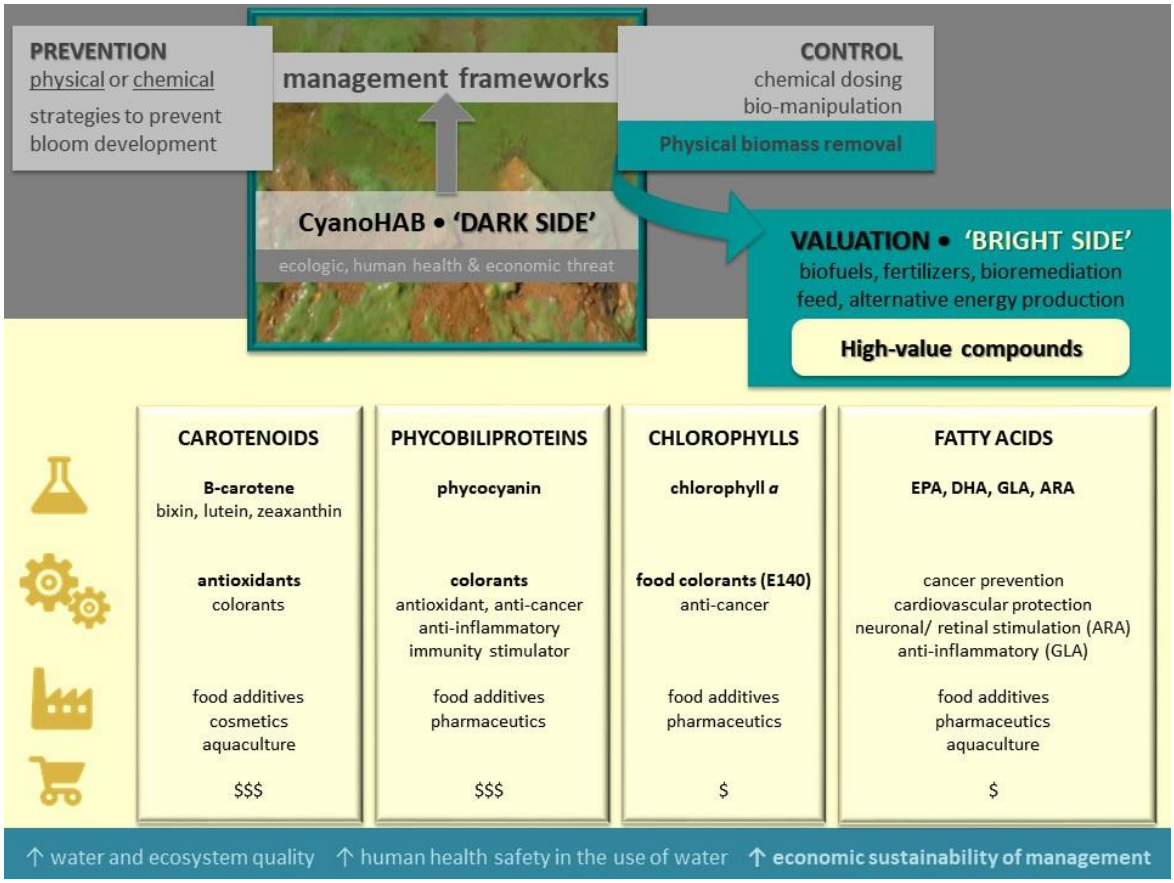

Figure II-2. Infographic overview of the proposed strategy for an improvement of management frameworks targeting cyanoHABs, which are growing driven by climate change. The central pillar of the proposal is the valorization of cyanobacteria biomass following control by physical removal, especially regarding the efficient recovery of compounds, along with their bioactivity, application sector and market value being indicated. High-value compounds that were not yet recognised for market size/value estimation were not included and are rather discussed in the text. Icons were draw in the free version of Iconion 2.7.

Still, pigments are the cyanobacteria products with the highest valorization potential, particularly carotenoids and phycobiliproteins, the market value depending on the location of production, the current marketing situations, and the product purity (Koller et al. 2014). According to authoritative platforms (https://www.gminsights.com, assessed on January 17th 2021, for market size and projections), the carotenoids market size reached more than USD 200 million in 2015 and is likely to exceed USD 300 million by 2024, with lutein reaching USD 40 million in 2015. Lutein can be sold in capsules of 18 mg (EU 27.78 for 30 capsules), to improve eye function and prevent macular degeneration (www.nutribio.pt; assessed on

March 24th 2021). Phycocyanin worth over USD 18.5 million in 2018 and the industry expects a consumption higher than 200 tons by 2025. One of the related products in the market is the Super Bluecell, from Vegafarma (EU 51.25 for 30 capsules). These capsules use phycocyanin from *Arthrospira* sp. (previously classified as *Spirulina* sp.) and are claimed to be relevant to prevent diseases and aging, protect the organism and prevent secondary effects of chemotherapy or radiotherapy (www.nutribio.pt; assessed on March 24th 2021). There is no specific information regarding chlorophylls, but the natural food colorants market, where these pigments are dominant, is projected to surpass USD 4.7 billion by 2024. The majority of chlorophyll sold in the market is presented as a liquid and the pigment is commonly extracted from plants like alfafa. Liquid chlorophyll can be dissolved in any drink, arguably to improve health by helping regenerating blood quality and detoxifying. It can be sold by EU 15.95 for 100 mL (www.amazon.de; assessed on March 24th 2021). The market for lipids (e.g. Omega 3) that can be sourced by cyanobacteria is smaller but still exceeding USD 2.3 billion in 2019 and estimated to reach USD 3.8 billion in 2026. An example of a product that is already in the market is ALGAE DHA, by Nordic Naturals. This Omega 3 supplement extracted from the microalgae *Schizochytrium* sp. is argued to support optimal brain, eye, and nervous system function - 60 capsules of 500 mg can cost EU 25.75 (www.iherb.com; assessed on March 24th 2021). This valorization route for cyanobacteria lipids is nevertheless apart from their appreciation to produce 3rd and 4th generation biofuel (Raheem et al. 2018). Indeed, many cyanobacteria polyunsaturated fatty acids such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), γ-Linolenic acid (GLA) and arachidonic acid (ARA), all having key roles in human metabolic and physiological processes (Karmali 1996; Horimoto and Ogawa 1997; Fan and Chapkin 1998; Holub 2009), can be used for nutraceutical, pharmaceutical and therapeutic applications (Figure II-2; FAO/WHO 1993; Ytrestøyl et al. 2015; Echeverría et al. 2017), such applications representing a much higher economic income than biofuels (Koller et al. 2014).

Besides the above applications entailing an already established market, cyanobacteria are also valuable for their polysaccharides (extracellular polymeric substances). These compounds can be used as bioflocculants by the water treatment industry (Tiwari et al. 2015); emulsifiers, stabilizers or thickening agents (De Philippis and Vincenzini 1998) in the food industry; bioactive substances, e.g. sulphated polysaccharides that can inhibit tumour invasion and metastasis (Hosikian et al. 2010); and bioremediation

agents due to their metal binding capacity (Shah et al. 2000). Cyanobacteria can also produce poly(hydroxyalkanoates) (Bhati et al. 2010), defined as potential substitutes to conventional plastic (Koller et al. 2011). These polyesters have similar applications as polypropylene, but are biodegradable, and related manipulation technologies are already widespread at the industrial scale (Gradíssimo et al. 2020). These compounds were already described in several cyanobacterial species (Bhati et al. 2010), and since cyanobacteria have smaller nutritional needs compared to heterotrophic bacteria, their use as industrial PHA producers is more appealing considering that the costs associated to the microbiological production of the latter are a constraint (Gradíssimo et al. 2020). Specific high-value metabolites have been extracted from cyanobacteria, with the most remarkable cases being (i) cyanovirin-N (CV-N), a protein produced by *Nostoc ellipsosporum* able to inactivate some primary strains of HIV-1; (ii) borophycin produced by *Nostoc* species with cytotoxicity against human epidermoid carcinoma and human colorectal adenocarcinoma cells; and (iii) cryptophycin, also isolated from *Nostoc* strains with fungicide activity and cytotoxic action against human tumour cells (Burja et al. 2001). The production of secondary metabolites is generally induced by stress conditions (Kultschar and Llewellyn 2018), which can meet environmental challenges such as increased salinity, drought or temperature consequent to global climate change trends.

Apart from extracted metabolites, whole cyanobacteria have been proven to be competent in many other fields. They are apparently good bioremediation agents for metal contamination (Fawzy and Mohamed 2017), can be used successfully as fertilizers (Grzesik and Kalaji 2017), as feed supplements in aquaculture or as hosts for the synthesis of nanoparticles (e.g. Lengke et al. 2007). The third-generation biofuels such as biohydrogen, biomethane, bioethanol and biodiesel, can be produced using cyanobacteria (Raheem et al. 2018) and their potential for integration in Microbial Fuel Cells for electricity production has been investigated (Zhao et al. 2012). Although the actual value of these applications is still largely unquantified, the markets involved represent a significant economic potential, more even considering that cyanobacteria do not compete with plants for (scarce) fertile soil.

II-6. Improving cyanobacteria exploitation: economic and sustainability aspects

The market of valuable products retrieved from cyanobacteria is increasing. This is being reflected in the increased number of industries producing and exploring this biomass,

as well as in research efforts (indicatively, a search in ISI® WoS by March 2021 using the terms ‘cyanobacteria’ and ‘biotechnology’ retrieved a total of 387 hits, 16% published from 2020 onwards for the Core Collection database, or a total of 2626 hits, 5% published from 2020 onwards when retrieving from all databases) and in the high pace of discovery of novel applications, as highlighted previously. Some examples of companies spread around the world working in this market are Transalgae (Israel), AlgaEnergy (Spain), Algae Systems (USA), Algenol (USA), IHI NeoG Algae (Japan), Pond Tech (Canada), Necton (Portugal), Cyanotech (Hawaii), Taiwan Chlorella (China). Multinational companies are also investing in this context, some heavily such as ExxonMobil in the field of fuel production (see the onset of collaborative efforts with research institutes in e.g. Vasudevan et al. (2012) and in the company’s dedicated website).

The use of natural biomass, especially nuisance biomass, to retrieve benefits while concomitantly offsetting environmental problems, is a smart innovation concept that allows building a win-win relationship between environmental and economic sustainability. These ‘mutualistic’ relationships are a key solution especially in a future where global climate change is evident and nuisances such as cyanobacteria are expected to grow worldwide. Realistically, regardless how exciting are academic exercises exploring the potential of natural products or supporting the need of improving environmental management to protect ecosystems and ensure the quality of natural resources, economic variables will always be of paramount importance in defining the success of any newly proposed solution. In this field, strategies that allow an integration of cyanobacteria biomass valorization in nuisance management frameworks towards environmental restoration and human-health protection (see Figure II-2 for an overview of this suggested approach) are definitively worth of further attention.

As previously detailed, control strategies based on the removal of cyanoHABs biomass are currently not particularly suitable, and we believe that this can be because they are not sufficiently explored. Our reasoning is essentially based on the valorization of removed biomass to cover the costs of operation, and depending on the design of valorization routes, the income may additionally compensate to a certain extent for the overall damage caused by the cyanoHABs (i.e. economic costs of ecological and public health threats). This perspective is illustrated in Figure II-2 as the change in focusing the “bright side” of cyanobacteria instead of their “dark side”. Such an add-on to nuisance management

frameworks was already suggested e.g. for the invasive macrofouling bivalve *Corbicula fluminea* by Rosa et al. (2014) and Domingues et al. (2020). An important remark stressed in these works where profitable features of invasive species are exposed regards the need to absolutely prevent further dispersion, while applying the proposed strategies to avoid the scale-up of noxious environmental and economic impacts. This principle should be extended to the valorization of cyanoHABs. Our proposal stems from the idea that cyanoHABs have potential to be valued, but it is strict in the sense that this should be done when blooms cannot be prevented and are already established. Moreover, its most prominent perspective is the promotion of the economic sustainability of blooms control and the concomitant stimulation of water quality improvement, which immediately tone down the relevance of business-based approaches requiring, for example, spatial and temporal stability of the biomass source; on the other hand, the framework is not preventive of an association between environmental management entities interested mostly in the control of the nuisance and business-driven sectors, depending on local regulation on the exploitation of natural resources, the availability of the biomass and the relationships allowed between the sectors.

Ideally, a process where multiple valuable products can be retrieved from the same biomass, as suitable in biorefinery processes, should be considered since this allows to magnify the incomes (Chisti 2007; Raheem et al. 2018). As suggested by Chisti (2007) for microalgae, cyanobacteria biomass can be used to concomitantly produce biodiesel, animal feed and biogas, the costs of feedstock production being reduced by natural availability, which contributes further to the efficiency of the valorization routes. Less positive aspects of the valorization of cyanoHABs within a biorefinery framework are two-fold. On the one hand, blooms are seasonal in temperate areas (note that the magnitude of this problem severely decreases in tropical and sub-tropical areas), but climate change (mostly rising temperatures and altered hydrologic patterns) is changing blooms occurrence (Paerl and Huisman 2009; Paerl and Paul 2012; Visser et al. 2016b), which translates in the extension of the periods through each year when this biomass is available. Indeed, related changes in the phytoplankton community composition have been observed (Visser et al. 2016b), as well as the geographical expansion of cyanobacteria (Paerl and Huisman 2009). Still, although cheap, cyanoHABs biomass is spatially and temporally variable, so as it is its quality and, consequently, the yields regarding different valuable products. A way to overcome both shortcomings is the establishment of culturing facilities that can be activated when natural

feedstocks cannot be collected whenever business-driven approaches are to be linked to nuisance management frameworks. The culturing of cyanobacteria/microalgae at the industrial scale is viable (see the example of Solazyme, Inc., San Francisco), and has potential for improvement, for example, through genetic engineering of the cultured strains (Wijffels et al. 2013) or by the implementation of integrated culturing systems that allow reducing the costs associated to the creation of culturing facilities. These systems can use wastewater treatment facilities, rich in nutrients, to produce algal biomass while retrieve part of the organic load from waters. Systems like this, transform organic waste in algal biomass, closing the cycle - see e.g. Rose et al. (2007) or Nagy et al. (2018) for the context, structure and possibilities of integrated algal ponding systems. These systems not only contribute generally to a sustainable development but also to improve the sanitation conditions, allowing waste recovery, recycle and reuse, enabling societies to live from its nature income rather than consuming its capital (Rose et al. 2007).

II-6.1. Rendering the concept concrete from collection to exploitation – a reasoned exercise

Realistically, economic dynamics cannot completely dissociate from environmental protection, and this in an important trigger to develop sustainable solutions for environmental problems. This concept of blooms valorization does not entail a traditional or even a straightforward approach, but it has the potential to effectively build a bridge to bring economical profit to the side of ecosystems conservation, hence favouring environmental protection and restoration. In the present section, we propose a structured theoretical framework to build this bridge, rendering the overall approach economically and environmentally productive, and thus sustainable. This framework is proposed and inspired by the circular economy philosophy, being composed of four sequential, internally flexible steps (Figure II-3): step 1 would comprise collection, transport and eventual storage of the blooms intended for valorization; step 2 would include biomass characterization; step 3 would comprise the selling of the biomass to extraction facilities; and step 4 would entail the application of the potential profit or Return (R) retrieved from the selling of the biomass.

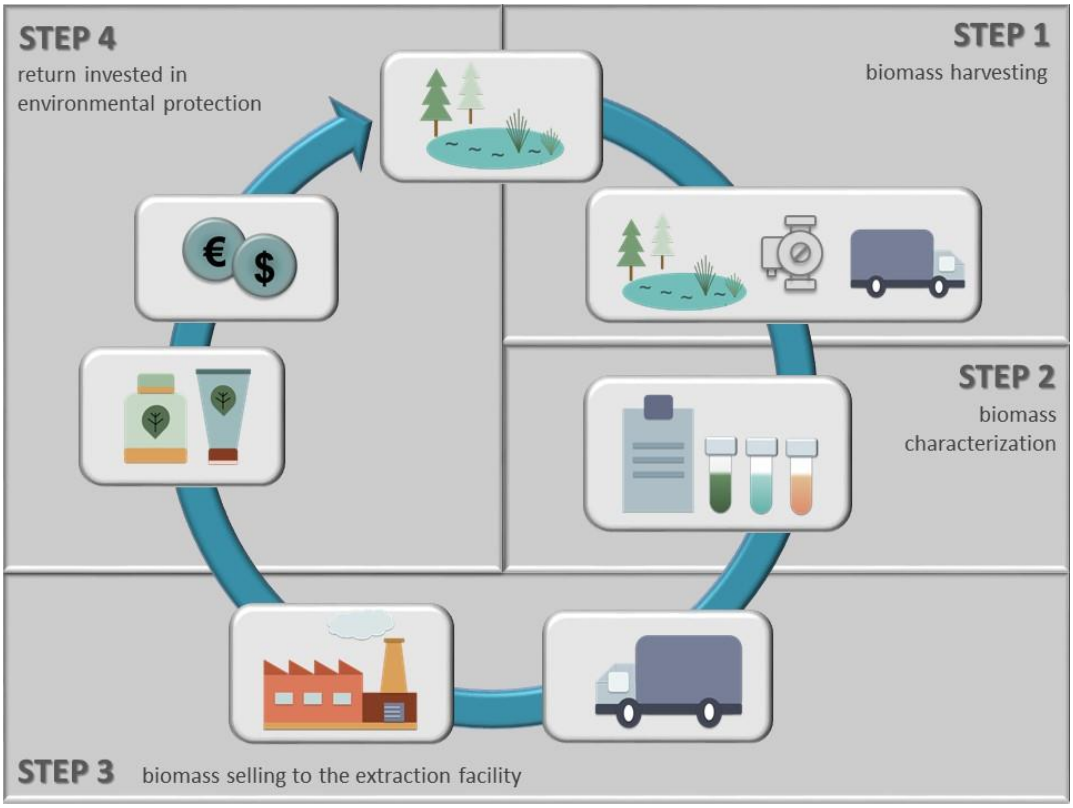

Figure II-3. Schematic illustration of the proposed concept of integrating the biotechnological exploitation (‘bright side’) of cyanobacteria natural blooms to improve the efficiency of management frameworks, as inspired by the circular economy philosophy. The scheme includes four sequential, internally flexible steps.

The sequence towards the sustainable valorization of cyanobacterial blooms obviously starts with the identification of an affected waterbody. Cyanobacterial biomass can be extracted (i.e. removed) by suction of water with circulator pumps coupled with sieves so that the retained cyanobacteria can be easily collected into a container in a truck (Step 1). Systems for extraction of blooms and concentration of the biomass are increasingly available, as illustrated by e.g. patent CN101602533B or the work of companies like AECOM (Levi 2018), or ASIO (www.asio.cz, assessed on April 1st 2021). The cells retained in the sieves can be scraped into an appropriate container for transport. The type of pump or oil-spill skimmer used for collecting the cyanobacteria should be carefully optimised as it constitutes an important part, if not the most significant part of the costs associated to the proposed valorization strategy. Furthermore, depending on the stage of the bloom being collected, sieving optimisation can be required. At an advanced stage (scum or nearly-scum

phase), cyanobacteria accumulate mostly in the surface, and pumping only from surface water layers prevents the capture of non-target organisms. When this is not the case, the sieving system requires optimisation, for example by assembling sequences of sieves of decreasing mesh size. While cyanobacteria should be retained by the finer sieve(s), the accumulated biomass being easily scrapped into the transport container, non-target organisms retained in larger upstream sieves can be easily returned to the ecosystem by back flushing. The cyanobacteria biomass should be then transported into a processing facility where it can be stored. The storage process (e.g. refrigerated chamber) prevents the degradation of the biomass and the production of bad odours, until further biomass characterization, i.e. toxin analysis (Step 2).

Step 2 is a critical step because the presence of toxins will determine the fate of the biomass. Safety measures are important when handling potentially toxic cyanobacteria. Protective equipment ranges from standard laboratory coats, gloves and safety glasses to breathing masks if there is a risk of inhalation (WHO 2021), although this equipment does not largely differ from basic safety equipment recommended for handling natural samples from any aquatic ecosystem that is not comprehensively known by operators. Naturally, the products extracted from a natural bloom may not be the same as the ones extracted from pure cultures of cyanobacteria. Compounds with high purity requirements such as those intended for food or pharmaceutical applications cannot be the primary targets of blooms collected *in natura*. However, there are suitable and economically attractive applications for such a raw biomass, including biofuels, fertilizers, but also the extraction of less purity-demanding compounds and even toxins. If toxins are present, the biomass should be treated carefully, and its destination could be the extraction of the toxins itself or the production of biofuels. These cyanotoxins can be used as biocides due to their allelopathic character, with application in algaecides, herbicides and insecticides (Berry 2008; Haque et al. 2017); high valorization potential can be illustrated by e.g. the selling of microcystin-LF (6 - 9 $\mu g.mL^{-1}$) by € $514.mL^{-1}$ (www.sigmaaldrich.com; assessed on April 1st 2021). It is worth noting in this context that there is at least one example of the successful separation of microcystins from phycocyanin using a natural bloom as a source of biomass (Shen et al. 2020), which reinforces the suitability of extracting valuable compounds even when toxins contaminate the raw biomass. If the biomass is potentially toxin-free, other uses can be immediately equated (see section II-5 for an overview) complying with the applicable regulation within

each market envisioned. Therefore, toxin screening is critical, and can be made through several approaches. Molecular approaches based on the identification of genes encoding for cyanobacterial toxins can play an important role as they are simple, rapid, cost effective, sensitive and specific, allowing the simultaneous analysis of several target gene products (Pearson and Neilan 2008). The presence of these genes can be easily assessed by PCR (Polymerase Chain Reaction) using primers targeting regions of the operons involved in the synthesis of microcystins (Tillett et al. 2001), cylindrospermopsin (Schembri et al. 2001), saxitoxins (Al-Tebrineh et al. 2010), anatoxins (Wood et al. 2010), and nodularins (Koskenniemi et al. 2007). However, the presence of target genes does not necessarily mean that they are transcribed and that transcripts are actually expressed for the actual toxins production by the cells. Still, this screening stage is relevant: if samples do not bear genes responsible for toxins production, then there is no need to invest in further stages (some more expensive) to confirm the presence of toxins in the sample; depending on the target genes present, then a selection of the downstream methods for the toxin quantification can be made, with the logical cost-efficiency gains. In this context, methods like the Enzyme Linked Immune Substrate Assays (ELISA) provide the concentration of a particular toxin in a tested sample. This is the most common biochemical technique for cyanotoxins screening and it is based on the coating of well plates or test tubes with toxin antibodies (Newcombe 2009). It is a relatively inexpensive, simple, fast, sensitive, specific and easy technique (WHO 1999). However, it also presents some limitations, as it does not distinguish toxin variants. Although this can be a limitation in some studies, in stage 2 of the proposed framework, a precise analysis of the toxin variants is generally unnecessary as the purpose of this biomass characterization stage is to support the decision on directing the raw biomass for different extraction companies, who then must design further assessment schemes depending on the application intended for the extracted product and also the extraction and purification processes used. Other options for the general characterization of the raw biomass may include analytical methods such as High-Performance Liquid Chromatography (HPLC) (e.g. Hiskia et al. 2017), Mass Spectrometry (MS) (e.g. Oehrle et al. 2010), or even but less used, the Nuclear Magnetic Resonance (NMR) (Sanseverino et al. 2017). An overview on the methods and recommendations can be found in the latest WHO guidelines (WHO 2021), in Chapter 14. The safety limits that should withstand for cyanotoxins in the collected biomass can be generally interpreted from those suggested by WHO for recreational waters. Still, the

requirements of each application should tune the definition of each specific set of limits, accounting to the planned processing stages since some of the steps involved (e.g. heat treatment, pH, use of specific solvents) can possibly inactivate some cyanotoxins or even allow the successful separation of the toxins from the target product extracted (Shen et al. 2020).

Once the biomass is characterized, the basic conditions are set for selling it to the most suitable company. A myriad of companies is available (see section II-6) and, according to their industrial processes, biomass will be processed and transformed to different products that can then enter in the value chain (Step 3). Finally, considering environmental ethic principles ruling worldwide, it is straightforward that a step 4 is in place to ensure compensatory measures. The most logical proposal is that the profit/return retrieved from the valorization of natural blooms (generally regarding the selling of the biomass to exploitation companies; see below) is re-invested in environmental protection in general or in control by physical removal of blooms (eventually synchronized with step 1 of the valorization framework) more specifically, picturing the closing of the cycle, as illustrated in Figure II-3.

In terms of framework management, the most efficient system would be to address the collection, transportation, storage and toxin analysis to local/regional entities with responsibilities towards environmental management of waterbodies (e.g. municipalities, public/non-profit water treatment companies/organizations, environmental management entities). This straightforwardly allows the use of the profit/return retrieved from biomass selling to exploitation companies either to compensate for the expenses in the physical removal of the blooms or to broadly apply in ecosystem protection and restoration measures. Assuming that such a straightforward approach is not universally realistic, a system of fares can be established in benefit of standard management entities in each country/region, so that private companies profiting from each of the steps of the framework can support environmental protection following the users-pay principle (OECD 2001). A final note is worth making on the need to tightly regulate the actions under the scope of valorization frameworks. In the case of our bloom valorization framework, regulation is critical to assure that the bloom is not assumed as a normal profitable asset, because this is not primarily a business model. Instead, the idea is restricted to the stimulation for an effective control of existing blooms when preventive management was not effective. Although we obviously do

not have real data to evaluate the economic viability of this approach, its preliminary assessment before any future implementation is critical (section II-6.1.1). This preliminary assessment was made without a specific assignment of the return to each stakeholder involved. This was done for simplification at such an early stage of the idea, but also because the structure of the consortia involved in its future implementation are very much dependent on the scale (national, regional or local), the overall governance structure regarding environmental management of natural resources at each country or region, and the managerial resources available, including personnel, infrastructure and budget. For example, depending on the context, dedicated financial support from governmental sources can assist the control by removal of cyanobacteria blooms in emergent situations (as reported e.g. for Lake Taihu, China (Kozacek 2014)). In such a case, this funding adds to the return that can be obtained through the valorization, reinforcing the stimulation towards effective environmental management of blooms in particular and/or environmental protection and restoration actions in general, since the scope for budget enlargement necessarily increases. In addition, it is worth mentioning the currently unaccounted benefits that can be gained regarding local and regional development through smart specialization as allowed by the biotechnology-based valorization approach suggested.

II-6.1.1. Economic viability of the proposed concept

The potential market of removing cyanobacteria from contaminated waters and commercially exploring the biomass was economically analysed, based on the creation different scenarios. Briefly, when an economic analysis is under development, three areas need to be fulfilled to complete the evaluation. It is required (i) to set up a target output or production scenarios, then (ii) to determine the sequence of unit operations/steps and their respective parameters and conditions, and finally, (iii) to collect the economic datasets to populate the model. Following this rationale, some attempts have been approached by some of us aiming to develop the best approach to evaluate the use of biotechnological processes on the valorization of different biomass sources (Passos et al. 2014; Martins et al. 2021). Specifically, in the recent work by Martins and collaborators (Martins et al. 2021), the valorization of a marine raw material (red macroalga, *Gracilaria gracilis*) through the exploitation of the economic and commercial potential of extracted and purified phycobiliproteins was successfully addressed. Using a similar approach, the parameters of

potential profit or Return (R) were defined and modelled herein. Briefly, this model/equation generates an R value that can be a positive or negative number, which represents the potential profit that can be generated from a product by considering its production, potential price, and production costs. The simplified version for the Return equation (Eq. II-1) used herein relates the Return to the concentration of cyanobacteria in the contaminated water (mass *per* unit of volume) (C_{cyano}), the potential selling price for this biomass (price *per* unit of mass) ($\$_{cyano}$), the associated administrative ($\$_{admin}$) and process ($\$_{proc}$) costs for harvesting the cyanobacteria (cost *per* unit of volume).

$$R = \left(C_{Cyano} \times \$_{Cyano} - \$_{Admin}\right) - \$_{Proc} \qquad \textbf{Eq.II-1}$$

As previously mentioned, different scenarios were created to provide a more complete evaluation. For a more comprehensive analysis, a large range of values for each variable was defined. It was decided to test for each of them the range from 0.001, then by increasing by a factor of 2 up to reaching the first value above of 100 (this limit was decided as it is possible to analyse any number of desired scenarios, but a limit must be established). These values were applied for each variable, and the units can be expressed in any currency, mass, and volume unit, as long as they are consistent throughout the equation. In the case of the example presented here the units were either in $g.L^{-1}$, Euro (EU).g^{-1}, or $EU.L^{-1}$. In total, 18 values for 4 variables provided a collection of 104 976 combinations. Within this range of values for R obtained by the 104 976 combinations the potential real (in practice) value can be found.

For this work, it was decided to use the value of EU 103 *per* 21 000 L (EU 0.0086 *per* 1 L) as a benchmark for the costs of harvesting cyanobacterial biomass from a contaminated waterbody (i.e. process costs, $\$_{proc}$). This is a median value charged by some Fire Departments in Portugal to fill private pools, following pumping from a natural reservoir and transporting 21 000 L of water. We argue that the costs of the Step 1, regarding the collection, transport and possibly short-term storage of the biomass (Figure II-3) should not be much higher/different that this reference value, considering our reasoning that public services with own resources should be involved at this stage (see section II-6.1). Notwithstanding this, most of the scenarios created use a cost higher than this (> EU 0.008

per 1 L), which will make this study a more robust tool to predict the potential real-life costs and Return.

Since the Return indicates the potential profit, it should have a positive value. In order to enhance the impact of the potential Return, the results are presented for the worst scenario calculated. This scenario was achieved by using the maximum value (EU 131.072.L^{-1}) for the variables $\$_{Admin}$ and $\$_{Proc}$. In this scenario, the values for C_{cyano} and $\$_{cyano}$ that provided the least positive R value were selected (Figure II-4). To preserve a positive value, the combinations of C_{Cyano} and $\$_{Cyano}$ will need to be maintained at the upper right area of Figure II-4. In terms of values, this means that a C_{Cyano} of 131.072 $g.L^{-1}$ can have a $\$_{Cyano}$ of EU 2.048.g^{-1}. The same relationship applies for the opposite, i.e. a value of $\$_{Cyano}$ of EU 131.072.g^{-1} can accommodate a low cyanobacterial concentration in water of 2.048 $g.L^{-1}$. It is critical to understand that these values can be read as high, but they are under the assumption of the worst scenario for $\$_{Admin}$ and $\$_{Prod}$. If these two variables ($\$_{Admin}$ and $\$_{Prod}$) values decrease, then the curve in Figure II-4 will shift towards the lower left, allowing room for different combinations of C_{Cyano} and $\$_{Cyano}$, particularly combinations with less favourable values.

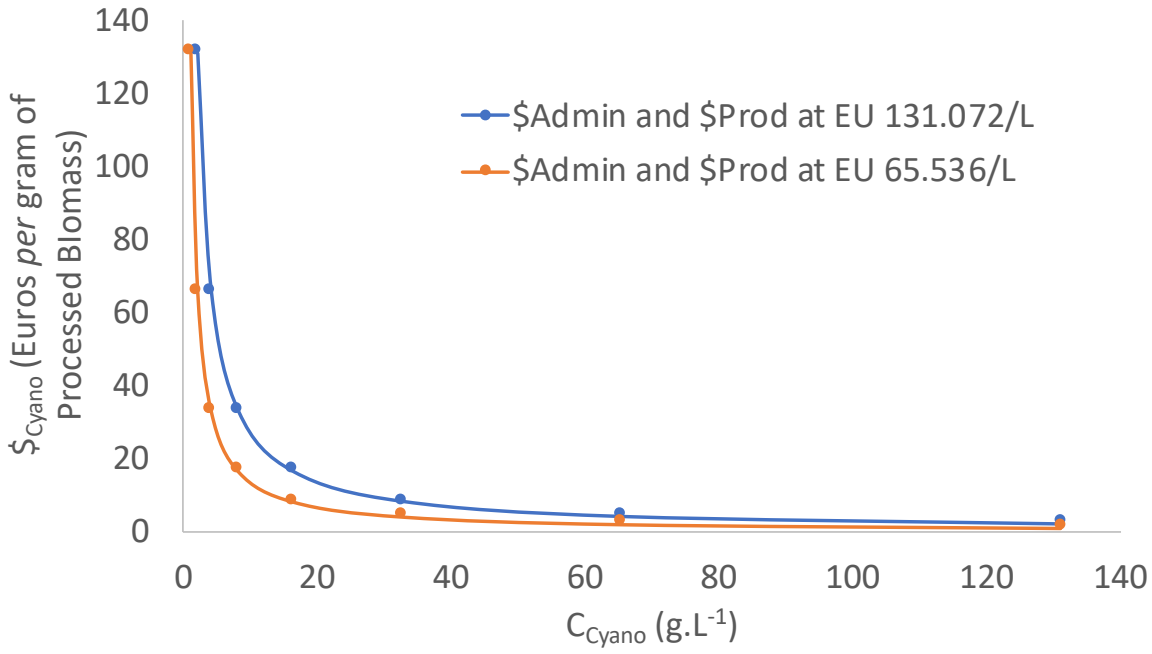

Figure II-4. Threshold for a positive Return (R) for a $\$_{Admin}$ and $\$_{Prod}$ at EU 65.536 per liter and EU 131.072 *per* liter. Combinations of $\$_{Cyano}$ and C_{Cyano} for a positive R need to be to the upper right of the curves.

This approach allows any interested stakeholder to estimate the Return and, potentially the profit resulting from the application of the suggested framework to natural cyanobacteria blooms considering their actual costs, which will assume specific values in between the wide range of scenarios used for each variable in the model, including the cyanobacteria concentration of each addressed bloom, largely depending on the species involved and the acting environmental conditions. Still, in order to render the approach more concrete and to further emphasize the applicability of the framework proposed herein, the economic analysis exercise can be continued by considering a specific extractable compound of interest. Indeed, the biomass collected from natural blooms can be explored for different bioproducts that can be used for different applications in different markets (depending on their toxin content). This can be incorporated in Eq. II-1 given the nature of the model, as long as sufficient data are available, which is not so common currently. Although there are many bioproducts that can be applied to the model, we are considering phycocyanin as a model product and as an example for which own information is available (Sintra et al. 2021). In this way, the Return analysis was added with two terms (Eq. II-2) to account (i) for the potential income of selling phycocyanin (C_{Phyco} x $\$_{Phyco}$); and (ii) for the production cost of this pigmented product ($\$_{Phyco\ Proc}$).

$$R = \left(C_{Cyano} \times \$_{Cyano} - \$_{Admin}\right) - \$_{Proc} + \left(C_{Phyco} \times \$_{Phyco} - \$_{Phyco\ Proc}\right) \qquad \textbf{Eq. II-2}$$

To populate Eq. II-2 and analyse the impact of including the profit and cost of phycocyanin production, benchmark values were established for the variables in order to decrease the number of calculated critical scenarios. Representing the worst-case scenario regarding the variables in Eq. II-1, C_{Cyano} and $\$_{Cyano}$ were fixed at the lowest level possible (0.001 grams of biomass *per* liter of water and EU 0.001 *per* gram of biomass, respectively), while $\$_{Admin}$ and $\$_{Proc}$ were fixed at the highest value analysed before (EU 131.072 *per* liter of processed water for both). For the new variables present only in Eq. II-2, C_{Phyco} was fixed at a reported value of 63 mg of phycocyanin *per* g of fresh biomass (Sintra et al. 2021), while $\$_{Phyco}$ was allowed to vary within the range comprising food- and analytical-grade prices (EU 0.13 and EU 15 *per* mg of phycocyanin) (Rito-Palomares et al. 2001; Sintra et al. 2021). The last variable is the production cost of obtaining phycocyanin ($\$_{Phyco\ Proc}$). Its value can change dramatically depending on the process used. In this way, and following the literature

(Martins et al. 2021), a convenient range of five values (EU 0.01, EU 0.1, EU 1, EU 10 and EU 100 *per* g of biomass) was tested. The required phycocyanin units conversion from EU *per* gram of biomass to EU *per* litre of processed water was made based on previous studies (100 grams *per* litre (Sintra et al. 2021)). Thus, and to emphasize the application of the proposed framework, the value used here was decreased to 1 gram *per* litre to show results under a restricted situation. Figure II-5 shows the two extremes of the scenarios calculated, evidencing R for phycocyanin obtained at food grade or at analytical grade. The R for any other purity grade in between will be located within the two lines. It is worth remarking that the rest of the variables were fixed at the most conservative value (worst-case scenario). In practice, it is expected that these values can be optimised, which will translate into an even higher potential Return.

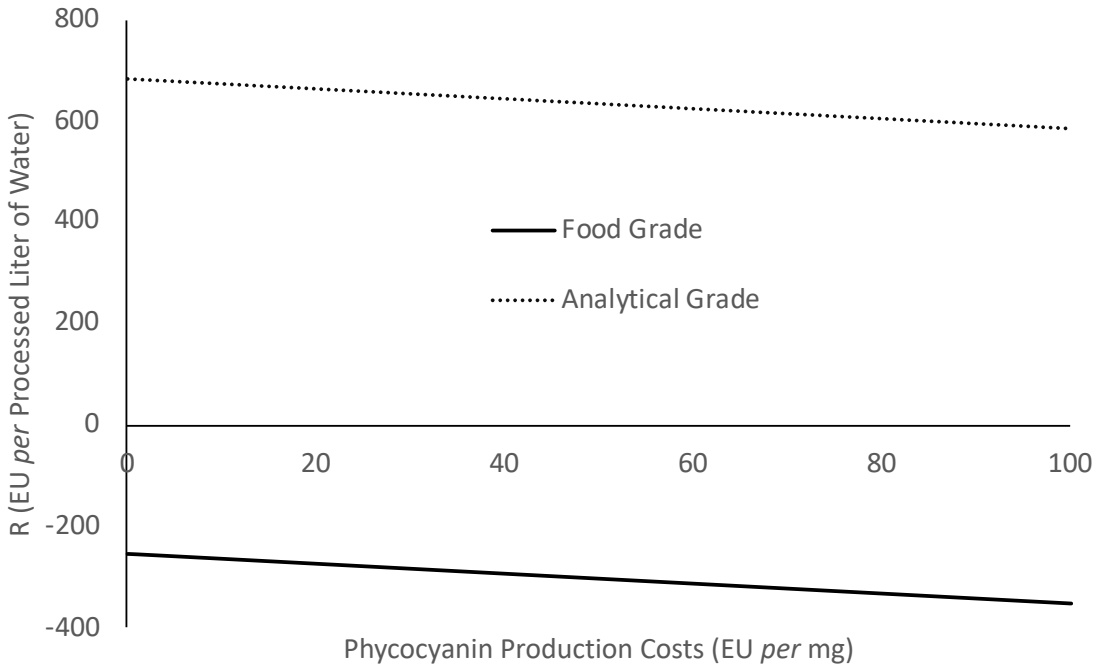

Figure II-5. Calculated Return (R) after the inclusion of phycocyanin production. Values for fixed variables are: $\$_{Admin}$ = EU 131.072 *per* L, $\$_{Proc}$ = EU 131.072 *per* L, C_{Cyano} = EU 0.001 g *per* L, $\$_{Cyano}$ = EU 0.001 *per* L, C_{Phyco} = 63 mg *per* g, $\$_{Phyco}$ = EU 0.13 and EU 15 *per* mg (for food and analytical grade, respectively). Results show limits for the potential R that can be obtained depending on the final purity, selling price and concentration of phycocyanin generated.

II-7. Conclusion

Climate change is increasing the frequency, duration and intensity of CyanoHABs, imposing growing impairment of the water supply quality, fisheries and recreational resources. Herein, a critical review on the nuisance potential of cyanoHABs (‘dark side’) and related management practices was followed by the conceptual proposal of integrating their biotechnological exploitation (‘bright side’) as a new axis to improve the efficiency of management frameworks. The basic protocol within this approach would be to value the cyanoHABs biomass physically removed from affected waterbodies rather than landfilling or directing it to (expensive) treatment, which is a traditional control strategy. Since cyanobacteria are rich in bioactive compounds of high commercial potential, they may be recognised as valuable alternative feedstocks for strategic sectors, under a multi-product scenario. We demonstrate that the economic income of such approach can cover the costs of control actions over existent cyanoHABs, maintaining the direct ecological, economic (in touristic areas), and human health safety benefits, but it can also be profitable. This was evidenced both at the level of the biomass delivery to production units and considering the full exploitation cycle using the valorization of phycocyanin as an example. Although the economic potential and viability of the overall concept explored in this work is already promising, there is further scope for growth if biorefinery frameworks for multi-product valorization from the same biomass are integrated (Slegers et al. 2020). As a novel proposal for the sustainable control of cyanobacteria blooms, this work is a stepping-stone rather than a final draft of a new framework. The present article demonstrates the suitability of the framework, but several aspects certainly need further development stages, both from the operational and the regulatory viewpoints. For example, the design of an efficient pumping system allowing the collection of the cyanobacterial biomass, while causing minimum disturbance in the biotic communities of the affected waterbody, is a primary aspect deserving attention, although most of the current strategies applied to the control of cyanobacteria blooms also have these kinds of drawbacks.

References

Ahmad AL, Mat Yasin NH, Derek CJC, Lim JK (2011) Optimization of microalgae coagulation process using chitosan. Chem Eng J 173:879–882. doi: 10.1016/j.cej.2011.07.070

Ahn C, Joung S, Jeon J, et al. (2003) Selective control of cyanobacteria by surfactin-containing culture broth of *Bacillus subtilis* C1. Biotechnol Lett 25:137–142. doi: 10.1023/a:1024508927361

Al-Tebrineh J, Mihali KT, Pomati F, Neilan B (2010) Detection of saxitoxin-producing cyanobacteria and *Anabaena circinalis* in environmental water blooms by quantitative PCR. Appl Environ Microbiol 76:7836–7842.

Amorim CA, de Moura-Falcão RH, Valença CR, et al. (2019) Allelopathic effects of the aquatic macrophyte *Ceratophyllum demersum* L. on phytoplankton species: contrasting effects between cyanobacteria and chlorophytes. Acta Limnol Bras. doi: https://doi.org/10.1590/s2179-975x1419

Atkins R, Rose T, Brown RS, Robb M (2001) The *Microcystis* cyanobacteria bloom in the Swan River - February 2000. Water Sci Technol 43:107–114. doi: 10.2166/wst.2001.0518

Backer LC (2002) Cyanobacterial harmful algal blooms (CyanoHABs): Developing a public health response. Lake Reserv Manag 18:20–31. doi: 10.1080/07438140209353926

Becker A, Herschel A, Wilhelm C (2006) Biological effects of incomplete destratification of hypertrophic freshwater reservoir. Hydrobiologia 559:85–100. doi: 10.1007/s10750-005-4428-3

Berry JP (2008) Cyanobacterial toxins as allelochemicals with potential applications as algaecides, herbicides and insecticides. Mar Drugs 6:117–146. doi: 10.3390/md20080007

Beutel MW, Horne AJ (1999) A review of the effects of hypolimnetic oxygenation on lake and reservoir water quality. Lake Reserv Manag 15:285–297. doi: 10.1080/07438149909354124

Bhati R, Samantaray S, Sharma L, Mallick N (2010) Poly-β-hydroxybutyrate accumulation

in cyanobacteria under photoautotrophy. Biotechnol J 5:1181–1185. doi: 10.1002/biot.201000252

Bingham M, Sinha SK, Lupi F (2015) Economic benefits of reducing harmful algal blooms in Lake Erie.

Bormans M, Webster IT (1997) A mixing criterion for turbid rivers. Environ Model Softw 12:329–333. doi: 10.1016/S1364-8152(97)00032-7

Buckler JH, Skelly TM, Luepke MJ (1988) Case study: The lake springfield sediment removal project. Lake Reserv Manag 4:143–152. doi: 10.1080/07438148809354389

Burja AM, Banaigs B, Abou-mansour E, et al. (2001) Marine cyanobacteria: a profilic source of natural products. Tetrahedron 57:9347–9377. doi: 10.1016/S0040-4020(01)00931-0

Cairns MJ, Krauss C, Johnston JH (2013) Recovery of phosphate from surface waters using a calcium silicate composite material for potential application in environmental remediation. TechConnect Briefs 3:252–255.

Cannon R, Shane M, Whitaker J (2008) Interaction of *Plectonema boryanum* (Cyanophyceae) and the LPP-cyanophages in continuous culture. J Phycol 12:418–421. doi: 10.1111/j.1529-8817.1976.tb02865.x

Cardozo KHM, Guaratini T, Barros MP, et al. (2007) Metabolites from algae with economical impact. Comp Biochem Physiol Part C 146:60–78. doi: 10.1016/j.cbpc.2006.05.007

Carreto JI, Carignan MO (2011) Mycosporine-like amino acids: Relevant secondary metabolites. Chemical and ecological aspects. Mar Drugs 9:387–446. doi: 10.3390/md9030387

Chen S, Carey CC, Little JC, et al. (2018) Effectiveness of a bubble-plume mixing system for managing phytoplankton in lakes and reservoirs. Ecol Eng 113:43–51. doi: 10.1016/j.ecoleng.2018.01.002

Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25:294–306. doi: 10.1016/j.biotechadv.2007.12.001

Chorus I, Falconer IR, Salas HJ, Bartram J (2000) Health risks caused by freshwater

cyanobacteria in recreational waters. J Toxicol Environ Heal Part BCritical Rev 3:323–347. doi: 10.1080/109374000436364

Coops H, Hosper SH (2002) Water-level management as a tool for the restoration of shallow lakes in the Netherlands. Lake Reserv Manag 18:293–298. doi: 10.1080/07438140209353935

Demeke A (2016) Cyanobacteria blooms and biological control methods. Int J Fauna Biol Stud 3:32–38.

Díaz GD, Li Q, Dashwood RH (2003) Caspase-8 and apoptosis-inducing factor mediate a cytochrome c-independent pathway of apoptosis in human colon cancer cells induced by the dietary phytochemical chlorophyllin. Cancer Res 63:1254–1261.

Domingues A, Rosa IC, da Costa JP, et al. (2020) Potential of the bivalve *Corbicula fluminea* for the remediation of olive oil wastewaters. J Clean Prod 252:119773. doi: 10.1016/j.jclepro.2019.119773

Dryden R, Wright S (1987) Predation of cyanobacteria by protozoa. Can J Microbiol 33:471–482. doi: https://doi.org/10.1139/m87-080

Echeverría F, Valenzuela R, Hernandez-Rodas MC, Valenzuela A (2017) Docosahexaenoic acid (DHA), a fundamental fatty acid for the brain: New dietary sources. Prostaglandins Leukot Essent Fat Acids 124:1–10. doi: 10.1016/j.plefa.2017.08.001

Eriksen NT (2008) Production of phycocyanin - a pigment with applications in biology, biotechnology, foods and medicine. Appl Microbiol Biotechnol 80:1–14. doi: 10.1007/s00253-008-1542-y

Fan YY, Chapkin RS (1998) Importance of dietary gamma-linolenic acid in human health and nutrition. J Nutr 128:1411–1414. doi: 10.1093/jn/128.9.1411

FAO/WHO (1993) Fats and oils in human nutrition. Rome

Fawzy MA, Mohamed AKSH (2017) Bioremediation of heavy metals from municipal sewage by cyanobacteria and its effects on growth and some metabolites of *Beta vulgaris*. J Plant Nutr 40:2550–2561. doi: 10.1080/01904167.2017.1380822

de Figueiredo D, Azeiteiro UM, Esteves S, et al. (2004) Microcystin-producing blooms - a serious global public health issue. Ecotoxicol Environ Saf 59:151–163. doi:

10.1016/j.ecoenv.2004.04.006

Gerphagnon M, Colombet J, Latour D, Sime-Ngando T (2017) Spatial and temporal changes of parasitic chytrids of cyanobacteria. Sci Rep 7:1–9. doi: 10.1038/s41598-017-06273-1

Gradíssimo DG, Xavier LP, Santos A V. (2020) Cyanobacteria polyhydroxyalkanoates: a sustainable alternative in circular economy. Molecules 25:4331. doi: 10.3390/molecules25184331

Gragnani A, Scheffer M, Rinaldi S (1999) Top-down control of cyanobacteria: A theoretical analysis. Am. Nat. pp 59–72

Griffiths DJ, Saker ML (2003) The Palm Island mystery disease 20 years on: A review of research on the cyanotoxin cylindrospermopsin. Env Toxicol 18:78–93. doi: 10.1002/tox.10103

Grzesik M, Kalaji HM (2017) Effectiveness of cyanobacteria and green algae in enhancing the photosynthetic performance and growth of willow (*Salix viminalis* L.) plants under limited synthetic fertilizers application. Photosynthetica 55:510–521. doi: 10.1007/s11099-017-0716-1

Hamilton DP, Wood SA, Dietrich DR, Puddick J (2013) Costs of harmful blooms of freshwater cyanobacteria. In: Sharma NK, Rai AK, Stal LJ (eds) Cyanobacteria An Econ. Perspect., Fisrt edit. John Wiley & Sons, Ltd, pp 245–256

Haque F, Banayan S, Yee J, Chiang YW (2017) Extraction and applications of cyanotoxins and other cyanobacterial secondary metabolites. Chemosphere 183:164–175. doi: 10.1016/j.chemosphere.2017.05.106

Harpster D (2018) Spotlight news magazine. In: Environ. Co. Tak. algae Probl. from top. https://www.swspotlight.com/articles/spotlight-news/environmental-company-takes-algae-problem-from-the-top/. Accessed 5 Apr 2021

Hiskia A, Spoof L, Kaloudis T, Meriluoto J (2017) Determination of cyanotoxins by High-Performance Liquid Chromatography with photodiode array. Handb Cyanobacterial Monit Cyanotoxin Anal. doi: 10.1002/9781119068761.ch21

Ho L, Dreyfus J, Boyer J, et al. (2012) Fate of cyanobacteria and their metabolites during

water treatment sludge management processes. Sci Total Environ 424:232–238. doi: 10.1016/j.scitotenv.2012.02.025

Holub BJ (2009) Docosahexaenoic acid (DHA) and cardiovascular disease risk factors. Prostaglandins Leukot Essent Fat Acids 81:199–204. doi: 10.1016/j.plefa.2009.05.016

Horimoto N, Ogawa T (1997) Arachidonic acid activation of potassium channels in rat visual cortex neurons. Neuroscience 77:661–671. doi: 10.1016/s0306-4522(96)00490-3

Hosikian A, Lim S, Halim R, Danquah MK (2010) Chlorophyll extraction from microalgae: A review on the process engineering aspects. Int J Chem Eng 1–11. doi: 10.1155/2010/391632

House J, Burch M (2002) Using algicides for the control of algae in Australia.

Huh JH, Ahn JW (2017) A perspective of chemical treatment for cyanobacteria control toward sustainable freshwater development. Environ Eng Res 22:1–11. doi: 10.4491/eer.2016.155

IPCC (2014a) Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA

IPCC (2014b) Climate Change 2014 - Impacts, Adaptation, and Vulnerability. Part A: Global and Sectorial Aspects. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA

Jančula D, MarŠálek B (2011) Critical review of actually available chemical compounds for prevention and management of cyanobacterial blooms. Chemosphere 85:1415–1422. doi: 10.1016/j.chemosphere.2011.08.036

Kamal A, Ahmad IZ (2014) Cyanobacteria “the blue green algae” and its novel applications: A brief review. Int J Innov Apllied Stud 7:251–261.

Karmali RA (1996) Historical perspective and potential use of n-3 fatty acids in therapy of cancer cachexia. Nutrition 12:S2–S4. doi: 10.1016/0899-9007(95)00070-4

Koller M, Gasser I, Schmid F, Berg G (2011) Linking ecology with economy: Insights into

polyhydroxyalkanoate-producing microorganisms. Eng Life Sci 11:222–237. doi: 10.1002/elsc.201000190

Koller M, Muhr A, Braunegg G (2014) Microalgae as versatile cellular factories for valued products. Algal Res 6:52–63. doi: 10.1016/j.algal.2014.09.002

Koskenniemi K, Lyra C, Rajaniemi-Wacklin P, et al. (2007) Quantitative real-time PCR detection of toxic *Nodularia* cyanobacteria in the Baltic Sea. Appl Environ Microbiol 73:2173–2179.

Kozacek C (2014) Circle of blue. https://www.circleofblue.org/2014/world/draft-world-stands-algae-dead-zones-ruin-water/. Accessed 4 Feb 2020

Kultschar B, Llewellyn C (2018) Secondary metabolites in cyanobacteria. In: Vijayakumar R, Raja SSS (eds) Second. Metab. - Sources Appl. pp 23–36

Kurmayer R, Christiansen G (2009) The genetic basis of toxin production in Cyanobacteria. Freshw Rev 2:31–50. doi: 10.1608/FRJ-2.1.2

Lengke MF, Fleet ME, Southam G (2007) Biosynthesis of silver nanoparticles by filamentous cyanobacteria from a silver(I) nitrate complex. Langmuir 23:2694–2699. doi: 10.1021/la0613124

Levi D (2018) Adressing an ecological crisis. https://aecom.com/blog/addressing-an-ecological-crisis. Accessed 23 Oct 2020

Lin Y, Chen A, Luo S, et al. (2019) Cyanobacterial bloom mitigation by sanguinarine and its effects on aquatic microbial community structure. Environ Pollut 253:497–506. doi: 10.1016/j.envpol.2019.07.060

Lin Y, Schiavo S, Orjala J, et al. (2008) Microscale LC-MS-NMR platform applied to the identification of active cyanobacterial metabolites. Anal Chim Acta 80:8045–8054. doi: 10.1021/ac801049k.Microscale

Lofton ME, McClure RP, Chen S, et al. (2019) Whole-ecosystem experiments reveal varying responses of phytoplankton functional groups to epilimnetic mixing in a eutrophic reservoir. Water 11:1–23. doi: 10.3390/w11020222

Macário IPE, Castro BB, Nunes MIS, et al. (2017) Correction to: Stepwise strategy for monitoring toxic cyanobacterial blooms in lentic water bodies (Environ Monit Assess,

10.1007/s10661-017-6292-9). Environ Monit Assess 189:620. doi: 10.1007/s10661-017-6361-0

Manage PM, Kawabata Z, Nakano S (2000) Algicidal effect of the bacterium *Alcaligenes denitrificans* on *Microcystis* spp. Aquat Microb Ecol 22:111–117. doi: 10.3354/ame022111

Martins M, Soares BP, Santos JHPM, et al. (2021) Sustainable strategy based on induced precipitation for the purification of phycobiliproteins. ACS Sustain Chem Eng 9:3942–3954. doi: https://doi.org/10.1021/acssuschemeng.0c09218

Matthijs HCP, Visser PM, Reeze B, et al. (2012) Selective suppression of harmful cyanobacteria in an entire lake with hydrogen peroxide. Water Res 46:1460–1472. doi: 10.1016/j.watres.2011.11.016

Merel S, Walker D, Chicana R, et al. (2013) State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environ Int 59:303–327. doi: 10.1016/j.envint.2013.06.013

Messineo V, Melchiorre S, Corcia AD, et al. (2010) Seasonal succession of *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* blooms with cylindrospermopsin occurrence in the volcanic Lake Albano, Central Italy. Environ Toxicol 25:18–27. doi: 10.1002/tox.20469

Metcalf JS, Codd GA (2012) Cyanotoxins. In: Whitton BA (ed) Ecol. Cyanobacteria II - Their Divers. Sp. Time. Springer Science + Business Media B.V., pp 651–675

Mishima T, Murata J, Toyoshima M, et al. (1998) Inhibition of tumor invasion and metastasis by calciumspirulan (Ca-SP), a novel sulfated polysaccharide derived from a blue-green alga, *Spirulina platensis*. Clin Exp Metastasis 16:541–550. doi: 10.1023/a:1006594318633

Mohamed ZA (2017) Macrophytes-cyanobacteria allelopathic interactions and their implications for water resources management - a review. Limnologica 63:122–132. doi: 10.1016/j.limno.2017.02.006

Moisander PH, Cheshire LA, Braddy J, et al. (2012) Facultative diazotrophy increases *Cylindrospermopsis raciborskii* competitiveness under fluctuating nitrogen availability. FEMS Microbiol Ecol 79:800–811. doi: 10.1111/j.1574-

6941.2011.01264.x

Nagy BJ, Makó M, Erdélyi I, et al. (2018) MAB2.0 project: Integrating algae production into wastewater treatment. EuroBiotech J 2:10–23. doi: 10.2478/ebtj-2018-0003

Naidenko OV, Cox C, Bruzelius N (2012) Troubled waters: Farm pollution threatens drinking water. Environmental Working Group, Washington DC 20009. In: Troubl. waters Farm Pollut. Threat. Drink. water. https://www.ewg.org/research/troubled-waters. Accessed 23 Mar 2021

Nakamura H (2019) Water chemical remediation for simultaneous removal of phosphate ion and blue-green algae from anthropogenically eutrophied pond. Water Chem. pp 1–17

Newcombe G (2009) International Guidance Manual for the Management of Toxic Cyanobacteria. London

NHMRC (2008) Guidelines for managing Risks in Recreational Water. Australian Government, National Health and Medical Research Council

Nie M, Deng Y, Nie S, et al. (2019) Simultaneous removal of bisphenol A and phosphate from water by peroxymonosulfate combined with calcium hydroxide. Chem Eng J 369:35–45. doi: 10.1016/j.cej.2019.03.046

Nürnberg GK, LaZerte BD, Olding DD (2003) An artificially induced *Planktothrix rubescens* surface bloom in a small kettle lake in Southern Ontario compared to blooms world-wide. Lake Reserv Manag 19:307–322. doi: 10.1080/07438140309353941

OECD (2001) Glossary of statistic terms. https://stats.oecd.org/glossary/detail.asp?ID=2827. Accessed 26 Oct 2020

Oehrle SA, Southwell B, Westrick J (2010) Detection of various freshwater cyanobacterial toxins using ultra-performance liquid chromatography tandem mass spectrometry. Toxicon 55:965–972. doi: 10.1016/j.toxicon.2009.10.001

Olaizola M (2000) Commercial production of astaxanthin from *Haematococcus pluvialis* using 25,000-liter outdoor photobioreactors. J Appl Phycol 12:499–506. doi: 10.1023/A:1008159127672

OPCW (2005) Organisation for the Prohibition of Chemical Weapons: Convention on the prohibition of the development, production, stockpiling and use of chemical weapons

and on their destruction. The Hague

Paerl H (2014) Mitigating harmful cyanobacterial blooms in a human- and climatically-impacted world. Life 4:988–1012. doi: 10.3390/life4040988

Paerl HW, Gardner WS, Havens KE, et al. (2016) Mitigating cyanobacterial harmful algal blooms in aquatic ecosystems impacted by climate change and anthropogenic nutrients. Harmful Algae 54:213–222. doi: 10.1016/j.hal.2015.09.009

Paerl HW, Huisman J (2009) Climate change: a catalyst for global expansion of harmful cyanobacterial blooms. Environ Microbiol Rep 1:27–37. doi: 10.1111/j.1758-2229.2008.00004.x

Paerl HW, Paul VJ (2012) Climate change: Links to global expansion of harmful cyanobacteria. Water Res 46:1349–1363. doi: 10.1016/j.watres.2011.08.002

Pandey VD, Pandey A, Sharma V (2013) Biotechnological applications of cyanobacterial phycobiliproteins. Int J Curr Microbiol Appl Sci 2:89–97.

Passos H, Freire MG, Coutinho JAP (2014) Ionic liquid solutions as extractive solvents for value-added compounds from biomass. Green Chem 16:4786–4815.

Pearson LA, Neilan BA (2008) The molecular genetics of cyanobacterial toxicity as a basis for monitoring water quality and public health risk. Curr Opin Biotechnol 19:281–288.

de Philippis R, Vincenzini M (1998) Exocellular polysaccharides from cyanobacteria and their possible applications. FEMS Microbiol Rev 22:151–175. doi: 10.1111/j.1574-6976.1998.tb00365.x

Pouria S, de Andrade A, Barbosa J, et al. (1998) Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. Lancet 352:21–26. doi: 10.1016/s0140-6736(97)12285-1

Pretty JN, Mason CF, Nedwell DB, et al. (2003) Environmental costs of freshwater eutrophication in England and Wales. Environ Sci Technol 37:201–208. doi: 10.1021/es020793k

Raheem A, Prinsen P, Vuppaladadiyam AK, et al. (2018) A review on sustainable microalgae based biofuel and bioenergy production: Recent developments. J Clean Prod 181:42–59. doi: 10.1016/j.jclepro.2018.01.125

Rito-Palomares M, Nuez L, Amador D (2001) Practical application of aqueous two-phase systems for the development of a prototype process for C-phycocyanin recovery from *Spirulina maxima*. J Chem Technol Biotechnol 76:1273–1280. doi: 10.1002/jctb.507

Rosa IC, Costa R, Gonçalves F, Pereira JL (2014) Bioremediation of metal-rich effluents: could the invasive bivalve work as a biofilter? J Environ Qual 43:1536–1545.

Rose PD, Wells C, Dekker L, et al. (2007) Salinity, Sanitation and Sustainability: a study in environmental biotechnology and integrated wastewater benefication in South Africa. Grahamstown

Sanseverino I, António DC, Loos R, Lettieri T (2017) Cyanotoxins: methods and approaches for their analysis and detection.

Sanseverino I, Conduto D, Pozzoli L, et al. (2016) Algal bloom and its economic impact. Eur Comm. doi: 10.2788/660478

Schembri MA, Neilan BA, Saint CP (2001) Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii*. Environ Toxicol 16:413–421.

Sekar S, Chandramohan M (2008) Phycobiliproteins as a commodity: Trends in applied research, patents and commercialization. J Appl Phycol 20:113–136. doi: 10.1007/s10811-007-9188-1

Shah V, Ray A, Garg N, Madamwar D (2000) Characterization of the extracellular polysaccharide produced by a marine Cyanobacterium, *Cyanothece* sp. ATCC 51142, and its exploitation toward metal removal from solutions. Curr Microbiol 40:274–278. doi: 10.1007/s002849910054

Shen Q, Li D, Li D, et al. (2020) Study on the safe disposal and resource utilization of cyanobacterial bloom biomass in Dianchi Lake, China. J Appl Phycol 32:1201–1213. doi: 10.1007/s10811-019-01995-3

Sintra TE, Bagagem SS, Ahsaie FG, et al. (2021) Sequential recovery of C-phycocyanin and chlorophylls from *Anabaena cylindrica*. Sep Purif Technol 255:117538. doi: 10.1016/j.seppur.2020.117538

Slegers PM, Olivieri G, Breitmayer E, et al. (2020) Design of value chains for microalgal

biorefinery at industrial scale: Process integration and techno-economic analysis. Front Bioeng Biotechnol 8:1048.

Smith MJ, Shaw GR, Eaglesham GK, et al. (2008) Elucidating the factors influencing the biodegradation of cylindrospermopsin in drinking water sources. Environ Toxicol 23:413–421. doi: 10.1002/tox.20356

Smith RB, Bass B, Sawyer D, et al. (2019) Estimating the economic costs of algal blooms in the Canadian Lake Erie Basin. Harmful Algae 87:101624. doi: 10.1016/j.hal.2019.101624

Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006) Commercial applications of microalgae. J Biosci Bioeng 101:87–96. doi: 10.1263/jbb.101.87

Srivastava A, Singh S, Ahn CY, et al. (2013) Monitoring approaches for a toxic cyanobacterial bloom. Environ Sci Technol 47:8999–9013. doi: 10.1021/es401245k

Steffensen DA (2008) Economic cost of cyanobacterial blooms. In: Hudnell HK (ed) Cyanobacterial Harmful Algal Bloom. State Sci. Res. Needs. Springer, pp 855–865

Svirčev Z, Lalić D, Savić GB, et al. (2019) Global geographical and historical overview of cyanotoxin distribution and cyanobacterial poisonings. Arch Toxicol 93:2429–2481. doi: 10.1007/s00204-019-02524-4

Tillett D, Parker DL, Neilan BA (2001) Detection of toxigenicity by a probe for the microcystin synthetase A gene (mcyA) of the cyanobacterial genus *Microcystis*: comparison of toxicities with 16S rRNA and phycocyanin operon (Phycocyanin Intergenic Spacer) phylogenies. Appl Environ Microbiol 67:2810–2818. doi: 10.1128/AEM.67.6.2810-2818.2001

Tiwari ON, Khangembam R, Shamjetshabam M, et al. (2015) Characterization and optimization of bioflocculant exopolysaccharide production by Cyanobacteria *Nostoc* sp. BTA97 and *Anabaena* sp. BTA990 in culture conditions. Appl Biochem Biotechnol 176:1950–1963. doi: 0.1007/s12010-015-1691-2

Vasudevan V, Stratton RW, Pearlson MN, et al. (2012) Environmental performance of algal biofuel technology options. Environ Sci Technol 46:2451–2459. doi: 10.1021/es2026399

Visser PM, Ibelings BW, Bormans M, Huisman J (2016a) Artificial mixing to control cyanobacterial blooms: a review. Aquat Ecol 50:423–441. doi: 10.1007/s10452-015-9537-0

Visser PM, Verspagen JMH, Sandrini G, et al. (2016b) How rising CO_2 and global warming may stimulate harmful cyanobacterial blooms. Harmful Algae 54:145–159. doi: 10.1016/j.hal.2015.12.006

Wang H, Zhong G, Yan H, et al. (2012) Growth control of cyanobacteria by three submerged macrophytes. Environ Eng Sci 29:420–425. doi: 10.1089/ees.2010.0286

Wang L, Liang W, Yu J, et al. (2013) Flocculation of *Microcystis aeruginosa* using modified larch tannin. Environ Sci Technol 47:5771–5777. doi: 10.1021/es400793x

Wang X, Wang X, Wei Z, Zhang S (2018) Potent removal of cyanobacteria with controlled release of toxic secondary metabolites by a titanium xerogel coagulant. Water Res 128:341–349. doi: 10.1016/j.watres.2017.10.066

Waterbury J, Valois F (1993) Resistance to co-occurring phages enables marine *Synechococcus* communities to coexist with cyanophages abundant in seawater. Appl Environ Microbiol 59:3393–3399.

Westrick JA, Szlag DC, Southwell BJ, Sinclair J (2010) A review of cyanobacteria and cyanotoxins removal/ inactivation in drinking water treatment. Anal Bioanal Chem 397:1705–1714. doi: 10.1007/s00216-010-3709-5

WHO (2003) Algae and cyanobacteria in fresh water. Guidel. safe Recreat. water Environ. World Health Organization, Geneva, Switzerland, pp 136–158

WHO (2021) Toxic cyanobacteria in water, 2nd ed. doi: https://doi.org/10.1201/9781003081449

WHO (1999) Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management. E & FN Spon, London

WHO (2018) WHO recommendations on scientific, analytical and epidemiological developments relevant to the parameters for bathing water quality in the Bathing Water Directive (2006/7/EC).

Wijffels RH, Kruse O, Hellingwerf KJ (2013) Potential of industrial biotechnology with

cyanobacteria and eukaryotic microalgae. Curr Opin Biotechnol 24:405–413. doi: 10.1016/j.copbio.2013.04.004

Wood SA, Heath MW, Kuhajek J, Ryan KG (2010) Fine-scale spatial variability in anatoxin-a and homoanatoxin-a concentrations in benthic cyanobacterial mats: implication for monitoring and management. J Appl Microbiol 109:2011–2018.

Ytrestøyl T, Aas TS, Åsgård T (2015) Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. Aquaculture 448:365–374. doi: 10.1016/j.aquaculture.2015.06.023

Zhao J, Li X-F, Ren Y-P, et al. (2012) Electricity generation from Taihu Lake cyanobacteria by sediment microbial fuel cells. J Chem Technol Biotechnol 87:1567–1573. doi: 10.1002/jctb.3794

CHAPTER III

Are Cyanobacteria a nearly immortal sources of high market value compounds?

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Contributions: I. P. E. M. wrote the manuscript; I. P. E. M. and T. V. acquired the experimental data and performed the data analysis; A. P. M. F. quantified the pigments; M. M. and S. F. helped with the data analysis; J. S., F. J. M. G., S. P. V., and J. L. P. supervised

Abstract: Cyanobacteria are increasingly used in biotechnological applications. However, a deeper understanding of the growth patterns and pigment production is required to support the selection of the most beneficial species and conditions for industrial production. In this work, the growth and pigment production (i.e. chlorophyll *a* and C-phycocyanin) of three cyanobacterium species was evaluated following a three-fold aim. First, we compared among a species commonly selected for exploitation (*Arthrospira platensis*) and two alternative species (*Anabaena cylindrica* and *Nostoc muscorum*) to gain an insight on how meaningful can species selection be for pigments exploitation. Then, we followed pigment production in the long-term to demonstrate that yields can be maximised if such baseline knowledge is available prior to the implementation of exploitation settings. Finally, we compared among different methods (spectrophotometry and fluorimetry) to understand whether there is an appropriate proxy of biomass increase and pigment production that can be used for monitoring purposes in exploitation settings. The three cyanobacteria species showed high longevity proving to be able to grow for more than 100 days without any additional supplementation. Yet, the maximum quantum yield of PSII (F_v/F_m) revealed that their photosynthetic efficiency varied over time with a clear decrease after two months in *A. cylindrica* and *A. platensis*. The pigment analysis showed a heterogeneous pattern during the growth period of all the three species, that could only be captured by the parameter F_v/F_m, but only for *A. cylindrica* and *N. muscorum*, in some stages of the culture period. *N. muscorum* was found to be the best chlorophyll *a* and C-phycocyanin producer, with the production peaking for all species at defined time periods within the growth profile. Overall, this study supports that exploitation efficiency is dependent on the selection of species and on the previous knowledge of pigments production dynamics of the selected species.

Keywords: cyanobacteria; growth curves; pigment production; chlorophyll *a*; C-phycocyanin

III-1. Introduction

Cyanobacteria can be considered as a source of compounds with biological activity and as a promising feedstock for many applications. Lipids, pigments, and proteins from cyanobacteria are known for their antibacterial, antifungal, antiviral, and antitumor activities (Eriksen 2008; Sekar and Chandramohan 2008). Moreover, their important role as CO_2 mitigators, biofertilizers, food supplements for human consumption and aquaculture, bioremediation agents, and bioenergy producers demonstrates the overall importance of cyanobacteria as a potential asset (Pandey et al. 2013; Kamal and Ahmad 2014). However, amongst more than 10 000 species of cyanobacteria, only *ca.* 1000 have been partly characterized and very few are currently cultured at an industrial scale (Raheem et al. 2018). This essentially means that the knowledge about the biotechnological potential of these organisms is still limited, but the market for cyanobacterial products is large and its value has been increasing: USD 18.5 million in 2018 for phycobiliproteins with industry expecting a consumption higher than 200 tons by 2025; and USD 4.7 billion by 2024 are expected for the market of food colorants, where chlorophylls are dominant (https://www.gminsights.com, assessed on May 17th 2021).

Pigments are to date the compounds produced by cyanobacteria with the highest commercial value, particularly carotenoids and phycobiliproteins (Koller et al. 2014). They have the potential to replace several synthetic dyes that can induce toxic effects and can be used as natural colorants for textiles, food, drugs, and cosmetics (Sekar and Chandramohan 2008). Phycobiliproteins are water-soluble fluorescent pigment-protein complexes that act as secondary light-harvesting components in the photosynthetic process (Pandey et al. 2013). Phycocyanin, a blue pigment-protein of the phycobiliprotein family, is mainly found in cyanobacteria and is conventionally defined as C-phycocyanin (Koller et al. 2014). Besides its use as natural dye, its biological activity makes it also suitable for pharmaceutical and biomedical applications. The antitumor activity against several types of cancer cell lines (Jiang et al. 2017), together with the antioxidant nature (Renugadevi et al. 2018), anti-inflammatory activity (González et al. 1999) and activity as a stimulator of the immune system (Romay et al. 1998), render phycobiliproteins as highly desirable. Like phycocyanin, chlorophyll *a* (Chl *a*) is a pigment that absorbs light mainly in the blue zone and to a minor extent in the red zone of the electromagnetic spectrum. This pigment can be used as food ingredient (E140) (Hosikian et al. 2010), in hygiene products as a deodorant, and also in

medical applications as a chemo-preventive agent (Montgomery and Nachtigall 1950; Díaz et al. 2003; Koller et al. 2014).

The industrial cultivation of cyanobacteria is already taking place in worldwide. However, its production is presently focused on a (too) short list of species (e.g. *Arthrospira* sp., *Aphanizomenon* sp., *Nostoc* sp.), which may not be the most adequate strains for the efficient extraction of the target molecules. Some studies have investigated the impact of the culturing parameters, such as nutrient concentrations as nitrogen supply (e.g. Loaiza et al. 2016), light (e.g. Poza-Carrión et al. 2001), and temperature (e.g. Kumar et al. 2011) on cyanobacteria' productivity. However, to our knowledge this is the first study investigating the longevity of cyanobacteria under straightforward culturing conditions with no nutrient re-supply as well as the fluctuations in the production of pigments throughout the growth cycle. These are two obvious variables for the optimization of cultures explored at larger scales for commercial purposes. In fact, nutrient supplementation is costly and on the other hand it can cause changes in physiology, which will reflect in an altered productivity. This includes the effect in the production of the molecule of interest and the corresponding expected extraction yield. In this context, the present study aimed at monitoring three filamentous cyanobacteria, *Anabaena cylindrica*, *Nostoc muscorum*, and *Arthrospira platensis*, regarding their growth kinetics, photosynthetic efficiency and pigment production. On the one hand, this monitoring endeavor was expected to demonstrate that an anticipated knowledge on the pigment yield dynamics can support the optimization of exploitation routines at an industrial level; on the other hand, it serves as the basis to gain a detailed insight on whether pigment exploitation can be guided by easily acquirable monitoring parameters based on spectrophotometric measurements or fluorimetry. The widely used species *A. platensis* was included in the study to provide performance comparability with the less conventional alternative species *A. cylindrica* and *N. muscorum* under the hypothesis that these latter may represent more efficient sources for pigments production, thus demonstrating the importance of a rational species selection when implementing exploitation settings.

III-2. Material and Methods

III-2.1. Cyanobacteria cultures and culturing conditions

Three species of filamentous cyanobacteria were used, *Anabaena cylindrica* PCC 7122, *Nostoc muscorum* UTAD_N213, and *Arthrospira platensis* UTEX LB 2340. Non-axenic cultures were maintained in borosilicate flasks, initially filled with 5 L of culture medium, in triplicate. For *A. cylindrica* and *N. muscorum*, the Woods Hole MBL synthetic medium was used (Nichols 1973; Table III-S2, Annex I) to provide its long-term support to optimal growth of these cyanobacterial strains in our laboratory. *A. platensis* was rather cultured in Spirulina medium (SAG, 2008; Table III-S2, Annex I), as it was found to provide optimal growth for this strain in preliminary trials compared to MBL (note that we aimed to compare between a conventional and alternative species, hence the adequacy of establishing comparable growth conditions). Borosilicate flasks (5 L capacity) were inoculated using 150 ml of an 9 day-old inoculum to an initial optical density (OD) at 750 nm of 0.05 ± 0.01, then incubated at 26 ± 2°C under a 16h-light/8h-dark photoperiod cycle, with a light intensity of 37 µmol photons m^{-2} s^{-1} (Quantum meter MQ-200, Apogee Instruments, Logan, Utah, USA), equal to 2300 lux, provided by cool white fluorescent tubes and constant aeration. Samples for growth rate, photosynthetic yield (2 mL) and pigment production (*ca.* 40 mL) (see below) were collected through the experimental period inside a flow chamber and using sterile material.

III-2.2. Spectrophotometric measurements

The culture growth was monitored three times a week by collecting optical density (OD) records at different wavelengths (440, 480, 620, 675, and 750 nm) to cover the absorption peaks of different pigments while controlling for total particulate matter. OD_{750} retrieved turbidity without the influence of pigments, thus it was interpreted *a priori* as a proxy for cell suspension concentration. The other wavelengths gave insights regarding specific pigments: 440 and 675 nm for Chl *a* (Agusti and Phlips 1992), 480 nm for carotenoids (Chen et al. 2017), and 620 nm for C-phycocyanin (Paswan et al. 2016).

III-2.3. Reliable endpoints to monitor culture growth and photosynthetic yield

An imaging chlorophyll fluorometer (Open FluorCAM 800-O/1010, Photon Systems Instruments; Brno, Czech Republic) was used to capture the maximum photosynthetic

quantum yield of Photosystem II (F_v/F_m) after 15 min of dark adaptation. The excitation light peaked at 621 nm with a 40 nm band width, and saturating pulses were applied with intensity of about 7000 µmol quanta m^{-2} s^{-1} and the duration of 0.8 s. Chl *a* fluorescence was captured by a CCD camera (CCD381) with a F1.2 (2.8–6 mm) objective resulting in images with 512 × 512 pixels and a spectral range of 695–780 nm. Images were processed using the FluorCam7 software (Photon Systems Instruments; Brno, Czech Republic). Fluorimetric measurements were made on each culture, three times a week, in triplicate. For this purpose, aliquots of 2 mL of each culture were transferred into 6-well plates. The ratio F_v/F_m was calculated following Eq. III-1.

$$\frac{F_v}{F_m} = \frac{F_m - F_o}{F_m} \qquad \textbf{Eq. III-1}$$

F_o is the minimum fluorescence in dark-adapted sample and F_m is the maximal fluorescence after exposure to a saturating light pulse. F_o is also known as a proxy of photosynthetic biomass, allowing monitoring the culture growth over time. As biomass and hence the fluorescence signal intensity increased over time, the sensitivity settings of the instrument were adjusted correspondingly. Measurements of the fluorescence signal before and after the adjustment were corrected accordingly. The resulted growth curve was used to estimate species-specific growth curve parameters within defined time periods.

Growth curves and corresponding parameters were obtained by fitting a logistic function to biomass data, by using MS Excel Solver (Eq. III-2):

$$N(t) = \frac{k}{1 + \frac{k - No}{No} e^{-r(t)}} \qquad \textbf{Eq. III-2}$$

N_0 is the initial cell density [F_o] at time 0, N(t) cell density [F_o] at time t, k is the maximum density [F_o], r is the growth rate [d^{-1}], and lag phase [d] the time before exponential growth starts. The doubling time [d] (Eq. III-3) and doubling *per* day [d^{-1}] were then calculated (Eq. III-4).

$$\text{Doubling time} = \frac{\ln(2)}{r} \qquad \textbf{Eq. III-3}$$

$$\text{Doubling per day} = \frac{1}{\text{Doubling time}} \quad \textbf{Eq. III-4}$$

III-2.4. Pigment quantification

The extraction and quantification of Chl *a* and C-phycocyanin was performed every 15 days for each cyanobacteria culture. Approximately 40 mL of each culture was recovered by centrifugation (4111 ×g for 5 min; Eppendorf 5810 R). The pelleted biomass was stored at -20 ºC until pigment quantification. Pure ethanol and 150 mM of sodium phosphate buffer (pH 7) was used to extract Chl *a* and C-phycocyanin, respectively (Saran et al. 2016; Yéprémian et al. 2016). These solid-liquid extractions (1:10, w:v) were performed at 35 ºC for 50 minutes, with an agitation of 1500 rpm in a thermomixer (Eppendorf ThermoMixer® C). All extractions were performed in triplicate. For the extraction of C-phycocyanin from *N. muscorum*, a second extraction step was needed, using the biomass left overnight after the first extraction, and applying the exact same conditions and the same amount of fresh solvent (150 mM sodium phosphate buffer, pH 7).

At the end of the solid-liquid extraction, the cell suspensions were centrifuged at 9500 ×g for 10 minutes in a VWR MicroStar 17 centrifuge, being the supernatant separated from the cell debris and used for pigments quantification. For each extract, the absorption spectra were collected between 200 and 700 nm using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek), in duplicate. The quantification of Chl *a* and C-phycocyanin was done using previously defined calibration curves (R^2 = 0.9805 and R^2 = 0.9912, built following optical density measurements at 667 nm and 615 nm, respectively). The yield of extraction ($mg_{pigment}\ g_{dry\ weight}^{-1}$) was calculated according to Eq. III-5.

$$\text{Yield } (mg_{pigment}\ g_{dry\ weight}{}^{-1}) = \frac{\text{pigment concentration} \times \text{extract volume}}{\text{mass of dry cyanobacteria}} \quad \textbf{Eq. III-5}$$

III-3. Results

III-3.1. Optical density measurements of cyanobacteria in the long-term

The profiles of the cultures monitored spectrophotometrically showed that all optical densities of the three cyanobacteria strains continuously, and generally exponentially, increased for more than 100 days, without reaching a stationary phase (panels a and b in Figures III-1-3). Despite the use of different wavelengths for this monitoring stage, all

showed the same tendency (Figures III-1-3a). In this way, we considered OD at 750 nm as a representative for further interpretation and detailed comparison among species. *A. cylindrica* and *A. platensis* showed similar $OD_{750\ nm}$ profiles. At the end of the experiment *A. platensis* showed the higher values of absorbance, followed by *A. cylindrica* and finally *N. muscorum*. Adjusted exponential equations described accurately the increase in absorbance of these species as denoted by the coefficients of determination higher than 0.98, obtained in all cases (Figures III-1-3 b).

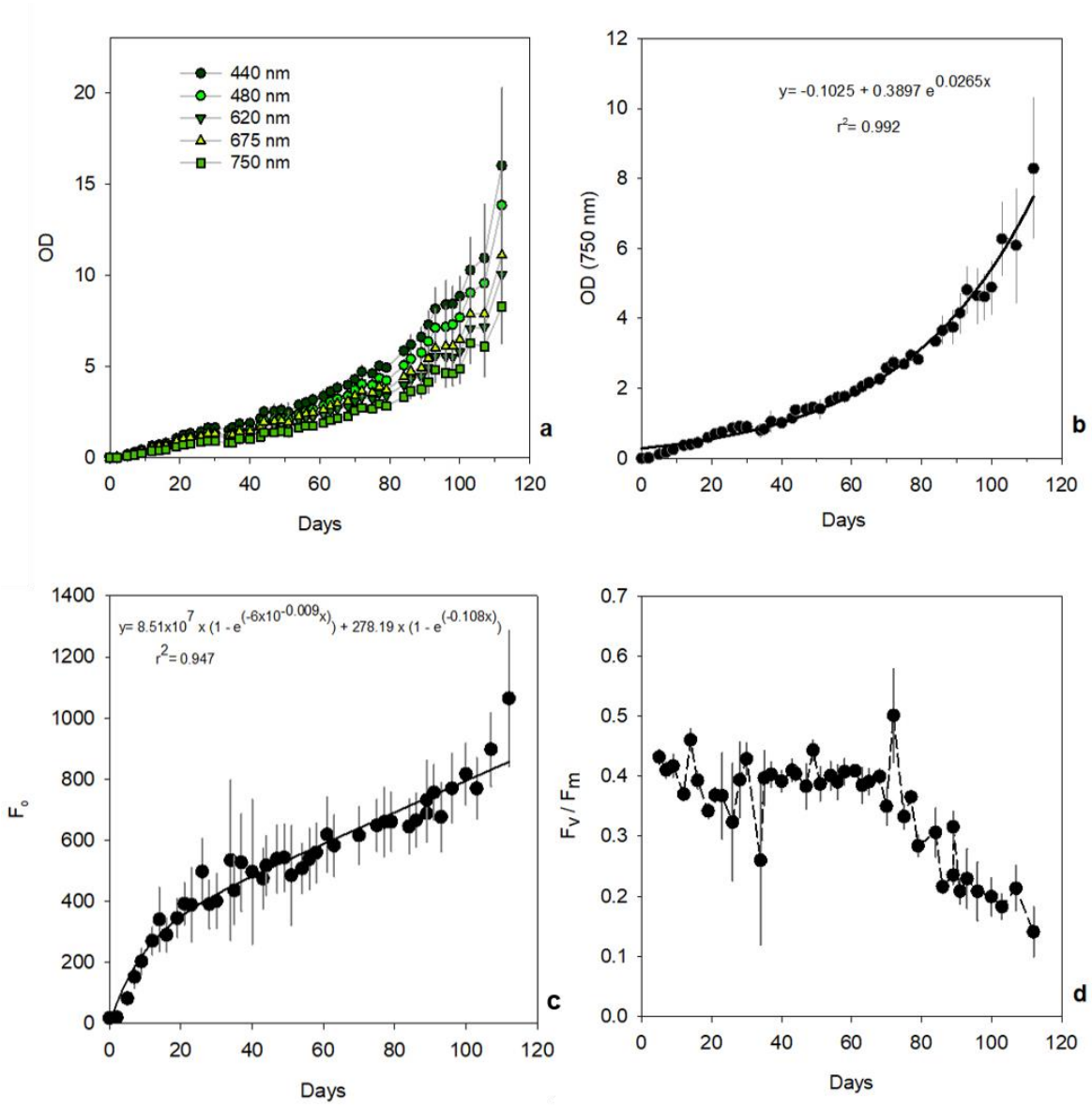

Figure III-1: Profiling of *A. cylindrica* throughout a monitoring period of 112 days, regarding (a) multiple OD measurements; (b) best fitted curve modelling through the least-squares method, based on OD_{750}; (c) best fitted curve based on the F_0 value; (d) dynamics of PSII efficiency (F_v/F_m) (only data with $F_0 > 100$ are depicted). In all graphics, marks represent the mean of three replicates and the

error bars represent the standard deviation. In panels a and d, a line was added joining the marks within each series when deemed necessary for clarity purposes, not reflecting any fitted model. In panels b and c, the solid black line represents the exponential model best fitted to the experimental data through the least-squares method.

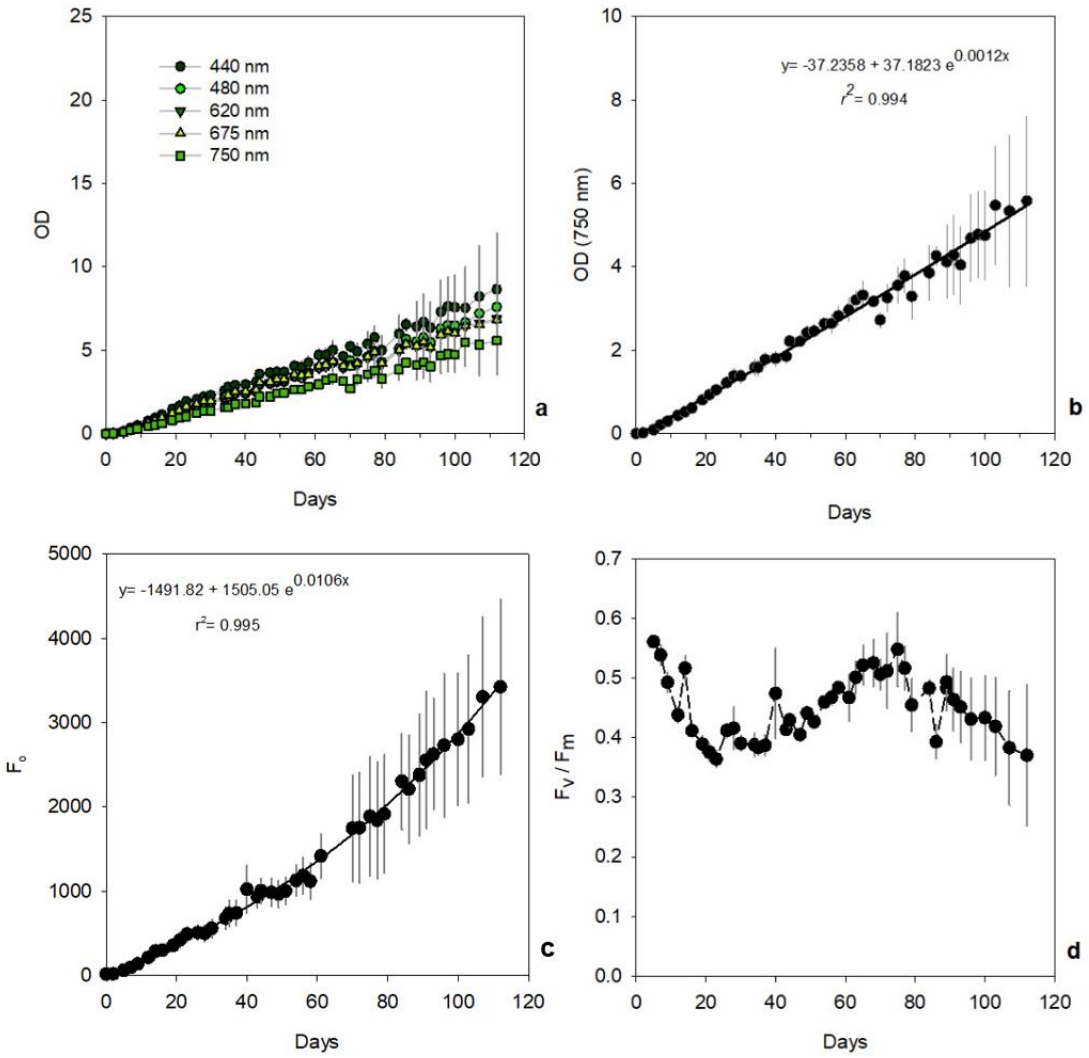

Figure III-2: Profiling of *N. muscorum.* throughout a monitoring period of 112 days, regarding (a) multiple OD measurements; (b) best fitted curve modelling through the least-squares method, based on OD_{750}; (c) best fitted curve based on the F_0 value; (d) dynamics of PSII efficiency (F_v/F_m) (only data with $F_0 > 100$ are depicted). In all graphics, marks represent the mean of three replicates and the error bars represent the standard deviation. In panels a and d, a line was added joining the marks within each series when deemed necessary for clarity purposes, not reflecting any fitted model. In panels b and c, the solid black line represents the exponential model best fitted to the experimental data through the least-squares method.

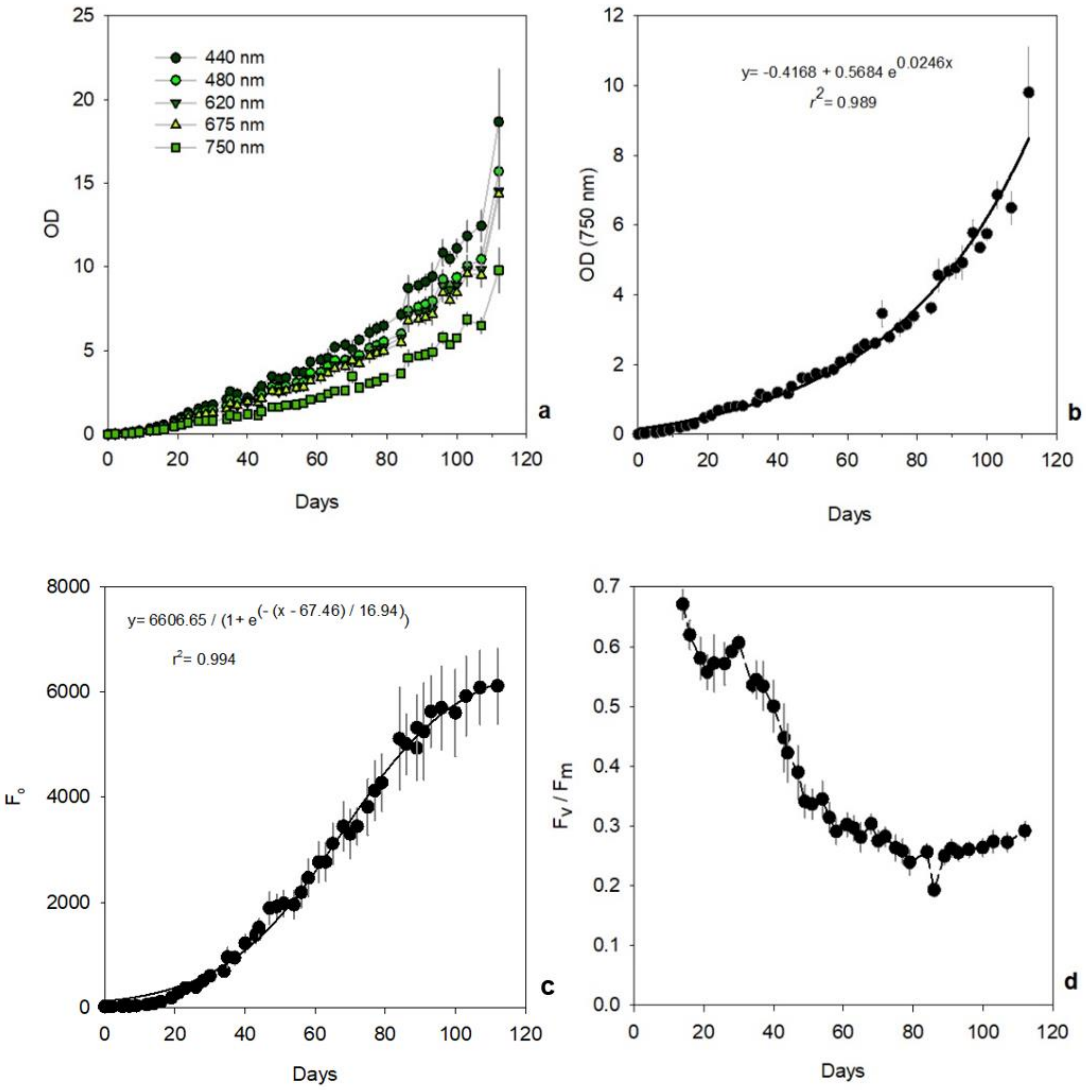

Figure III-3: Profiling of *A. platensis* throughout a monitoring period of 112 days, regarding (a) multiple OD measurements; (b) best fitted curve modelling through the least-squares method, based on OD_{750}; (c) best fitted curve based on the F_0 value; (d) dynamics of PSII efficiency (F_v/F_m) (only data with $F_0 > 100$ are depicted). In all graphics, marks represent the mean of three replicates and the error bars represent the standard deviation. In panels a and d, a line was added joining the marks within each series when deemed necessary for clarity purposes, not reflecting any fitted model. In panels b and c, the solid black line represents the model best fitted to the experimental data through the least-squares method.

III-3.2. Growth and health status of cyanobacteria in the long-term

Regardless the common use of optical density to describe cell growth, this technique does not differentiate living from dead cells. For this reason, other parameters (i.e. F_o and F_v/F_m) with better physiological relevance, where also monitored during the experiments. The values of F_o were used as a proxy of the photosynthetically active biomass, due to its high sensitivity and specificity towards Chl *a*. These data, illustrated in Figures III-1-3c, also showed that all the species continuously grew through 112 days. Despite the fluctuations within and between species, it is clear that all cyanobacteria were capable of withstanding growth for this large period of time without changing culture conditions and, remarkably, without nutrient supplementation. In the last days of the experiment, *A. platensis* started to stabilize its growth, indicating the reaching of a stationary phase (Figure III-3c). However, *A. platensis* was the species showing better biomass outcomes compared to the other two species. *A. cylindrica* was the one that reached lower values of F_o, denoting a flatter growth dynamics (Figure III-1c).

A detailed inspection of the F_o values through time (Figures III-1-3c) allowed the identification of what resembles a short plateau stage occurring between day 40 and 60 (especially visible in the case of *N. muscorum* and *A. platensis*), followed by a renewed acceleration of biomass production (Figures III-1-3c). This specific feature found in the cyanobacteria growth data prevented the feasible adjustment of the appropriate logistic function for *A. cylindrica* and consequently the comparative analysis of growth dynamics. Therefore, in order to estimate growth parameters for species comparison, we artificially limited the fitting to data retrieved in the first half of the experimental period (until day 56). In this first period, the growth of all cyanobacterium species is feasibly described by logistic equations (r^2= 0.919 – 0.993; Figure III-4 and Table III-1). Under these limited circumstances, *A. platensis* was the species with the longest lag phase of 1.73 days while no lag phase was verified for the other two species. This resulted in the lowest growth rate during the first 23 days, then compensated by a sharp acceleration (Figure III-4). As a result *A. platensis* presented a doubling time of 5.57 days, which allowed it to reach the highest value of maximum growth (K = 2425.61 [rel.unit]) when comparing among the three species (Table III-1). On the contrary, *A. cylindrica* showed the highest increase in biomass during the first 20 days, but after that the growth decelerated and the species recorded the lowest values of maximum growth (K = 471.17 [rel.unit]). This fast initial increase in biomass

translated into the highest growth rate (0.24 d^{-1}) and doubling cells *per* day (0.34 d^{-1}). *N. muscorum* behaviour was intermediate between *A. platensis* and *A. cylindrica*.

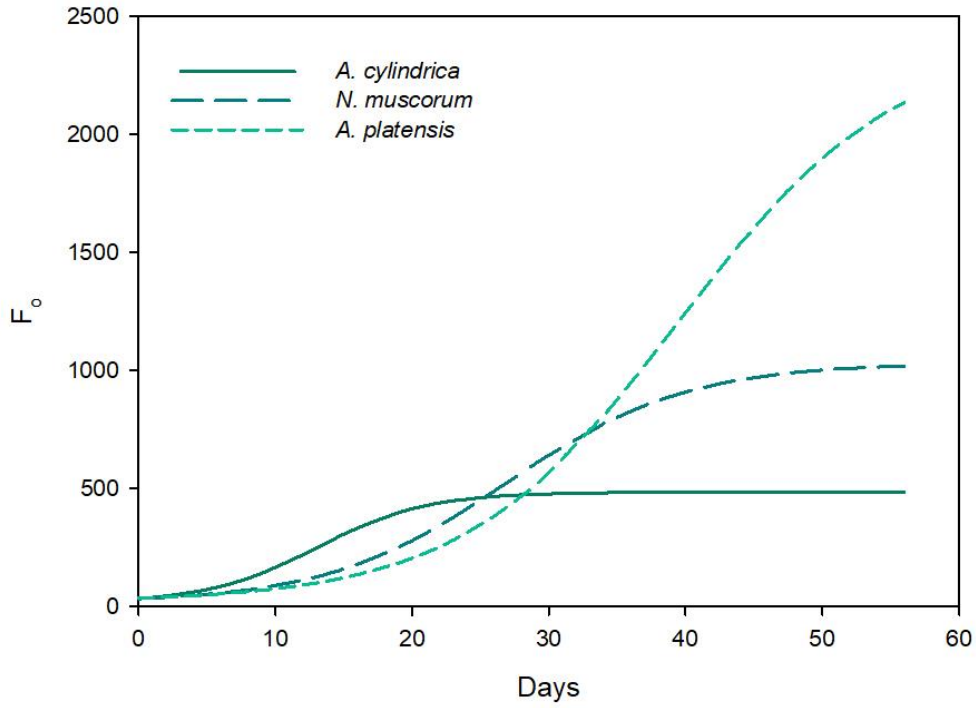

Figure III-4: Growth kinetics estimated for the three species using F_o values following the fitting of logistic models to the experimental data retrieved through the first 56 days of the experience.

Table III-1: Growth parameters of the three species estimated following the fitting of logistic equations to experimental data retrieved through the first 56 days of the experiment. K stands for the maximum growth, r for growth rate, and lag for the lag phase; these parameters are expressed in days.

	A. cylindrica	***N. muscorum***	***A. platensis***
K [rel. unit]	471.17	1653.55	2425.61
r (d^{-1})	0.24	0.10	0.12
Lag (d)	0.00	0.00	1.73
Doubling time (d)	2.94	7.11	5.57
Doublings per day (d^{-1})	0.34	0.14	0.18
Model equation	y= 471.17/(1+471.17-17.21/17.21 $e^{-0.24x}$)	y= 1653.55/(1+1653.55-16.78/16.78 $e^{-0.10x}$)	y= 2425.61/(1+2426.61-20.57/20.57$e^{-0.12x}$)
Model correlation (r^2)	0.919	0.956	0.993

The maximal PSII efficiency (F_v/F_m) was used as proxy for photosynthetic efficiency, thus an indicator of health status of the cultures. The F_v/F_m differed appreciably among the cultures and through the experiment (Figures III-1-3d). *A. cylindrica* started with an F_v/F_m record of 0.43 at day 5, which remained stable until approximately day 70. After that day, F_v/F_m started to decrease until reaching the value of 0.14 in the last day of the experiment. In contrast, *N. muscorum* showed a periodic pattern. While starting with F_v/F_m values of 0.56 at day 5, the records slowly decreased to 0.36 at day 23, then increased to a maximum value of 0.55 at day 75 and decreasing again to 0.37. *A. platensis* was the species showing the most prominent declining of F_v/F_m throughout the experimental period: it recorded initial higher values of F_v/F_m (0.67) at day 14, declining to average values of 0.27 during the last days.

III-3.3. Pigment production

Despite the continuous growth observed for the three cultures (Figures III-1-3), this trend did not hold for pigment production (Figure III-5). Yields of C-phycocyanin and Chl *a* have markedly fluctuated throughout the monitored period for all cyanobacteria but following different patterns depending on the species. The concentration of C-phycocyanin was always higher than the one of Chl *a*. More specifically, the production of C-phycocyanin by *A. cylindrica* was generally stable during the first half of the experiment (reaching 136.6 $mg_{C\text{-}phycocyanin}\ g_{dry\ weight}^{-1}$) and started to decrease at day 56. At the first days, *A. cylindrica* was the best producer of C-phycocyanin, but was then surpassed by *N. muscorum*. C-phycocyanin production peaked in two specific moments of the growth of the other two species: at day 30 and then more expressively at day 85 for *N. muscorum*; at day 45 and then more expressively at day 85 for *A. platensis*. The highest C-phycocyanin yield was recorded by *N. muscorum* throughout the culturing period, reaching a maximum of 249.4 $mg_{C\text{-}phycocyanin}\ g_{dry\ weight}^{-1}$. Clearly, *N. muscorum* was the best Chl *a* and C-phycocyanin producer, with a peak around days 28 and 70, for Chl *a* (*ca.* 12 $mg_{Chlorophyll\ a}\ g_{dry\ weight}^{-1}$) and at days 28 and 84 for C-phycocyanin (*ca.* 201.5 and 249.4 $mg_{C\text{-}phycocyanin}\ g_{dry\ weight}^{-1}$). The C-phycocyanin production by *N. muscorum*, started to decrease after day 84 and at the end of the experiment it reached a value similar to the ones achieved by day 14 (*ca.* 99.9 $mg_{C\text{-}phycocyanin}\ g_{dry\ weight}^{-1}$). *A. platensis* showed a similar profile for C-phycocyanin, with two

moments of higher production (day 43 with *ca.* 61.4 $mg_{C\text{-}phycocyanin}$ $g_{dry\ weight}^{-1}$, and day 84 with *ca.* 77.3 $mg_{C\text{-}phycocyanin}$ $g_{dry\ weight}^{-1}$). However, the production of Chl *a* by *A. platensis* showed a different pattern, increasing continuously throughout time, reaching the highest yield at the end of the experiment of *ca.* 4.1 $mg_{Chlorophyll\ a}$ $g_{dry\ weight}^{-1}$. *A. cylindrica* reached its maximum Chl *a* concentration after the first peak reached by *N. muscorum*, at day 56 (*ca.* 10.1 $mg_{Chlorophyll\ a}$ $g_{dry\ weight}^{-1}$), and then the production decreased until reaching approximately 4.1 $mg_{Chlorophyll\ a}$ $g_{dry\ weight}^{-1}$ by the end of the monitoring period, which was approximately the same concentration observed at the beginning of the experiment, by day 14.

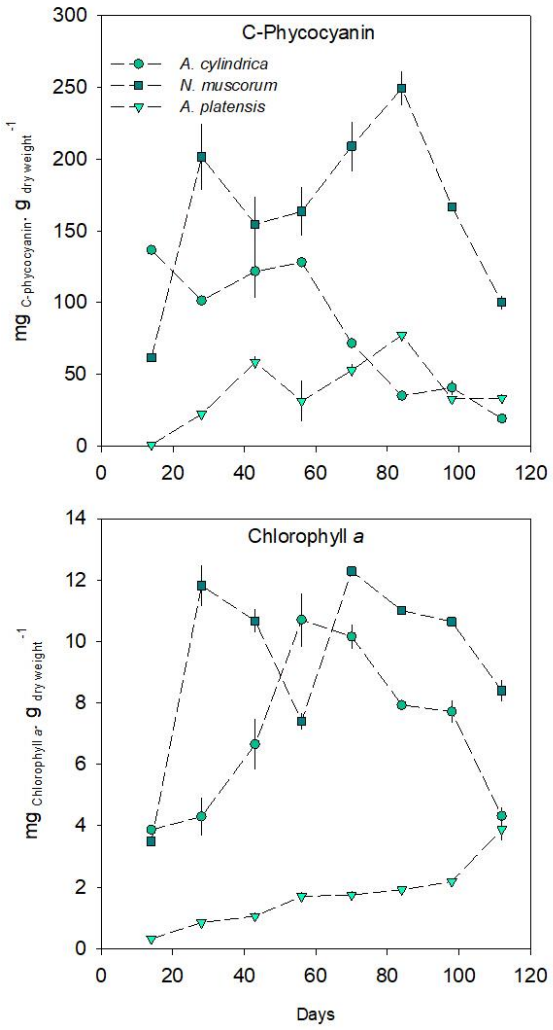

Figure III-5: Yield of C-phycocyanin and chlorophyll *a* for the three species throughout a monitoring period of 112 days. In all, marks represent the mean of three replicates and the error bars represent the standard deviation. A line was added joining the marks within each series for clarity purposes.

III-4. Discussion

The ground explored in this work entails the interplay between the patterns of growth and pigment production in cyanobacteria. The production of C-phycocyanin and Chl *a* did not show a clear pattern during the growth period of each species, and the production dynamics was clearly different among species. This dynamics is of strategic importance to improve pigment production at a commercial scale, because it would allow to identify the growth state where compound production is the highest, leading to an improved harvesting efficiency. Despite the relevance of a deep understanding of the growth dynamics of species exploited for biotechnological applications, very few studies have been published addressing cyanobacteria growth curves in detail. In this way, and to the best of our knowledge, this is one of the few works demonstrating the extraordinary longevity of cyanobacterium cultures (more than 100 days with no nutrient supplementation), which is a promising feature for different areas where these organisms can be exploited. Another example of the longevity can be found for the terrestrial cyanobacterium *Nostoc* sp., which is able to grow for more than 140 days with no supplementation (Arai 2009). At an industrial scale, such feature is very interesting because the culture medium does not need to be replaced or supplemented frequently, allowing to save costs in consumables and in labour.

As many studies demonstrate, different species have different pigment contents and their production is dependent on the culture conditions (Poza-Carrión et al. 2001; Loaiza et al. 2016; Jaiswal et al. 2018) and on the growth phase (Jaiswal et al. 2018). Knowledge on which species is the best producer of a specific compound of interest and on the growth phase when such production is maximal, are critical variables. However, such a systematic development of the exploitation process seems to have been neglected, at least considering what is pictured in the dedicated literature. The majority of the studies do not evaluate the production of pigments through the growth curves, considering only specific days conveniently defined (Kumar et al. 2011; Simeunovic et al. 2012; Khazi et al. 2018) or the exponential phase before an hypothesised stationary or decline phase as it would be typical for microalgae (Tiwari et al. 2015; Loaiza et al. 2016). Interestingly, the species studied in the present work demonstrate a continuous increase in absorbance that lasts for at least the 112 days of the experiment. These data from optical density are generally corroborated with the data from F_o (the relation between these two variables is depicted in Figure III-S1, Table III-S3 (Annex I)) showing an increase in fluorescence during the whole experiment,

although *A. platensis* decelerated its growth in the last days (Figure III-3c) and *A. cylindrica* presented a less impressive cell biomass (Figure III-1c), though with a growing trend.

The minimal fluorescence F_o can be used as a proxy for microalgal biomass (e.g. Honeywill et al. 2002; Jesus et al. 2006), allowing to feasibly model a curve which resembles more a biological growth curve (i.e. bearing an exponential and then an approximation to a stationary phase) than that allowed by the data obtained from OD (showing a continuous growth acceleration, with no apparent biological significance). Indeed, high cell densities and particular cell morphologies can interfere with absorbance methods while the data from F_o provided interesting results. Additionally to this overall picture, the growth curves of the three species showed a small plateau near days 40-60, and then restarted to grow, especially recognisable in *N. muscorum* and *A. platensis*.

The importance of this finding can be reasoned within at least two contexts. First, at a more practical level, it shows that there are two phases of accelerated growth (or exponential growth stages) within the growth curves of these cyanobacteria species. These are undistinguishable using typical monitoring tools such as OD, but they can be accurately identified using fluorescence measurements, greatly supporting the sustainable large-scale exploitation of cyanobacteria. For example, we could identify *A. cylindrica* as the most demanding species in terms of initial nutrient supply as this was the species showing the highest growth rates before reaching the first plateau and that reaching the plateau (or the system's carrying capacity) sooner. The most standard species *A. platensis*, on the contrary, faced a *lag* phase (recognised only using F_o measurements) before the first exponential phase. However, *A. platensis* was clearly the best performer regarding biomass production in the long-term as recognisable from maximum growth rates and the F_o records at the end of the experiment. Regardless of the differences among species, a note is worth making regarding the short-term growth rates found in this study for the three species (0.10-0.24; Table III-1), which are lower than the typical records found in the literature for cyanobacteria, e.g. 0.89 for *Anabaena flos-aquae*, 1.15 for *Microcystis* sp., 0.7 and 2.8 for *Synechocystis* sp. PCC6803 (Gotham and Rhee 1981; Kim et al. 2015). Distinct species, culture conditions and assessment periods likely explain the differences noted with the literature. The second arena where the finding of the intermediate plateau gains relevance is its biological or ecophysiological meaning. This intermediate plateau suggests that growth becomes limited at some point, likely due to essential nutrients limitation (e.g. iron and

molybdenum; Rueter and Petersen 1987; Gonzáles et al. 2018), but then the cyanobacteria are able to overcome through some metabolic shift and restart growth which was indeed confirmed in parallel metabolomics profiling studies (discussed later in Chapter V), which reinforces further the importance of using a monitoring tool that can identify the specific features of the growth allowing to predict the production of target compounds. Indeed, specific compounds of interest can be preferably or exclusively available for exploitation before or after the metabolic shift indicated by the growth plateau. It is worth remarking at this point that *N. muscorum* was the best pigment producer but not the best biomass yielder, which demonstrates that biomass production, is not a feasible proxy for pigment production. Thus, monitoring strategies intending to predict pigment production need to be carefully optimised to avoid lowering exploitation efficiencies.

Fluorimetry techniques can also give important insights on assessing the photosynthetic efficiency. These techniques allow an indication of the maximum quantum yield of the PSII and consequently insights on the photosynthetic efficiency, through the measurement of the parameter F_v/F_m. The F_v/F_m of the cultures tested herein was not constant during the entire experiment, with exception of *N. muscorum*, where the yield remained quite stable (0.45 ± 0.05). Common values of F_v/F_m for cyanobacteria range within 0.4 - 0.6 (Misumi et al. 2016), but the species of this study showed a higher range due to the long-term culturing (0.14 - 0.50 for *A. cylindrica*; 0.37 - 0.56 for *N. muscorum*; and 0.19 - 0.67 for *A. platensis*). The photosynthetic efficiency is a valuable parameter as it can allow the evaluation of the fitness of a culture. If cells are experiences stress, the yield decreases. Such stress conditions could be linked to the production of secondary metabolites or pigments like carotenoids, with high commercial value (Kultschar and Llewellyn 2018). However, this technique presents some limitations in the case of cyanobacteria, since the absolute level of F_v/F_m is not a reliable indicator of PSII function (Campbell et al. 1998; Ogawa et al. 2017). This happens because, in cyanobacteria, the phycobiliprotein fluorescence also contributes to F_o, especially at high concentrations, and the PSII accounts for only a small proportion of total Chl *a* (Campbell et al. 1998). This can cause a downward distortion of the levels of F_v/F_m, and consequently on conclusions regarding the photosynthetic activity (Campbell et al. 1998). Although bearing in mind these limitations, fluorimetry could be a useful tool to compare cultures within the same group, and these observations may reflect different cellular strategies to cope with nutrient depletion.

Comparing the F_v/F_m data with the production of pigments, no relationship was found for *A. platensis*. However, some inferences could be made for *A. cylindrica* when comparing these two endpoints. The C-phycocyanin and Chl *a* from *A. cylindrica* started to decrease approximately after day 60, which was in the accordance with a reduction of the values of F_v/F_m; however, until day 60 there is an increment in Chl *a*, which is not translate into F_v/F_m values. These data showed that for *A. cylindrica* F_v/F_m relates only to the production of C-phycocyanin. Regarding *N. muscorum*, there were two peaks in C-phycocyanin and Chl *a* production, around day 20 and day 80, but only one peak was observed for F_v/F_m around the day 80. These correlation between F_v/F_m data and pigment production was expected, since values of F_o, F_m, and F_v/F_m are influenced by the pigment concentration (Ting and Owens 1992). However, this correlation does not hold for *A. platensis*. Although presenting some limitations, F_v/F_m data showed that, despite the worst health status observed in the last days (especially in the case of *A. cylindrica*, Figure III-1d and *A. platensis*, Figure III-3d), the cells of the three species were alive during the 112 days. This highlights the remarkable capability of the cyanobacteria to cope with nutrient limitation. In this arena, metabolomic studies could help to explain the mechanisms of cyanobacteria to thrive in the long-term under nutrient starvation and a potential disturbance in osmotic balance, caused by the consumption of nutrients and the excretion of specific metabolites. Indeed, a possible path the organisms can trigger to tackle this challenge is the "salt-out" strategy, i.e. they produce compatible solutes to lower internal water potential in the presence of high external salinity (Bremer 2000).

When looking specifically to the pigment production profiles, it is clear that higher concentrations of C-phycocyanin compared to Chl *a* are produced, which was expected since phycobiliproteins are the major constituents of cyanobacteria that can reach up to 60% of the total protein content (Bogorad 1975). *N. muscorum* was clearly the best Chl *a* and C-phycocyanin producer. Jaiswal et al. (2018) quantified the pigment content of four cyanobacterium species over a period of 28 days and observed that the Chl *a* content decreased over time, while C-phycocyanin reached the highest level at day 14. In contrast with the present study, Jaiswal et al. (2018) likely observed higher production of both Chl *a* and C-phycocyanin in *Anabaena variabilis* (Chl *a* = 20 - 25 $\mu g.mg^{-1}$ DW; C- phycocyanin = 41.07 $\mu g.mg^{-1}$ DW) than in *Nostoc muscorum* (Chl *a* = 15 – 20 $\mu g.mg^{-1}$ DW; C-phycocyanin = 25 - 30 $\mu g.mg^{-1}$ DW), with lower C-phycocyanin concentration when

compared to the present study. Similarly, Loaiza et al. (2016) found that two strains of *Anabaena* are best Chl *a* and C-phycocyanin producers than two *Nostoc* strains. However, the values obtained in the exponential phase by Loaiza et al. (2016) (*Nostoc* sp.: Chl *a* = 2.37 - 2.56 μg.mL^{-1}, C-phycocyanin = 11.50 - 14.01 μg.mL^{-1}; *Anabaena* sp.: Chl *a* = 15.04 - 18.09 μg.mL^{-1}, and C-phycocyanin = 85.46 -102.90 μg.mL^{-1}) are also much lower than the ones obtained here (maxima in the present study: 99 μg.mL^{-1} Chl *a* and 1004 μg.mL^{-1} C-phycocyanin for *N. muscorum*; 80 μg.mL^{-1} Chl *a* and 1019 μg.mL^{-1} C-phycocyanin for *A. cylindrica*), even in the first extraction corresponding to day 14. The increase in *A. platensis* pigment content during the first days was corroborated by the study of Kumar et al. (2011), although these authors only evaluated their growth during 25 days. *Arthrospira* is one of the most well-known cyanobacteria, widely cultured at the industrial scale and commercialized. It is a source of valuable products such as proteins, essential amino acids, vitamins (e.g. B12), C-phycocyanin, β-carotene, and γ-linolenic acid (Stanic-Vucinic et al. 2018). However, in our study, the Chl *a* production by *A. platensis* was the lowest throughout the experiment among the three species compared. Other works (e.g. Simeunovic et al. 2012) also show that *Arthrospira* strains are worse phycobiliprotein producers when compared to *Anabaena* and *Nostoc* strains. C-phycocyanin from *A. platensis* is already widely used in several food products (e.g. gums, candies, frosting, ice cream and frozen desserts, coatings, and toppings) due to its blue colour, which is very difficult to find in nature (Stanic-Vucinic et al. 2018). This means that there is a market demand for C-phycocyanin and that species like *N. muscorum* could be a valuable alternative to *Arthrospira*. Other interesting species to include in studies regarding pigment production are *Synechocystis* sp. and *Synechococcus* sp.. *Synechocystis* sp. is a commonly used model in genetic editing studies, and this technology can be applied to enhance pigment production, namely specific carotenoids (i.e. myxoxanthophyll and zeaxanthin) (Lagarde et al. 2000). The marine cyanobacterium *Synechococcus* sp. presents different phenotypes with dominance of different pigments (Sliwinska-Wilczewska et al. 2020). Functional genomic information is also available for this species, and the genes involved in pigment production are also well known (Xia et al. 2018). In this sense, we suggest that industry should enlarge the scope of production to an array of species instead of focussing on a single one. The reasoning is two-fold. First, culturing conditions are mostly the same for most cyanobacteria thus the logistics behind stepping into multi-species culturing within a production facility is certainly not

complicated. Second, as proven by this limited comparison among three cyanobacteria species, because a given compound of interest would be produced better by a specific species or strain, at a given phase of the culture growth profile under the defined culturing conditions. The results presented herein are consistent with those obtained by Simeunovic et al. (2012), who observed that the qualitative and quantitative contents of different types of phycobiliproteins in cyanobacteria are dependent on both the strain and culture media. This is exactly why the systematic assessment of yields of strains of interest through time is a daunting, but a critical task prior to the implementation of industrial exploitation processes to ensure their economic sustainability. In addition, selecting a parameter that correlates feasibly to the production of pigments is very important and would allow a better monitoring of the cultures to select the best period for the pigment extraction.

III-5. Conclusion

In this work, growth and pigment production of three cyanobacterium species were assessed and compared. All three species showed impressive results as they were able to grow without supplementation for more than 100 days, raising questions on whether the consumption of nutrients is very low or if there are some cells dying and providing the nutrients for the remaining. The use of fluorimetric tools allowed understanding that although the cyanobacteria continuously grew, there was a degradation of their health status, especially prominent for *A. platensis*. In terms of biomass outcome, *A. platensis* showed the best profile. However, *N. muscorum*. was proven to be the most interesting species for industrial exploitation concerning pigments, since it presents high growth rates, as well as the best production of Chl *a* and C-phycocyanin.

During the growth period, pigment analysis showed a heterogeneous pattern, with peak moments of production for Chl *a* and C-phycocyanin. Unfortunately, the parameters used in these work (i.e. optical density, F_o, and F_v/F_m), did not relate consistently to pigment production, apart from F_v/F_m applying to *A. cylindrica* and *N. muscorum*. in some stages of the culture period. Calibration studies involving these parameters and other biomass proxies (e.g. weight or cell number) towards finding a platform for a feasible prediction of pigment production dynamics would be worth of future investment with practical application in the industry. It is worth remarking that optical density is a very commonly used parameter for the purposes, but the present study demonstrates the inadequacy of such a strategy.

References

Agusti S, Phlips EJ (1992) Light absorption by cyanobacteria: Implications of the colonial form. Limnol Ocean 37:434–441.

Arai M (2009) Cyanobacteria for Space agriculture on Mars. Biol Sci Sp 23:203–210. doi: 10.2187/bss.23.203

Bogorad L (1975) Phycobiliproteins and complementary chromatic adaptation. Annu Rev Plant Physiol 26:369. doi: doi.org/10.1146/annurev.pp.26.060175.002101

Bremer E (2000) Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes in bacteria. Bact Stress responses 79–97. doi: 10.1016/S1095-6433(00)80031-8

Campbell D, Hurry V, Clarke AK, et al. (1998) Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. Microbiol Mol Biol Rev 62:667–83. doi: 10.1128/MMBR.62.3.667-683.1998

Chen J, Wei D, Pohnert G (2017) Rapid estimation of astaxanthin and the carotenoid-to-chlorophyll ratio in the green microalga *Chromochloris zofingiensis* using flow cytometry. Mar Drugs 15:1–23. doi: 10.3390/md15070231

Díaz GD, Li Q, Dashwood RH (2003) Caspase-8 and apoptosis-inducing factor mediate a cytochrome c-independent pathway of apoptosis in human colon cancer cells induced by the dietary phytochemical chlorophyllin. Cancer Res 63:1254–1261.

Eriksen NT (2008) Production of phycocyanin - a pigment with applications in biology, biotechnology, foods and medicine. Appl Microbiol Biotechnol 80:1–14. doi: 10.1007/s00253-008-1542-y

Gonzáles A, Fillat MF, Bes MT, et al. (2018) The challange of iron stress in Cyanobacteria. In: Tiwari A (ed) Cyanobacteria. IntechOpen, pp 137–144

González R, Rodríguez's S, Romay C, et al. (1999) Anti-inflammatory activity of phycocyanin extract in acetic acid-induced colitis in rats. Pharmacol Res 39:55–59. doi: 10.1006/phrs.1998.0409

Gotham IJ, Rhee G -Y (1981) Comparative kinetic studies of nitrate-limited growth and nitrate uptake in phytoplankton in continuous culture. J Phycol 17:309–314. doi: 10.1111/j.0022-3646.1981.00309.x

Honeywill C, Paterson DM, Hagerthey SE (2002) Determination of microphytobenthic biomass using pulse-amplitude modulated minimum fluorescence. Eur J Phycol

37:485–492. doi: 10.1017/S0967026202003888

Hosikian A, Lim S, Halim R, Danquah MK (2010) Chlorophyll extraction from microalgae: A review on the process engineering aspects. Int J Chem Eng 1–11. doi: 10.1155/2010/391632

Jaiswal A, Koli DK, Kumar A, et al. (2018) Pigments analysis of cyanobacterial strains. Int J Chem Stud 6:1248–1251.

Jesus B, Perkins RG, Mendes CR, et al. (2006) Chlorophyll fluorescence as a proxy for microphytobenthic biomass: Alternatives to the current methodology. Mar Biol 150:17–28. doi: 10.1007/s00227-006-0324-2

Jiang L, Wang Y, Yin Q, et al. (2017) Phycocyanin: A potential drug for cancer treatment. J Cancer 8:3416–3429. doi: 10.7150/jca.21058

Kamal A, Ahmad IZ (2014) Cyanobacteria "the blue green algae" and its novel applications: A brief review. Int J Innov Apllied Stud 7:251–261.

Khazi MI, Demirel Z, Dalay MC (2018) Evaluation of growth and phycobiliprotein composition of cyanobacteria isolates cultivated in different nitrogen sources. J Appl Phycol 30:1513–1523. doi: 10.1007/s10811-018-1398-1

Kim HW, Park S, Rittmann BE (2015) Multi-component kinetics for the growth of the cyanobacterium *Synechocystis* sp. PCC6803. Environ Eng Res 20:347–355. doi: 10.4491/eer.2015.033

Koller M, Muhr A, Braunegg G (2014) Microalgae as versatile cellular factories for valued products. Algal Res 6:52–63. doi: 10.1016/j.algal.2014.09.002

Kultschar B, Llewellyn C (2018) Secondary metabolites in Cyanobacteria. Second Metab - Sources Appl. doi: 10.5772/intechopen.75648

Kumar M, Kulshreshtha J, Singh GP (2011) Growth and biopigment accumulation of cyanobacterium *Spirulina platensis* at different light intensities and temperature. Brazilian J Microbiol 42:1128–1135. doi: 10.1590/S1517-838220110003000034

Lagarde D, Beuf L, Vermaas W (2000) Increased production of zeoxanthin and other pigments by apllication of genetic engineering techniques to *Synechocystis* sp. strain PCC6803. Appl Environ Microbiol 66:64–72.

Loaiza R, Vera P, Aiello-Mazzarri C, Morales E (2016) Comparative growth and biochemical composition of four strains of *Nostoc* and *Anabaena* (Cyanobacteria, Nostocales) in relation to sodium nitrate. Acta Biológica Colomb 21:347–354. doi:

10.15446/abc.v21n2.48883

Misumi M, Katoh H, Tomo T, Sonoike K (2016) Relationship between photochemical quenching and non-photochemical quenching in six species of cyanobacteria reveals species difference in redox state and species commonality in energy dissipation. Plant Cell Physiol 57:1510–1517. doi: 10.1093/pcp/pcv185

Montgomery RM, Nachtigall HB (1950) Oral administration of chlorophyll fractions for body deodorization. Postgrad Med 8:401. doi: doi.org/10.1080/00325481.1950.11694031

Nichols HW (1973) Growth media-freshwater. In: Stein JR (ed) Handb. Phycol. methods Cult. methods growth Meas. Cambridge University Press, Cambridge, UK, pp 16–17

Ogawa T, Misumi M, Sonoike K (2017) Estimation of photosynthesis in cyanobacteria by pulse-amplitude modulation chlorophyll fluorescence: problems and solutions. Photosynth Res 133:63–73. doi: 10.1007/s11120-017-0367-x

Pandey VD, Pandey A, Sharma V (2013) Biotechnological applications of cyanobacterial phycobiliproteins. Int J Curr Microbiol Appl Sci 2:89–97.

Paswan MB, Chudasama MM, Mitra M, et al. (2016) Fluorescence quenching property of C-phycocyanin from *Spirulina platensis* and its binding efficacy with viable cell components. J Fluoresc 26:577–583. doi: 10.1007/s10895-015-1742-7

Poza-Carrión C, Fernández-Valiente E, Piñas FF, Leganés F (2001) Acclimation of photosynthetic pigments and photosynthesis of the cyanobacterium *Nostoc* sp. strain UAM206 to combined fluctuations of irradiance, pH, and inorganic carbon availability. J Plant Physiol 158:1455–1461. doi: 10.1078/0176-1617-00555

Raheem A, Prinsen P, Vuppaladadiyam AK, et al. (2018) A review on sustainable microalgae based biofuel and bioenergy production: Recent developments. J Clean Prod 181:42–59. doi: 10.1016/j.jclepro.2018.01.125

Renugadevi K, Nachiyar CV, Sowmiya P, Sunkar S (2018) Antioxidant activity of phycocyanin pigment extracted from marine filamentous cyanobacteria *Geitlerinema* sp. TRV57. Biocatal Agric Biotechnol 16:237–242. doi: 10.1016/j.bcab.2018.08.009

Romay C, Armesto J, Remirez D, et al. (1998) Antioxidant and anti-inflammatory properties of C-phycocyanin from blue-green algae. Inflamm Res 47:36–41. doi: 10.1007/s000110050256

Rueter JG, Petersen RR (1987) Micronutrient effects on cyanobacterial growth and

physiology. New Zeal J Mar Freshw Res 21:435–445. doi: 10.1080/00288330.1987.9516239

SAG (2008) Maintenance of Cultures - Spirulina Medium Recipe-Vers. 10. 2008. Sammlung von Algenkulturen Göttingen Cult. Collect. Algae

Saran S, Puri N, Jasuja ND, et al. (2016) Optimization, purification and characterization of phycocyanin from *Spirulina platensis*. Int J Appl Pure Sci Agric 2:15–21.

Sekar S, Chandramohan M (2008) Phycobiliproteins as a commodity: Trends in applied research, patents and commercialization. J Appl Phycol 20:113–136. doi: 10.1007/s10811-007-9188-1

Simeunovic JB, Markovic SB, Kovac DJ, et al. (2012) Filamentous cyanobacteria from Vojvodina region as source of phycobiliprotein pigments as potencial natural colorants. Food Feed Res 39:23–31.

Sliwinska-Wilczewska S, Konarzewska Z, Wisniewska K, Konik M (2020) Photosynthetic pigments changes of three phenotypes of picocyanobacteria *Synechococcus* sp. under different light and temperature conditions. Cells 9:1–19. doi: 10.3390/cells9092030

Stanic-Vucinic D, Minic S, Nikolic MR, Velickovic TC (2018) *Spirulina* phycobiliproteins as food components and complements. In: Jacob-Lopes E (ed) Microalgal Biotechnol. IntechOpen, pp 129–149

Ting CS, Owens TG (1992) Limitations of the pulse-modulated technique for measuring the fluorescence characteristics of algae. Plant Physiol 100:367–373. doi: 10.1104/pp.100.1.367

Tiwari ON, Indrama T, Singh KO, et al. (2015) Enumeration, pigment analysis and nitrogenase activity of cyanobacteria isolated from unexplored rice fields of Manipur, India falling under Indo-Burma biodiversity hotspots. Int J Curr Microbiol Appl Sci 4:666–680.

Xia X, Liu H, Choi D, Noh JH (2018) Variation of *Synechococcus* pigment genetic diversity along two turbidity gradients in the China seas. Microb Ecol 75:10–21. doi: 10.1007/s00248-017-1021-z

Yéprémian C, Catherine A, Bernard C, et al. (2016) Chlorophyll *a* extraction and determination. Handb Cyanobacterial Monit Cyanotoxin Anal. doi: https://doi.org/10.1002/9781119068761.ch34

CHAPTER IV

Blue is not enough: Biological activity of C-phycocyanin extracts from *Anabaena cylindrica*

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Contributions: I. P. E. M. wrote the manuscript; I. P. E. M. and T. V. acquired the experimental data; I. P. E. M. performed the data analysis; A. P. M. F. and T. R. D. extracted the C-phycocyanin; M. M. helped with the data analysis; H. O., F. J. M. G., S. P. V., and J. L. P. supervised

Abstract: Natural products are under the spotlight due to the increasing awareness of consumers. Among these products is phycocyanin (C-PC), a blue colorant that can be extracted from cyanobacteria - a poorly explored feedstock - and applied in several fields, due to its argued health benefits. *Arthrospira* (previously called *Spirulina*) is nowadays the major source of C-PC, but within the scope of pursuing for alternative sources, the cyanobacterium *Anabaena cylindrica* was used in the present study to extract this colorant. The biological activities of two types of extracts (raw and purified, both in fresh and in lyophilized forms) were analysed. The raw extracts present better results than the pure counterparts, presenting higher antioxidant activity, better biocompatibility, and antitumor activity. However, when compared to literature records, C-PC extracts from *A. cylindrica* produced less impressive results. This leads to a new hypothesis and to a simple comparison among the antioxidant activity of C-PC extracts from different biomass sources. These second-stage results showed that different cyanobacteria species produced different C-PC extracts, with different antioxidant profiles, and highlighted that, even when the extracts are blue, they may present differential composition and therefore, different biological activity. This study proves that the direct link often made between biological activities and C-PC can be speculative, and thus should be avoided.

Keywords: cyanobacteria, C-phycocyanin, antioxidant, antimicrobial, biocompatibility, antitumor

IV-1. Introduction

Natural products are a huge attraction nowadays. The increasing awareness of the consumers over health and environment boosts the market of natural compounds and stimulates the search for alternative processes with lower environmental impacts. Cosmetic and food sectors are key arenas that have been invaded by this trendy consideration of natural products, and cyanobacteria have been promising model organisms in this context. Cyanobacteria are included in the set of feedstocks poorly explored, although they are rich in bioactive compounds like amino acids, pigments, lipids, polysaccharides, or proteins, known for their antibacterial, antifungal, antiviral, or antitumor activities (Eriksen 2008; Sekar and Chandramohan 2008). Cyanobacteria may also produce cyanotoxins, which can be used in algaecides, herbicides, and insecticides (Berry 2008; Haque et al. 2017). According to the effects in mammals, these toxins can be classified as neurotoxins, hepatotoxins, cytotoxins, irritants and gastrointestinal toxins. For the *Anabaena* genus, microcystin, anatoxin-a, homoanatoxin-a, cylindrospermopsin, saxitoxin, lipopolysaccharides, and BMAA (β- Methylamino-L-alanine) are amongst the cyanotoxins already described (Metcalf and Codd 2012; Ilieva et al. 2019). While microcystin is one of the most frequent and dangerous hepatotoxin, anatoxin-a, homoanatoxin-a, saxitoxins and BMAA are neurotoxins, cylindrospermopsin is classified as a cytotoxin, and lipopolysaccharides produce irritant effects (Metcalf and Codd 2012; Ilieva et al. 2019). However, not all strains are toxic and the production of toxins only occurs under specific conditions, which are not fully elucidated yet (Pearson et al. 2016).

Cyanobacteria present a characteristic bluish colour, which is given by C-phycocyanin (C-PC). PC belongs to water-soluble fluorescent pigment-protein complexes (phycobilisomes), which act as secondary light-harvesting components in the photosynthetic process and are assembled in regular arrays on the outer surface of the thylakoid membranes (Glazer 1994). Besides PC (blue), phycoerythrin (purple), phycoerythrocyanin (orange), and allophycocyanin (bluish green) can also be found in cyanobacteria. These different colours are originated mainly by the covalent bound prosthetic groups (open-chain tetrapyrrole chromophores) with A, B, C, and D rings named phycobilins (Glazer 1994). These compounds act in photosynthetic light harvesting and their absorption maxima is located at wavelengths where chlorophylls have low extinction coefficients: approximately 650-655 nm for allophycocyanin, 615-640 nm for PC, 575 nm for phycoerythrocyanin, and 565-575

nm for phycoerythrin (Bryant et al. 1979).

The appealing colour of PC features its primary potential as a natural dye. Due to its colour, C-PC can be used as a food pigment replacing current synthetic pigments (e.g. in chewing gums, candies or cold recipes), and in cosmetic products like lipsticks and eyeliners (Spolaore et al. 2006). Indeed, natural blue pigments are rare. To produce this colour, the only options at the moment are anthocyanins, from plants (but their colour is pH dependent), fungi and microorganisms (yet production is a response to specific stimuli), gardenia blue, from *Gardenia jasminoides* Ellis (*Gardenia augusta* Merrill), and PC from cyanobacteria and algae (Stanic-Vucinic et al. 2018). Among these options, C-PC is the one that offers brightness, brilliance, and shade closer to the synthetic pigment Brilliant Blue FCF (Blue 1 or E133) (Stanic-Vucinic et al. 2018). However, only blue anthocyanins are allowed to be used in food products according to the European legislation (E163) (EU 2021), while C-PC is still under consideration as per the info contained in the EU food additives database (https://webgate.ec.europa.eu/foods_system, assessed on November 26th 2021). Nevertheless, the use *Arthrospira* extracts is already approved and regulated by the U.S. Food & Drug Administration (CRF 2021). Besides its colour, its fluorescence properties allow its use as a label for antibodies, receptors, and other biological molecules (Sekar and Chandramohan 2008). C-PC is also known for its antitumor activity for several types of cell lines (Jiang et al. 2017), as well as for its capacity as an antioxidant (Renugadevi et al. 2018), anti-inflammatory (González et al. 1999), and as an immune system stimulator (Romay et al. 1998). The use of C-PC in such different applications is highly dependent on its purity, defined as the ratio between its absorbance at 620 nm and the total proteins' absorbance measured at a wavelength of 280 nm. Values above or equal to 0.7 indicate that C-PC can be applied for example in foods as a colorant; values of or above 3.9 specify reactive grade C-PC; and a value of or above 4.0 specifies analytical grade C-PC (Rito-Palomares et al. 2001). In practice, this means that a higher purity ratio is obtained when fewer contaminants are present in the extract, being the use of purification techniques required depending on the specific purity demanded by a given application. Besides purity, the stability and the activity of PC are essential features for its biotechnological application, while denaturation, precipitation, and discoloration can be important problems to be dealt with (Wu et al. 2016).

Inspired by the argued health benefits of PC in general and C-PC in particular, which are currently translating into significant commercial interests mostly in the nutraceutics field

(e.g. note the growth of the marked for *Arthrospira*, the major source of C-PC, is expected to grow at a CAGR of 13.2% from 2021 to 2028, reaching $ 968.6 million by 2028; www.globenewswire.com assessed on July 14th 2021), we studied specific biological activities of C-PC extracted from the cyanobacterium *Anabaena cylindrica*. The specific aim of this work was to understand if it is possible to achieve the same property (i.e. biological activity) using C-PC extracts (raw and purified), which are naturally less expensive to produce than the pure pigment. Freshly prepared and lyophilized purified and raw extracts were tested for their antioxidant and antimicrobial activities, biocompatibility, and antitumor activity.

IV-2. Material and methods

IV-2.1. Cyanobacteria cultivation

A. cylindrica PCC 7122 was cultured in 5-L Schott flasks containing sterilized liquid MBL - Woods Hole culture medium (Nichols 1973), in an incubation chamber at 20 ± 2 °C, under a 16h light:8h dark photoperiod using 2300 lx provided by cool white fluorescent tubes. After 13 days in culture, the biomass was harvested and concentrated through centrifugation at 4 ºC (4111 ×*g*; Eppendorf 5810 R). The fresh biomass was stored briefly at -20 ºC until further use.

IV-2.2. Extracts production and purification

The frozen biomass was left at room temperature for 90 min protected from the light and then homogenized in sodium phosphate buffer 150 mmol.L^{-1} at pH 7, using a solid-liquid ratio of 1:10. The extraction was performed in an Eppendorf Thermomixer Comfort equipment at 1500 rpm and 35 ºC during 50 min. After the extraction time, the cell suspension was centrifuged at 12000 ×*g* for 10 min in a VWR microstar 17 centrifuge. The pellet was discarded and the supernatant was directly used as the raw extract.

An aliquot of the same supernatant was purified according to the procedure described by Martins and collaborators (2021) to produce the purified extract for further characterization and testing. Briefly, 20 % (w/v) of ammonium sulfate was added and the mixture was left overnight at 4 ºC. The precipitated proteins were recovered by centrifugation at 900 ×*g* for 15 min in a VWR microstar 17 centrifuge. The supernatant was discarded, and the pellet was resuspended in the same initial volume of ultrapure water. An

ultrafiltration system was performed to the recovered protein fraction after the precipitation. A 500 µL sample was added in each Amicon Ultra-0.5 mL Centrifugal Filter Unit 100 K. The sample was centrifuged at 14000 ×*g* during 15 min in a VWR microstar 17 centrifuge. The permeate was discarded and 480 µL of ultrapure water was added to the concentrate and centrifuged in the same conditions, being this last step repeated twice. Lastly, 480 µL of ultrapure water was added to recover the concentrated sample after a centrifugation for 2 min, at 1000 ×*g*, and a purified blue extract was obtained.

For each extract, the absorption spectra were collected (200-700 nm; UV-Vis microplate reader Synergy HT, BioTeK Instruments Inc., Winooski, VT, USA), at least in duplicate and using ultrapure water as a blank. C-PC concentration in the extracts was quantified based on a previously defined calibration curve (R^2 = 0.9912) built following optical density measurements at 615 nm.

Raw and the purified extracts were used in the following steps, either fresh (used immediately after the extraction process) or lyophilized (Scanvac cool safe, LaboGene ApS).

IV-2.3. Extracts' antioxidant activity

The antioxidant activity of all extracts was assessed through the ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) method, using ascorbic acid as the standard antioxidant. Two controls were performed: one with ultrapure water for purified extracts and ascorbic acid, and another control with sodium phosphate buffer for the raw extracts. The ABTS radical cation was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate at room temperature, in the dark, for 12 h. This ABTS solution was diluted with phosphate buffer at 75 mM (pH 7.4) to an absorbance of 0.70 ± 0.05 at 734 nm. The diluted ABTS solution was added to each sample and mixed thoroughly. After a period of incubation of 10, 30, or 60 min at room temperature in the dark, absorbance at 734 nm was recorded (Shimadzu, UV 1800, Kyoto, Japan). The scavenging activity was calculated through Eq. IV-1,

$$\%\, Scavenging\ activity = \frac{A0 - A1}{A0} \times 100 \qquad \textbf{Eq. IV-1}$$

where A_0 is the absorbance of the control, and A_1 the absorbance of the sample.

IV-2.4. Extracts' antimicrobial activity

All extracts were tested against 11 bacterial strains, both gram-negative and gram-positive (*Micrococcus luteus* ATCC 4698*, E. coli* ATCC 25922*, Pseudomonas aeruginosa* ATCC 27853*, Bacillus cereus* ATCC 14579*, Staphylococcus aureus* ATCC 25923*, Salmonella enterica* ATCC 14028*, Klebsiella pneumoniae* ATCC 13883*, Aeromonas* sp.*, Proteus mirabilis* ATCC 43071*, Enterococcus faecalis* ATCC 29212*,* and *Bacillus sphaericus* ATCC 12488), using the Kirby-Bauer Disk Diffusion test. Briefly, Mueller-Hinton agar plates were uniformly inoculated with a bacterial suspension, and then filter paper disks containing the extracts were placed on the agar surface. Standard antibiotic disks for gram-positive and gram-negative bacteria (10 µg of Penincillin and Chloramphenicol, Oxoid, Hampshire, England) were also added to the plates, to allow comparing the inhibition halo. Ultrapure water and sodium phosphate buffer were also tested, to discard the possible influence/interference of the solvents. The plates were incubated at 37 ºC, and inhibition zones were observed after 24, 48, and 72 h. All tests were conducted independently and in triplicate.

IV-2.5. Skin biocompatibility and antitumor activity of the extracts

Immortalized human keratinocyte HaCaT cells, used herein to assess skin biocompatibility, were obtained from Cell Lines Services (Eppelheim, Germany), and human melanoma MNT-1 cells, used herein as a proxy to assess antitumoral activity, were provided by Dr. Manuela Gaspar (iMed.ULisboa, Portugal). HaCaT and MNT-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % of fetal bovine serum (FBS) and 1 % of L-glutamine, penicillin–streptomycin and fungizone (Life Technologies, Grand Island, NY, USA). Both were incubated under a humidified atmosphere at 37 ºC and with 5 % of CO_2. The cell morphology was monitored using an inverted microscope Nikon Eclipse 80i (Nikon, Tokyo, Japan).

The cytotoxic effects of all extracts were assessed through the colorimetric MTT assay (Twentyman and Luscombe 1987). Briefly, HaCaT and MNT-1 cells were seeded in 96-well plates and allowed to adhere. After adhesion, cells were incubated (37 ºC in 5 % CO_2) for 72 h with a range of six extract concentrations (0.6, 3.0, 15.0, 60.0, 120.0, and 300.0 µg.mL^{-1} for purified extracts, and 0.8, 4.2, 21.0, 83.0, 166.0, and 416.0 µg.mL^{-1} for raw extracts), after extract sterilization by filtration through a 0.22 µm CA syringe filter.

After exposure, 50 µL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in PBS at pH 7.2) was added to each well. After 4 h of incubation, the medium was replaced with 150 µL of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. The plate was shaken for approximately 2 h in the dark. Cell viability was measured through the reading of optical density of reduced MTT at 570 nm (Synergy HT microplate reader; BioTeK Instruments Inc., Winooski, VT, USA).

Cell viability was expressed in percentage, relative to the control. The controls were differentially set up for lyophilized and fresh extracts. One control treatment with only DMEM was used when testing lyophilised extracts, because the lyophilized extracts were reconstituted in DMEM. However, for fresh extracts, 6 different control concentrations were also established, in order to appropriately correct for differences in volume among extracts with different C-PC concentration. These controls were established corresponding to each tested concentration, were DMEM was added to ultrapure water (for purified extracts), and to sodium phosphate buffer (for raw extracts) in the exact same volume as used in the treatment with the extract (see Figure IV-S1 and Table IV-S3 for the influence of different solvent concentrations in cell viability). In this case, the cell viability found in each extract treatment was expressed regarding the respective control.

IV-2.6. Data analysis

Data regarding antioxidant activity and cell viability were graphically expressed as the mean ± standard deviation (SD) of at least three independent experiments. The effect of the lyophilized extracts in cell viability was statistically addressed using a one-way ANOVA approach followed by the post-hoc Dunnet test when applicable to distinguish extract concentrations from the control. Significant differences in cell viability between each fresh extract treatment and the corresponding control were also assessed through a one-way ANOVA followed by the post-hoc Dunnet test. An alpha level of 0.05 was considered in these analyses.

IV-3. Results

IV-3.1. C-PC extraction

C-PC was successfully extracted from the cultures of *A. cylindrica*, through the use of sodium phosphate buffer. This simple extraction step produced the raw extract, while a second purification step leads to the production of the purified extract. The extracted C-PC was characterized by UV–visible spectrometry and exhibited a maximum absorbance at 615 nm (Figure IV-1).

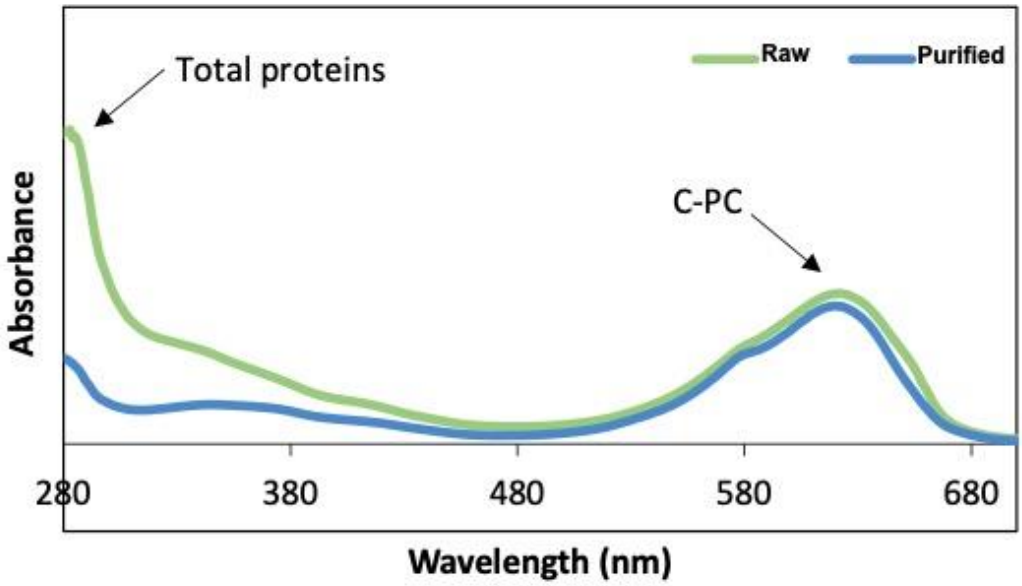

Figure IV-1: UV visible spectrum of the raw extract (green) and the purified extract (blue) of C-PC from *A. cylindrica*.

IV-3.2. Antioxidant activity

All extracts presented antioxidant activity, with the exception of the fresh purified extract tested at 500 µg.mL^{-1}. There was a clear increase in the percentage of the scavenging activity with increasing C-PC concentration, which represents an increase in the antioxidant activity (Figure IV-2). The raw extracts (both in fresh and in lyophilized forms) presented higher antioxidant activity than the purified counterpart, with the highest record being found for the raw extract at 2500 µg.mL^{-1} that reached more than 40 % of scavenging activity (comparable to the control with ascorbic acid at 200 µg.mL^{-1}; Figure IV-2). The antioxidant activity also increased in general with the time of exposure used in the ABTS assay (Table IV-S1, Annex II). In order to clarify the results obtained, a follow-up experiment was carried

out concerning the antioxidant activity of C-PC extracts from different species, as detailed in the Discussion section.

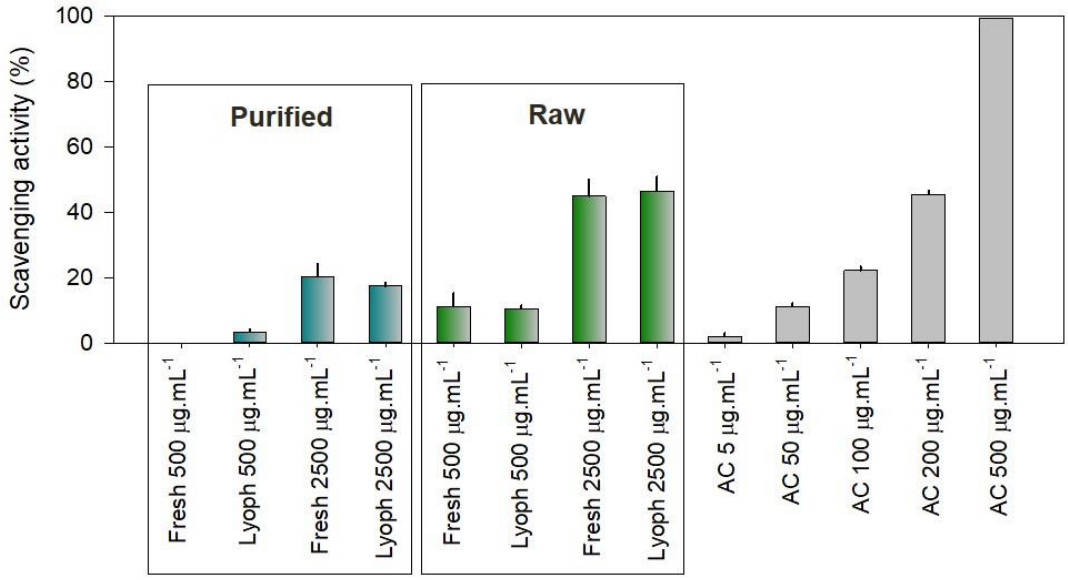

Figure IV-2: Antioxidant activity represented as the mean (n = 3) percent of scavenging of the tested extracts at 500 µg.mL^{-1} and 2500 µg.mL^{-1}, read after 60 minutes of exposure. Blue bars represent purified extracts, while green bars represent raw extracts. AC stands for ascorbic acid, which was used as a positive control. Error bars represent the standard deviation.

IV-3.3. Antimicrobial activity

The antimicrobial activity was tested against 11 pathogenic bacterial strains. None of the extracts or the solvents showed antimicrobial activity. However, all replicates were validated through the identification of an inhibition halo present on the antibiotic disks (see Table IV-S2, Annex II).

IV-3.4. Biocompatibility with human skin cells

The biocompatibility of the extracts was interpreted from the percentage of HaCaT cells viability following exposure compared to the control (Figure IV-3). Concerning the purified extract, the lyophilized form showed better biocompatibility than the fresh form. However, the cell viability decreases significantly (see ANOVA summaries in Table IV-S4, Annex II) with the increase in C-PC concentration for both extracts, up to a minimum value of 68 % of cell viability at 300 µg.mL^{-1}, after 72 h of exposure. The fresh form was slightly less biocompatible, reaching the minimum value of 64 % in cell viability at 60 µg.mL^{-1}.

Regarding raw extracts, a better biocompatibility was achieved. Indeed, at the lowest concentration (0.8 $\mu g.mL^{-1}$) an increase in the cell viability of 20 % was found. Again, the lyophilized form was slightly more biocompatible than the fresh one. As for the purified extract, a significant decrease in cell viability was recorded with the increase in C-PC concentration. A minimum of 55 % of cell viability was observed for the fresh extract, compared to a slightly higher record of 65 % of cell viability found for the equivalent lyophilized extract at the highest concentration tested (416 $\mu g.mL^{-1}$).

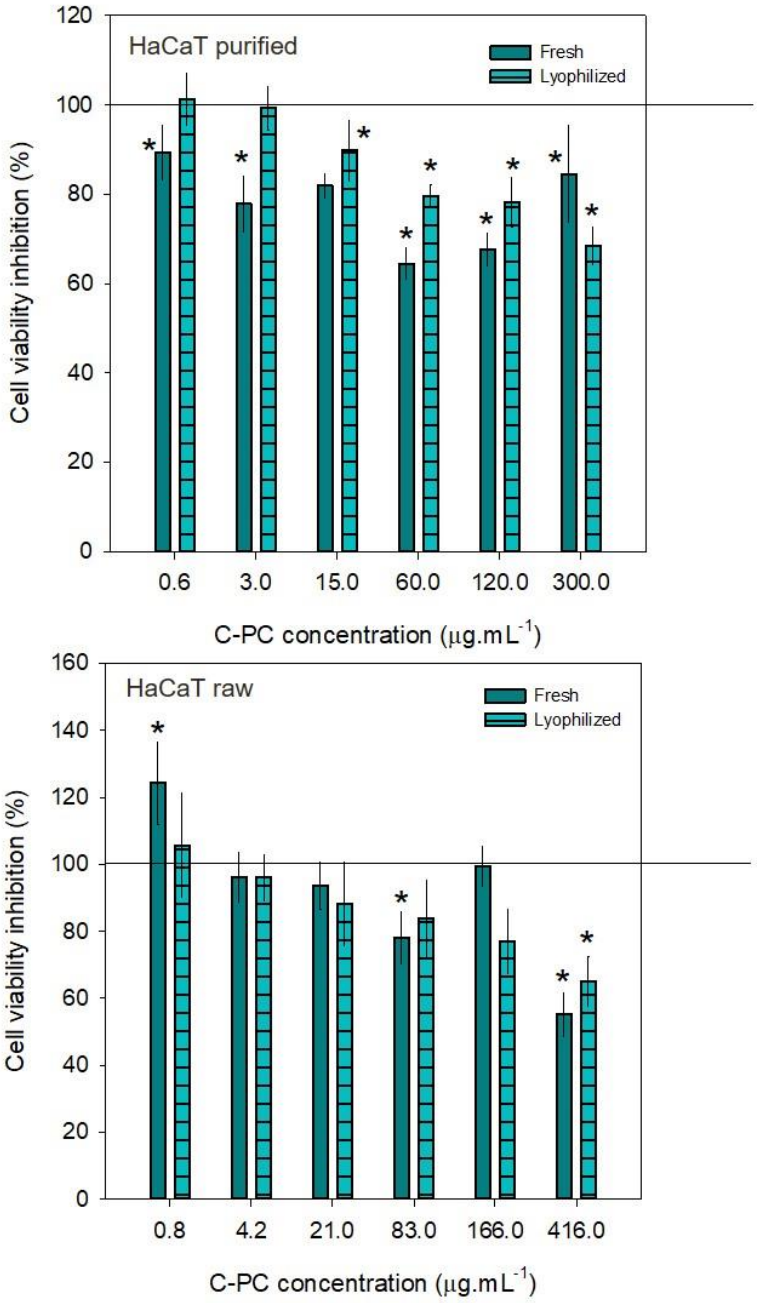

Figure IV-3: Cell viability of purified and raw extracts (in the fresh and lyophilized form) for HaCaT cells, after 72 h of exposure. For fresh extracts, cell viability is expressed as the percent inhibition relative to the control with ultrapure water in the case of purified extracts and sodium phosphate buffer in the case of raw extracts. For lyophilized extracts, cell viability is expressed as the percent inhibition relative to the control with DMEM medium. The results are expressed as the mean (bars) ± standard deviation (error bars) of nine replicates. The horizontal bar at 100 % marks the experimental control viability. The asterisks denote statistically significant differences between the control and treatments with lyophilized extracts or between each fresh extract and the corresponding control (Dunnet test; p <0.05).

IV-3.5. Antitumor activity with melanoma cells

The antitumor activity of the extracts was assessed using a human melanoma cell line (MNT-1), as indicated by the recorded percent of cell viability relative to reference viability records achieved in the control. Antitumor activity was achieved only with raw lyophilized extract (at 4.2, 21.0, 83.0, and 166.0 $\mu g.mL^{-1}$), as shown in Figure IV-4. No antitumor activity was promoted by the fresh extracts, neither by the purified extracts, as interpretable from cell viability generally above the control levels (standard assumption of 100 % viability for the control treatments) in Figure IV-4. In fact, an increase in cell viability was observed in the fresh form of both purified and raw extracts compared to the control, although with no statistical significance; the higher increase (approximately 20 %) was found at 3 and 120 $\mu g.mL^{-1}$ of purified extracts.

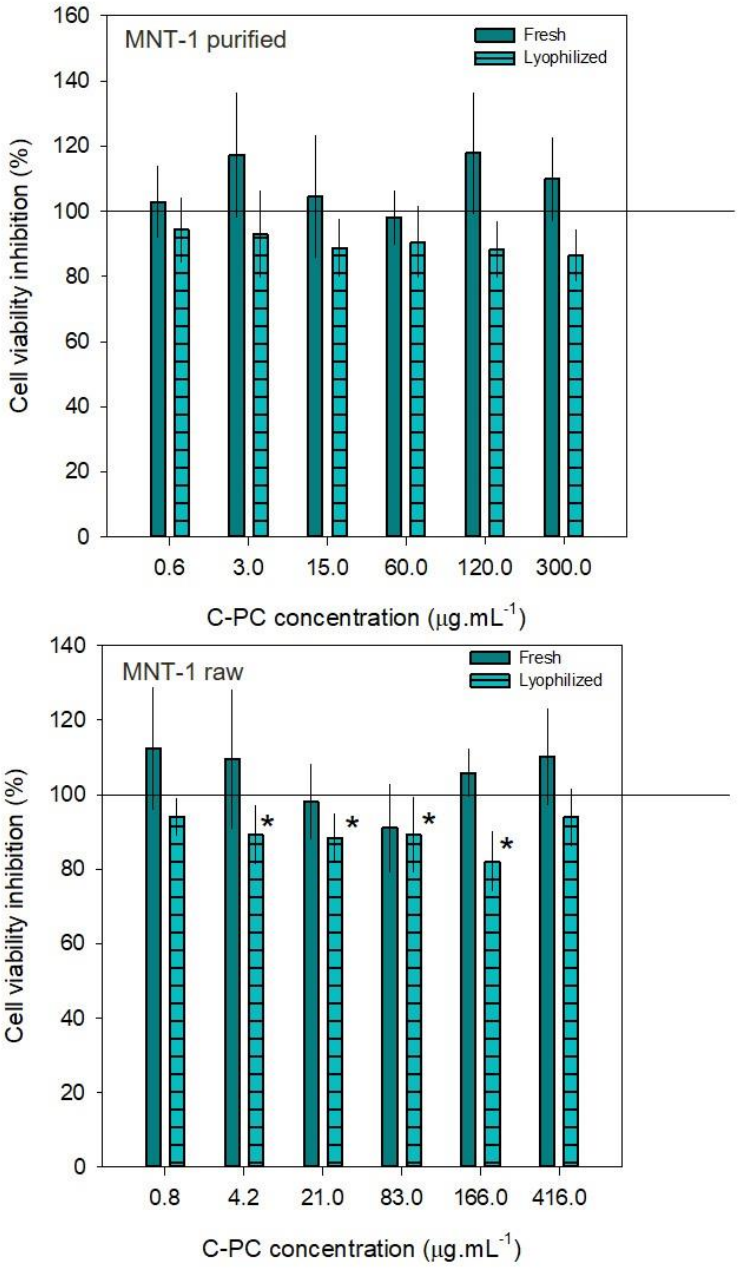

Figure IV-4: Cell viability of purified and raw extracts (in the fresh and lyophilized form) for MNT-1 cells, after 72 h of exposure. For fresh extracts, cell viability is expressed as % of cells regarding the control with ultrapure water in the case of purified extracts and sodium phosphate buffer in the case of raw extracts. For lyophilized extracts, cell viability is expressed as % of cells regarding the control with DMEM medium. The results are expressed as the mean (bars) ± standard deviation (error bars) of nine replicates. The horizontal bar at 100 % represents the standard assumption of the experimental control viability. The asterisks denote statistically significant differences between the control and treatments with lyophilized extracts or between each fresh extract and the corresponding control (Dunnet test; p <0.05).

IV-4. Discussion

The benefits of PC have been widely explored, but whether cyanobacteria are good C-PC producers is a question that has not been systematically addressed. Indeed, the yield of production of C-PC from different species with different culture conditions has been reported (Kumar et al. 2011; Maurya et al. 2014), through which exciting information on the biological activity of cyanobacteria extracts (Basha et al. 2008; Mohite et al. 2015; Shanmugam et al. 2017; Jiang et al. 2018; Renugadevi et al. 2018; Jang and Kim 2021) was delivered. The different *A. cylindrica* C-PC extracts studied herein showed much less impressive results, which triggered this underlying picture behind our study reflecting that the blue attribute is not enough to define an advantageous valorization of biologically active cyanobacteria biomass.

The antioxidant activity is a very interesting feature for a natural extract, because of the wide range of applications suiting such extracts, within e.g. the food, pharmaceutical, and cosmetic markets (Renugadevi et al. 2018). Antioxidants can delay or inhibit the process of oxidation, playing an important role in the defense mechanism against the attack of free radicals. Its activity relies on their increased ability to donate a hydrogen atom from the aromatic hydroxyl group to a free radical and/or to the capacity of their aromatic structures to support an unpaired electron (Fratelli et al. 2020). The highest antioxidant activity of a C-PC extract produced by *A. cylindrica* in the present study was shown by the raw extract at 2500 $\mu g.mL^{-1}$, with approximately 40 % of scavenging activity. This is much lower than the records by Renugadevi et al. (2018) with a C-PC extract from *Geitlerinema* sp. TRV57 (200 $\mu g.mL^{-1}$) reaching a scavenging activity of 78.75 % (DPPH test) or a hydrogen peroxide radical scavenging activity of up to 95.72 %. However, less expressive results can be also found in the literature, using C-PC extracts from *Arthrospira platensis* - e.g. 50 % scavenging activity with 560 $\mu g.mL^{-1}$ of C-PC (ABTS test) (Wu et al. 2016) and 25.21 % scavenging activity with 10.8 $mg.g^{-1}$ of C-PC (DPPH test) (Jerley and Prabu 2017). It is worth noting that the results regarding the antioxidant activity are difficult to compare since different protocols can be applied, representing different mechanisms, redox potentials, pH values, and solvent dependencies (Apak et al. 2013). Also, within the same assay, there is a discrepancy in the units used for the expression of results (e.g. % of scavenging activity, or Trolox equivalent units), which can largely prevent feasible comparisons among studies.

The discovery of new antibiotics is also a major line of research currently, and natural products have been privileged sources of new drug molecules. Microorganisms and plants have been particularly under the spotlight in this context (Bérdi 2005), and cyanobacteria are known to produce antimicrobial compounds, like C-PC. For instance, C-PC extracted from *Arthrospira* species showed antimicrobial activity against *Staphylococcus aureus, Shigella* spp., *Pseudomonas aeruginosa, Escherichia coli,* and *Salmonella typhi* (Mohite et al. 2015). C-PC extracted from *Oscillatoria* sp. also showed antimicrobial activity against *Pseudomonas* sp., *Staphylococcus aureus,* and *Klebsiella* sp. (Shanmugam et al. 2017). However, in the present study, the C-PC sourced by *A. cylindrica* did not present any antimicrobial activity, which suggests that either the antimicrobial potential found for the extracts that were previously tested was due to components other than C-PC, or that C-PC antimicrobial properties are species-specific. In those previous studies, C-PC was extracted from different species, with slightly different procedures. While Mohite et al. (2015) applied the freeze-thaw method followed by an extraction step with a phosphate buffer (pH 7), Shanmugam et al. (2017) applied Tris-HCl buffer followed by precipitation with ammonium sulphate, after the freeze-thaw step. Although these extraction procedures allow obtaining an extract rich in C-PC, the phycobiliprotein will not be the single constituent of the extract. This can concur to explain the lack of antimicrobial activity of the extracts analyzed in the present study.

The applicability of C-PC in the cosmetic sector justifies the study of the biocompatibility of both the pigment and extracts rich in the pigment with human skin. The biocompatibility of commercial C-PC from *Arthrospira* with human keratinocytes (HaCaT cell line) was already reported, using a concentration range of 5 - 80 $\mu g.mL^{-1}$ (Jang and Kim 2021). Moreover, not only C-PC did not show any toxicity to HaCaT, as it exerted a protection against UV-B radiation, preventing wrinkle formation, helping to restore the physical barrier function of skin, and suppressing oxidative stress (Jang and Kim 2021). However, the extracts of C-PC used in the present study present some toxicity to HaCaT cells. This toxicity was especially evident with purified extracts, which is not in accordance with the referred previous studies carried out with the pure commercial pigment. With raw extracts, better results were achieved, and at 0.8 $\mu g.mL^{-1}$ the fresh form significantly increased the cell viability. This is a promising result since it suggests an increase in cell proliferation, which is very interesting from the point of view of research on skin

regeneration.

The antitumor activity of C-PC was already reported for melanoma cells (A375 cell line) (Hao et al. 2018), breast cancer cells (MDA-MB-231 cells) (Jiang et al. 2018), and hepatocyte carcinoma (HepG-2 cells) (Basha et al. 2008), for instance. For the MNT-1 cells (also melanoma cells), addressed herein for the first time, only raw extracts showed antitumor activity, although with small reductions in cell viability. The best result was achieved with 166.0 $\mu g.mL^{-1}$ (80 % of cell viability). This record is less impressive than those reported by other authors - e.g. *ca.* 40 % A375 cell viability with 8 $\mu g.mL^{-1}$ (Hao et al. 2018); less than 50 % MDA-MB-231 cell viability with 300 $\mu g.mL^{-1}$ (Jiang et al. 2018); and 38 % HepG 2 cell viability with 7 $\mu g.mL^{-1}$ (Basha et al. 2008). Still, our results are interesting because although C-PC is known for having an antitumor activity, the raw extracts are the ones responsible for this activity, rather than the purified ones. Within these extracts, the lyophilized form achieved better results than the fresh form. Furthermore, although some of the present extracts showed toxicity to HaCaT cells, the conditions that showed antitumor activity also present good biocompatibility with HaCaT (i.e. 4.2, 21.0, 83.0, and 166.0 $\mu g.mL^{-1}$ of lyophilized raw extract), and should be focus of future studies and characterization.

Comparing purified and raw extracts, it is possible to observe that the raw extracts produced better results than the purified extracts, namely higher antioxidant capacity, better biocompatibility and antitumor activity. However, at this point, it is honest to recognize that *Anabaena* extracts are not particularly efficient in promoting desirable biological activities. A possible explanation could be the lack of stability of C-PC after extraction. This is of key importance since stability of proteins can be disturbed by several factors, including C-PC origin (Chen and Berns 1978), extraction temperature (Dejsungkranont et al. 2017), light exposure (Colla et al. 2016), extraction process (Jaeschke et al. 2021), type of solvent (İlter et al. 2018), pH (Silveira et al. 2008), biomass/solvent ratio and biomass form (Jaeschke et al. 2021). The protein structure has a protective effect on the chromophore, which is responsible for the stability of C-PC. The structure of C-PC is an open-chain tetrapyrrole chromophore (phycobilin) covalently linked to protein molecules. Interferences in this tetrapyrrole structure (i.e. in the chromophore) caused by compounds like oxygen, free radicals, and acids, promote C-PC degradation, with colour fading and antioxidant activity loss (Jaeschke et al. 2021). Therefore, stabilizing agents are normally added after extraction

to ensure stability (Stanic-Vucinic et al. 2018). Also, in aqueous solutions, proteins partially dissociate in discrete aggregates (e.g. trimmers and monomers), exposing phycobilin to reactant species (Jaeschke et al. 2021). In fact, it was already reported by Fratelli et al. (2020) that different extraction methods lead to different antioxidant activities of C-PC extracted from *Arthrospira*. Both ultrasound-assisted extraction (UAE) (Hadiyanto 2016) and pressurized liquid extraction (Herrero et al. 2005; Ruiz-Domínguez et al. 2021) are common methods for the extraction of phycobiliproteins, however, it is reported that UAE did not protect the bioactivity of C-PC (Fratelli et al. 2020). Among the extraction methods compared by Fratelli et al. (2020), solvent extraction is the one that results in higher C-PC antioxidant activity when compared to the other extraction methods evaluated. The solvent extraction with buffer phosphate is a common method and already used by other authors with cyanobacteria (e.g. Mohite et al. 2015; Wu et al. 2016). These literature evidences supported our methodological options for the extraction process and consequently suggest that the poor biological activities we monitored are mostly related to the species used.

IV-4.1. On the species-specificity of C-PC biological activity

As evident from the above, the source of the extract (i.e. the species) is a remarkable constraint to C-PC biological activities. Although not often reasoned, this is logical given the differential metabolic composition among species, which defines the composition of extracts and their C-PC load. Moreover, although extracts are blue, it is noteworthy that C-PC would not be their single constituent. This means that the biological activities described for several C-PC extracts may or may not be strictly driven by C-PC, and it is reasonable to hypothesize that synergic relations between C-PC and other molecules composing the extracts are rather responsible for the activity records. In such a scenario, the selection of the species to produce the extracts is extremely important, because it will dictate its biological activity, and hence the success of a given application. To elaborate on this hypothesis, formulated on the basis of the outcome of the present study, and to support our discussion, we extended our experimentation. Four different sources (*A. cylindrica, Nostoc* sp., *Arthrospira platensis*, and dried *Arthrospira* sp. from a commercial source) were used to produce several C-PC extracts following similar procedures as described in the methodology section for the raw extracts, using water and sodium phosphate buffer as solvents. In some biomasses a second extraction step was added in order to evaluate if it was possible to

recover more C-PC. After this second extraction step the extracts with quantified C-PC were also subject to the evaluation of the antioxidant activity. Therefore, in this small experience the effect of two different solvents in four different biomasses was assessed, as well as the influence of the addition of a second extraction step. These extracts, differing as to the source species and the solvent used in extraction bare distinct antioxidant activity, although comprising similar C-PC concentration (Figure IV-5, Table IV-S5). The different colors of the extracts are an immediate indication of distinct composition, which translates into different antioxidant activity records. *Arthrospira platensis* extracts are the ones with higher activity, especially following extraction with water. Although both water and sodium phosphate buffer successfully extracted C-PC, equivalent extracts are differentially active, meaning that the observed activity cannot be exclusively attributed to the C-PC presence. Moreover, sequential extractions of the same biomass also promote different activity, although the concentration in C-PC of the extracts is the same (see the example of *Arthrospira* from a commercial source with 500 $\mu g_{C\text{-}PC}.mL^{-1}$). In this case, a second step on the extraction procedure lead to an extract with the same C-PC concentration (500 $\mu g.mL^{-1}$), but with a small scavenging activity. This demonstrates that the extraction procedure has a role in the activity, because the same concentration of C-PC produced different scavenging activity records.

In these extractions, sodium azide (NaN_3) was also added to the solvents. This is an anti-microbial agent used to enhance storage stability and maintain the quality of C-PC (Jaeschke et al. 2021). As stated above, the use of stabilizers can play a role in bioactive abilities. However, this addition did not produce better antioxidant extracts (see *A. cylindrica* raw extracts at 500 $\mu g.mL^{-1}$ C-PC in Figure IV-2, and with buffer at 500 $\mu g.mL^{-1}$ C-PC in Figure IV-5).

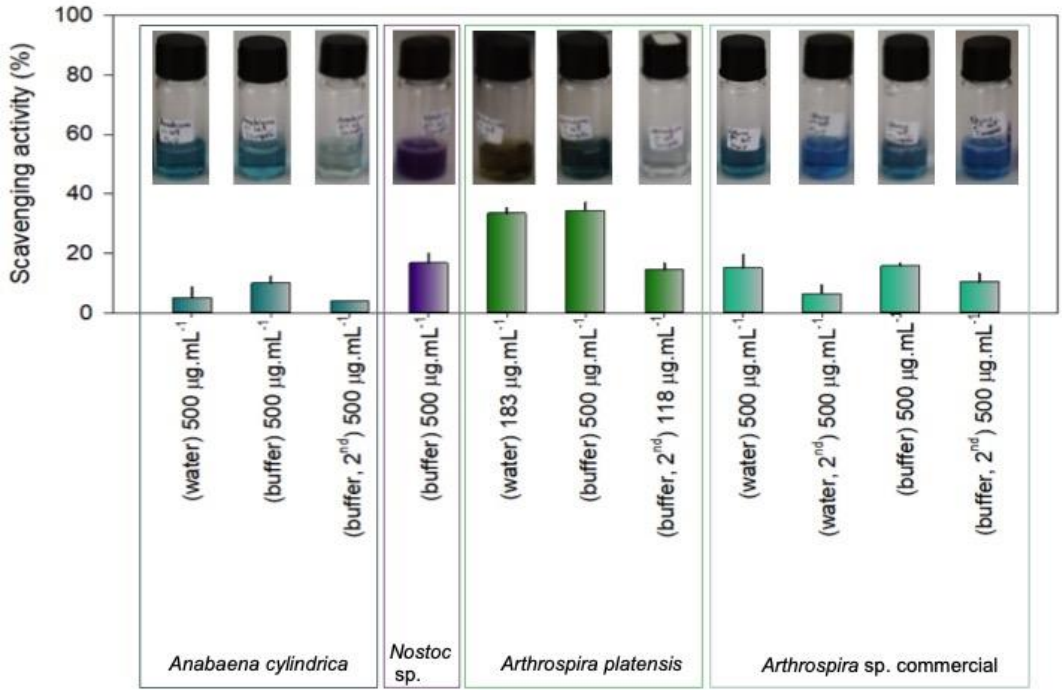

Figure IV-5: Antioxidant activity represented as the percent of scavenging activity of several C-PC extracts obtained from different species (*A. cylindrica*, *Nostoc* sp., *Arthrospira platensis*, and dried *Arthrospira* sp. from a commercial source), bearing different but comparable C-PC concentrations, read after 60 min exposure in the ABTS assay. Water and sodium phosphate buffer were used as solvents for the extraction. Some biomasses were subject to a second sequential extraction and are identified as 2nd.

This exercise demonstrated that the extract colour does not necessarily translates directly into antioxidant activity, thus suggesting that the link often made between many biological activity attributes and C-PC is rather speculative. In recent years, many efforts have been made to understand the conditions that promote the production of C-PC and to select the best method for its extraction. However, being blue is not enough, and efforts should be made to characterize the constituents of the extracts. This will allow understanding, which is or are the components of the extracts that are responsible for a specific biological activity, and could help to demystify some of the features that may have been wrongly attributed to C-PC.

IV-5. Conclusion

In the present work, C-PC extracts from *A. cylindrica* were analyzed regarding some common biological activities (i.e. antioxidant, antimicrobial, biocompatibility, and antitumor against human cell lines). When comparing raw *vs* purified extract, raw extracts were found more interesting, as they showed higher antioxidant activity, better biocompatibility, and even antitumor activity in some concentrations. Still, these extracts underperformed compared to literature reports and raised this question on whether all cyanobacteria are good C-PC producers. Extended experimental confirmed that C-PC extracts from different cyanobacteria species present differential antioxidant activity. This supported our motto, as the C-PC presence (or the blue coloring) is clearly not the single variable constraining extracts' biological activity, and recall for the need to reformulate the common assumption of C-PC bioactivity attributes. Biological activities in C-PC containing extracts are most likely the result of synergic interactions between the components of the extracts, rather than reflect only the presence of the pigment.

References

Apak R, Gorinstein S, Böhm V, et al. (2013) Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). Pure Appl Chem 85:957–998. doi: 10.1109/ICHR.2010.5686322

Basha OM, Hafez RA, El-Ayouty YM, et al. (2008) C-Phycocyanin inhibits cell proliferation and may induce apoptosis in human HepG2 cells. Egypt J Immunol 15:161–167. doi: 10.1016/j.algal.2013.01.004

Bérdi J (2005) Bioactive microbial metabolites. J Antibiot (Tokyo) 58:1–26.

Berry JP (2008) Cyanobacterial toxins as allelochemicals with potential applications as algaecides, herbicides and insecticides. Mar Drugs 6:117–146. doi: 10.3390/md20080007

Bryant DA, Guglielmi G, de Marsac NT, et al. (1979) The structure of cyanobacterial phycobilisomes: a model. Arch Microbiol 123:113–127. doi: 10.1007/BF00446810

Chen C-H, Berns DS (1978) Comparison of the stability of phycocyanins from thermophilic, mesophilic, psychrophilic and halophilic algae. Biophys Chem 8:203–213. doi:

https://doi.org/10.1016/0301-4622(78)87002-1

Colla LM, Bertol CD, Ferreira DJ, et al. (2016) Thermal and photo-stability of the antioxidant potential of *Spirulina platensis* powder. Brazilian J Biol 77:332–339. doi: 10.1590/1519-6984.14315

CRF (2021) *Spirulina* extract, 73.530. Title 21 – Food and Drugs, Chapter I – part 500.

Dejsungkranont M, Chen HH, Sirisansaneeyakul S (2017) Enhancement of antioxidant activity of C-phycocyanin of *Spirulina* powder treated with supercritical fluid carbon dioxide. Agric Nat Resour 51:347–354. doi: 10.1016/j.anres.2017.12.001

Eriksen NT (2008) Production of phycocyanin - a pigment with applications in biology, biotechnology, foods and medicine. Appl Microbiol Biotechnol 80:1–14. doi: 10.1007/s00253-008-1542-y

EU (2021) Regulation (EC) No 1333/2008 of the European Parliament and of the Council, amended at 16 July 2021.

Fratelli C, Burck M, de Amarante MCA, Braga ARC (2020) Antioxidant potential of nature's "something blue": Something new in the marriage of biological activity and extraction methods applied to C-phycocyanin. Trends Food Sci Technol 107:309–323. doi: https://doi.org/10.1016/j.tifs.2020.10.043

Glazer AN (1994) Phycobiliproteins - a family of valuable, widely used fluorophores. J Appl Phycol 6:105–112. doi: 10.1007/BF02186064

González R, Rodríguez's S, Romay C, et al. (1999) Anti-inflammatory activity of phycocyanin extract in acetic acid-induced colitis in rats. Pharmacol Res 39:55–59. doi: 10.1006/phrs.1998.0409

Hadiyanto SH (2016) Response surface optimization of ultrasound assisted extraction (UAE) of phycocyanin from microalgae *Spirulina platensis*. Emirates J Food Agric 28:227–234. doi: 10.9755/ejfa.2015-05-193

Hao S, Li S, Wang J, et al. (2018) Phycocyanin reduces proliferation of melanoma cells through downregulating GRB2/ERK signaling. J Agric Food Chem 66:10921–10929. doi: 10.1021/acs.jafc.8b03495

Haque F, Banayan S, Yee J, Chiang YW (2017) Extraction and applications of cyanotoxins and other cyanobacterial secondary metabolites. Chemosphere 183:164–175. doi: 10.1016/j.chemosphere.2017.05.106

Herrero M, Simó C, Ibáñez E, Cifuentes A (2005) Capillary electrophoresis-mass

spectrometry of *Spirulina platensis* proteins obtained by pressurized liquid extraction. Electrophoresis 26:4215–4224. doi: 10.1002/elps.200500230

Ilieva V, Kondeva-Burdina M, Georgieva T, Pavlova V (2019) Toxicity of cyanobacteria. Organotropy of cyanotoxins and toxicodynamics of cyanotoxins by species. Pharmacia 66:91–97. doi: 10.3897/pharmacia.66.e37035

İlter I, Akyıl S, Demirel Z, et al. (2018) Optimization of phycocyanin extraction from *Spirulina platensis* using different techniques. J Food Compos Anal 70:78–88. doi: 10.1016/j.jfca.2018.04.007

Jaeschke DP, Teixeira IR, Marczak LDF, Mercali GD (2021) Phycocyanin from *Spirulina*: A review of extraction methods and stability. Food Res Int 143:110314. doi: 10.1016/j.foodres.2021.110314

Jang YA, Kim BA (2021) Protective effect of *Spirulina*-derived C-phycocyanin against ultraviolet B-induced damage in HaCaT cells. Medicina (B Aires) 57:1–12.

Jerley AA, Prabu DM (2017) Purification, characterization and antioxidant properties of C-Phycocyanin from *Spirulina platensis*. Scrut Int Res J Agric Plant Biotechnoloy Bio Prod 4:1–9.

Jiang L, Wang Y, Liu G, et al. (2018) C-Phycocyanin exerts anti-cancer effects via the MAPK signaling pathway in MDA-MB-231 cells. Cancer Cell Int 18:1–14. doi: 10.1186/s12935-018-0511-5

Jiang L, Wang Y, Yin Q, et al. (2017) Phycocyanin: A potential drug for cancer treatment. J Cancer 8:3416–3429. doi: 10.7150/jca.21058

Kumar M, Kulshreshtha J, Singh GP (2011) Growth and biopigment accumulation of cyanobacterium *Spirulina platensis* at different light intensities and temperature. Brazilian J Microbiol 42:1128–1135. doi: 10.1590/S1517-838220110003000034

Martins M, Soares BP, Santos JHPM, et al. (2021) Sustainable strategy based on induced precipitation for the purification of phycobiliproteins. ACS Sustain Chem Eng 9:3942–3954. doi: https://doi.org/10.1021/acssuschemeng.0c09218

Maurya SS, Maurya JN, Pandey VD (2014) Factors regulating phycobiliprotein production in cyanobacteria. Int J Curr Microbiol Appl Sci 3:764–771.

Metcalf JS, Codd GA (2012) Cyanotoxins. In: Whitton BA (ed) Ecol. Cyanobacteria II - Their Divers. Sp. Time. Springer Science + Business Media B.V., pp 651–675

Mohite YS, Shrivastava ND, Sahu DG (2015) Antimicrobial activity of C-phycocyanin from

Arthrospira platensis isolated from extreme haloalkaline environment of Lonar Lake. IOSR J Environ Sci Toxicol Food Technol 1:40–45.

Nichols HW (1973) Growth media-freshwater. In: Stein JR (ed) Handb. Phycol. methods Cult. methods growth Meas. Cambridge University Press, Cambridge, UK, pp 16–17

Pearson LA, Dittmann E, Mazmouz R, et al. (2016) The genetics, biosynthesis and regulation of toxic specialized metabolites of cyanobacteria. Harmful Algae 54:98–111. doi: 10.1016/j.hal.2015.11.002

Renugadevi K, Nachiyar CV, Sowmiya P, Sunkar S (2018) Antioxidant activity of phycocyanin pigment extracted from marine filamentous cyanobacteria *Geitlerinema* sp. TRV57. Biocatal Agric Biotechnol 16:237–242. doi: 10.1016/j.bcab.2018.08.009

Rito-Palomares M, Nuez L, Amador D (2001) Practical application of aqueous two-phase systems for the development of a prototype process for C-phycocyanin recovery from *Spirulina maxima*. J Chem Technol Biotechnol 76:1273–1280. doi: 10.1002/jctb.507

Romay C, Armesto J, Remirez D, et al. (1998) Antioxidant and anti-inflammatory properties of C-phycocyanin from blue-green algae. Inflamm Res 47:36–41. doi: 10.1007/s000110050256

Ruiz-Domínguez MC, Fuentes JL, Mendiola JA, et al. (2021) Bioprospecting of cyanobacterium in Chilean coastal desert, *Geitlerinema* sp. molecular identification and pressurized liquid extraction of bioactive compounds. Food Bioprod Process 128:227–239. doi: 10.1016/j.fbp.2021.06.001

Sekar S, Chandramohan M (2008) Phycobiliproteins as a commodity: Trends in applied research, patents and commercialization. J Appl Phycol 20:113–136. doi: 10.1007/s10811-007-9188-1

Shanmugam A, Sigamani S, Venkatachalam H, et al. (2017) Antibacterial activity of extracted phycocyanin from *Oscillatoria* sp.. J Appl Pharm Sci 7:62–67. doi: 10.7324/JAPS.2017.70310

Silveira ST, Quines LKM, Burkert CAV, Kalil SJ (2008) Separation of phycocyanin from *Spirulina platensis* using ion exchange chromatography. Bioprocess Biosyst Eng 31:477–482. doi: 10.1007/s00449-007-0185-1

Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006) Commercial applications of microalgae. J Biosci Bioeng 101:87–96. doi: 10.1263/jbb.101.87

Stanic-Vucinic D, Minic S, Nikolic MR, Velickovic TC (2018) *Spirulina* phycobiliproteins

as food components and complements. In: Jacob-Lopes E (ed) Microalgal Biotechnol. IntechOpen, pp 129–149

Twentyman PR, Luscombe M (1987) A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer 56:279–285. doi: 10.1038/bjc.1987.190

Wu HL, Wang GH, Xiang WZ, et al. (2016) Stability and antioxidant activity of food-grade phycocyanin isolated from *Spirulina platensis*. Int J Food Prop 19:2349–2362. doi: 10.1080/10942912.2015.1038564

CHAPTER V

Metabolic composition of the cyanobacterium *Nostoc muscorum* as a function of culture time: a ^{1}H NMR metabolomics study

I. P. E. Macário; T. Veloso; J. Romão; F. J. M. Gonçalves; J. L. Pereira; I. F. Duarte; S. P. M. Ventura. Metabolic composition of the cyanobacterium *Nostoc muscorum* as a function of culture time: a ^{1}H NMR metabolomics study. *Submitted to Algae Research*

Contributions: I. P. E. M. wrote the manuscript; I. P. E. M. and T. V. acquired the experimental data and performed the data analysis; J. R. helped with the data analysis; I. F. D. performed the NMR spectra aquisition and processing and helped with the data analysis; F. J. M. G., S. P. V., and J. L. P. supervised

Abstract: Cyanobacteria are often considered a factory of added-value compounds. However, knowledge about the array of interesting compounds that could be extracted from these prokaryotic organisms is still very limited. Nuclear Magnetic Resonance (NMR) spectroscopy is a widely used technique for metabolic profiling that allows an overview of the main metabolites present in complex biological matrices. In this work, high resolution ^{1}H NMR was applied to the freshwater cyanobacterium *Nostoc muscorum*, and used to screen the production of metabolites demanded by several biotechnological applications. The growth and metabolic profile of *N. muscorum*. were monitored during 112 days. This species showed an incredible longevity, being able to grow for more than one hundred days without any medium supplementation and withstanding the capacity as a source of valuable compounds. During the period of study, several interesting metabolites were detected, such as several sugars and oligosaccharides, lipids (e.g. glycolipids, ω-3 and ω-6 fatty acids), amino acids, including mycosporin-like, peptides, and pigments (e.g. chlorophyll *a* and carotenoids). Owing to the long-term monitoring implemented in this study, the production of these compounds could be associated to specific moments of the growth of *N. muscorum*, providing new insights into the most appropriate harvesting time points for the biotechnological exploitation of specific molecules.

Keywords: cyanobacteria, *Nostoc muscorum*, metabolome, NMR spectroscopy, bioactive compounds

V-1. Introduction

The search of natural compounds with biological activity is an active and growing area of research. While bacterial and fungal microorganisms (*Actinomycetes* and *Hyphomycetes*) have been the most widely explored sources of bioactive compounds for a long time, in recent years, the focus largely shifted to microalgae and cyanobacteria (Singh et al. 2005). Cyanobacteria are prokaryotic organisms, which synthesize a plethora of secondary metabolites in response to environmental conditions. Many of these metabolites can be harvested and exploited for a multitude of applications, which supports the biotechnological potential of these microorganisms. Recent reviews on cyanobacteria metabolites highlight their use as chelating agents, biofuels, biocides, cosmetics, pharmaceuticals, fertilizers, and foods, just to mention a few (e.g. Smith & Thanh Doan, 1999; Singh et al., 2016; Haque et al., 2017; Kultschar & Llewellyn, 2018; Huang & Zimba, 2019; Macário et al., 2021). The molecules themselves can be assorted into a vast collection of chemical groups, including fatty acids, terpenoids, phenolics, phytohormones, alkaloids, isoprenoids, polysaccharides and photoprotective compounds (Martínez-Francés and Escudero-Oñate 2018). However, from more than 10 000 cyanobacteria species, only a thousand were analysed for some kind of metabolite characterization and a much smaller number of species is currently cultured at the industrial scale for commercial exploitation (Raheem et al. 2018). This means that our knowledge about the biotechnological potential of these organisms is still very limited, configuring a new avenue that is worth exploring.

Nostoc is a genus of filamentous nitrogen-fixing cyanobacteria (i.e. diazotrophic species). It can be found in terrestrial and aquatic environments, often forming macroscopic or microscopic colonies (Potts 2002). Specialized cells like heterocysts (N_2-fixing cells) and akinetes (resistance cells) are present in its filaments or trichomes (Borowitzka 2018). Several species of the *Nostoc* genus (e.g. *Nostoc flagelliforme, Nostoc muscorum*, *Nostoc sphaeroides*) have been used as edible food items in China, other Asian countries and in South America (Gao 1998; Johnson et al. 2008). These species of *Nostoc* are also known to produce interesting compounds with a wide range of biological activities. Remarkably, cyanovirin-N (CV-N) is a protein discovered in *Nostoc ellipsosporum* able to inactivate some primary strains of HIV-1 (Burja et al. 2001). Borophycin is a metabolite confirmedly produced by the marine strains *Nostoc linckia* and *Nostoc spongiaeforme* var. *tenue*, which shows cytotoxicity against human epidermoid carcinoma and human colorectal

adenocarcinoma cell lines (Burja et al. 2001). Cryptophycin was isolated from some strains of *Nostoc*, and was shown to have activity as a potent fungicide and as a cytotoxic compound against human tumour cell lines (Burja et al. 2001). These examples, among many others, support further the interest on studying this genus for potential biotechnological exploitation.

The biological activity of both documented and novel cyanobacteria metabolites is of significant relevance to various applications, including in the medical field. Thus, there is growing interest in detecting these molecules in different sample species and assessing the dynamics of their production. By enabling the detection, identification, and quantification of a multitude of metabolites in complex biological matrices, metabolomics methods are exquisitely powerful in this respect. The most popular analytical techniques employed in metabolomics studies are Mass Spectrometry (MS), typically preceded by some kind of chromatographic separation, and Nuclear Magnetic Resonance (NMR) spectroscopy (Schwarz et al. 2013). Although the relatively low sensitivity of ^{1}H NMR generally precludes the detection of compounds in sub-micromolar concentrations, this technique readily provides structural and quantitative information on a wide range of metabolites, from different chemical families, in a rapid and non-destructive way (Fan and Lane 2008; Dona et al. 2016). Accordingly, NMR-based metabolomics has been proven useful in the metabolic characterization of several algal species (Nylund et al. 2011; Gupta et al. 2013; Ito et al. 2013; Kumar et al. 2015; Arora et al. 2018) and cyanobacteria (Kim et al. 2006; Zea Obando et al. 2016; Iglesias et al. 2020).

The present study reports the first NMR metabolomics investigation of freshwater cyanobacteria from the *Nostoc* genus, with special emphasis on prospecting the presence of compounds that can be worth of further exploitation. The hypotheses in this work were two-fold. First, we explored the presence of biotechnologically interesting metabolites based on previous evidence for the genus (see above). Second, and based on general principles of the growth dynamics of these organisms as constrained by abiotic (e.g. metabolic products accumulation) and biotic (e.g. density-dependent competition for nutrients), we assumed that the metabolic profile would change through the growth period in the long-term, this being critical to define the most appropriate harvesting moments for the biotechnological exploitation of different molecules of interest. To address these hypotheses, we monitored the changes in the metabolome in a culture growing for more than one hundred days with no renewal or nutrient supplementation.

V-2. Material and methods

V-2.1. Cyanobacteria culture and growth monitoring

The filamentous cyanobacterium *Nostoc muscorum* UTAD_N213. was cultured in triplicate, in 5-L Schott flasks with 5 L of Woods Hole MBL culture medium (Nichols 1973). A 9-days inoculum was used to onset the cultures, which achieved an initial optical density (440 nm) of 0.08 ± 0.01 (UV-Vis spectrophotometer, Schimadzu UV 1800). The cultures were incubated with constant aeration at 26 ± 2 (°C) under a 16 h-light/8 h-dark photoperiod cycle; light intensity during the day period was set to 2300 lux provided by cool white fluorescent tubes. The cyanobacteria were kept under these conditions for a period of 112 days with no medium renewal or supply. Samples were collected three times *per* week, in a flow chamber and using sterile material for preventing any additional bacterial contamination (these cultures are hardly axenic in the laboratory or in scaled-up cultivation systems), for growth kinetics monitoring based on optical density reading at 440 nm. Due to the frequent sampling, no appropriate volumes were available for sampling and analysis after 112 days.

V-2.2. Sample collection and treatment for NMR analysis

Two technical sample replicates from each replicated culture were collected every two weeks for NMR analysis (see section V-2.3), in a total of 6 replicates *per* timepoint. Samples (15-20 mL) of each homogenized culture were harvested under sterile conditions and vacuum-filtered through a nylon membrane (0.45 µm pore size, 47 mm diameter; Whatman). A washing step with 10 mL of 0.9 % NaCl was run at room temperature, then the membrane was scrapped to recover the retained cells into a pre-washed (MeOH) glass tube containing ~100 mg of cooled pre-washed (MeOH) glass beads (0.5 mm; Scientific Industries). Solvents (MeOH:$CHCl_3$:H_2O; 1:1:0.7) were added to the biomass (indicative volumes are given for 20 mg of biomass) or to the blank samples (filter with no cells washed with NaCl) set in parallel, and extractions were run inside an icebox. Briefly, cold MeOH 80 % (-20 ºC; 800 µL) was added to the biomass, vortexed, cooled down on ice, and vortexed again (for some samples, an extra step of ultrasounds bath with ice for samples to dissolve was considered). Each tube was then added with cold $CHCl_3$ (-20 ºC; 320 µL) twice and cold ultrapure water (4 ºC; 288 µL), with 2-min intermediate vortexing steps. The tubes were placed on ice for 15 min and centrifuged at 4111 ×*g* (4 ºC; 15 min). The upper aqueous phase

was then transferred to a microtube and dried in a speedvac (Eppendorf concentrator with UNIJET II vacuum pump), while the lower organic phase was placed into an amber glass vial and dried under nitrogen. All samples were stored at -80 ºC. For NMR analysis, dried aqueous and organic phases were reconstituted with deuterated phosphate buffer (pH 7.4 containing 0.1 mM TSP-d_4; 600 µL) or deuterated dimethyl sulfoxide (DMSO-d_6 containing 0.01 % TMS; 600 µL), respectively. The samples were then shortly vortexed, homogenised (2 min at 2500 rpm; Digital Disruptor Genie, Scientific Industries), placed in a cold ultrasound bath for 180 s for complete dissolution, and transferred to 5 mm NMR tubes (550 µL).

V-2.3. NMR spectra acquisition and processing

NMR spectra were acquired on a Bruker Avance III HD 500 spectrometer (University of Aveiro, Portuguese NMR Network) operating at 500.13 MHz for ^{1}H observation, at 298 K, using a 5 mm TXI probe. Standard 1D spectra (Bruker pulse programs 'noesypr1d', with water suppression, for aqueous extracts, and 'zg' for organic extracts) were recorded with a 7002.8 Hz spectral width, 32 k data points, a 2 s relaxation delay and 512 scans. Spectral processing (TopSpin 4.0) comprised exponential multiplication with 0.3 Hz line broadening, zero filling to 64 k data points, manual phasing, baseline correction, and chemical shift calibration to the TSP or TMS signal at 0 ppm. To assist spectral assignment, 2D experiments have also been recorded for selected samples, namely: i) ^{1}H-^{1}H total correlation (TOCSY) spectra, which show the spin systems corresponding to scalar coupled protons in a molecule, giving a unique 2D pattern; ii) ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC) spectra, which shows proton-carbon single bond correlations, being especially useful for assigning singlets that have no TOCSY correlations; and iii) *J*-resolved spectra where the ^{1}H signals multiplicity and coupling constants are made explicit along the F1 axis.

V-2.4. Data analysis

The first stage of data analysis consisted of assigning the signals detected in the spectra of aqueous and organic phases of extracted biomass. Compound annotation was based on matching 1D and 2D spectral information to several literature reports (Hauksson et al. 1995; Marcolongo et al. 2006; Pontis et al. 2007; Kobayashi et al. 2013; Lafountain et

al. 2013; Sallans et al. 2013) and reference spectra available in Chenomx (Edmonton, Canada), BBIOREFCODE-2–0–0 (Bruker Biospin, Rheinstetten, Germany), and HMDB (Wishart et al. 2018). Then, representative signals of assigned compounds, free of major overlap, were selected for integration in Amix-Viewer 3.9.15. The areas of selected signals were then normalized by the total spectral area (excluding residual solvent signals) and used to assess quantitative changes over time, through multivariate analysis (performed in Metaboanalyst 5.0, https://www.metaboanalyst.ca) and univariate statistics. Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were applied to normalized and unit variance-scaled data to assess, respectively, grouping trends (as visualized in the scores scatter plot), and the most important variables accounting for sample discrimination across time of growth (variable importance to the projection, VIP ≥ 1). Additionally, Spearman rank correlation analysis was employed to search for the most time-correlated variables ($|r| > 0.6$; $p < 0.05$). One-way ANOVA with post-hoc Tukey tests was applied to assess the effect of time in the production of each metabolite with a VIP score ≥ 1 and/or $|r| > 0.6$. An alpha level of 0.05 was considered in all analyses.

V-3. Results

V-3.1. Growth of the N. muscorum *culture in the long-term*

The growth of the *N. muscorum* was monitored through 112 days using the optical density as a proxy of biomass. An adjusted exponential equation described accurately the increase in absorbance as denoted by the coefficient of determination higher than 0.98 (Figure V-1). These data reflect a continuous increase in absorbance throughout the entire experiment, with higher standard deviations being recorded for later time points (after day 80), which likely relates to the increased turbidity caused by high sample concentrations. Notably, although there was no nutrient reinforcement throughout the study period, the cultures never reached a clear stationary phase. These data were previously confirmed by more feasible proxies for culture growth based on fluorescence parameters described in Chapter III.

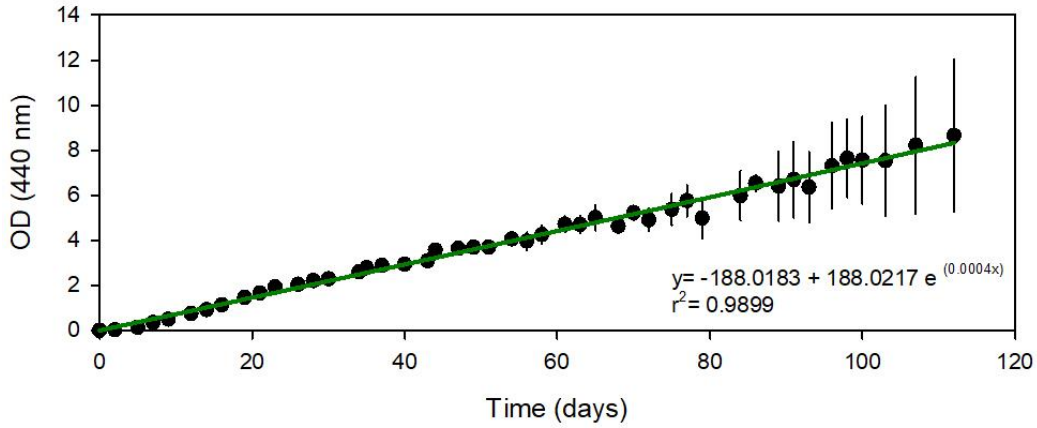

Figure V-1. Growth kinetics of *N. muscorum* throughout a monitoring period of 112 days. Marks represent the mean of 3 replicates and the error bars represent the standard deviation. The best fitted exponential model was added as a solid green curve. Note that OD measurements were always made using controlled sample dilutions resulting in readings up to a threshold of 1.000.

V-3.2. Metabolic profile of N. muscorum

The 1D ^{1}H NMR spectra of aqueous and organic extracts (Figure V-2) revealed complementary information on the biomass constituents, which were identified based on the sets of 2D spectra collected for each sample type, as detailed in the experimental section. Additionally, some assignments were further confirmed through in-house NMR analysis of standard solutions and spiking experiments (addition of standard to sample to confirm a specific assignment based on signal increments). The compounds detected in aqueous extracts comprised amino acids and small peptides, monosaccharides, disaccharides and oligosaccharides, organic acids, nucleotides and compatible solutes (betaines, glucosylglycerol). The organic extracts contained mainly lipids and photosynthetic pigments (chlorophyll and carotenoids). The list of assigned resonances is shown in Supplementary Table V-S1 (Annex III). In some cases, the information retrieved from spectral analysis was insufficient to provide definite assignments, hence, some compounds were identified in more general terms, namely: i) peptides 1, 2 and 3 (NMR profile consistent with bound amino acids, but specific identity not determined); ii) AXP (AMP, ADP and/or ATP) and UXP (UMP, UDP and UTP); iii) glycolipids (galactosyl, glucosyl and glyceryl moieties, as in Marcolongo et al. (2006) and Sallans et al. (2013)); iv) sucrose-containing oligosaccharides, as in Pontis et al. (2007); v) betaines 1, 2 and 3 (3 singlets with ^{1}H and ^{13}C chemical shifts characteristic of these amino acid derivatives). Overall, 36 signals were integrated and their

areas, which are proportional to compound concentrations, were used as input for multivariate analysis.

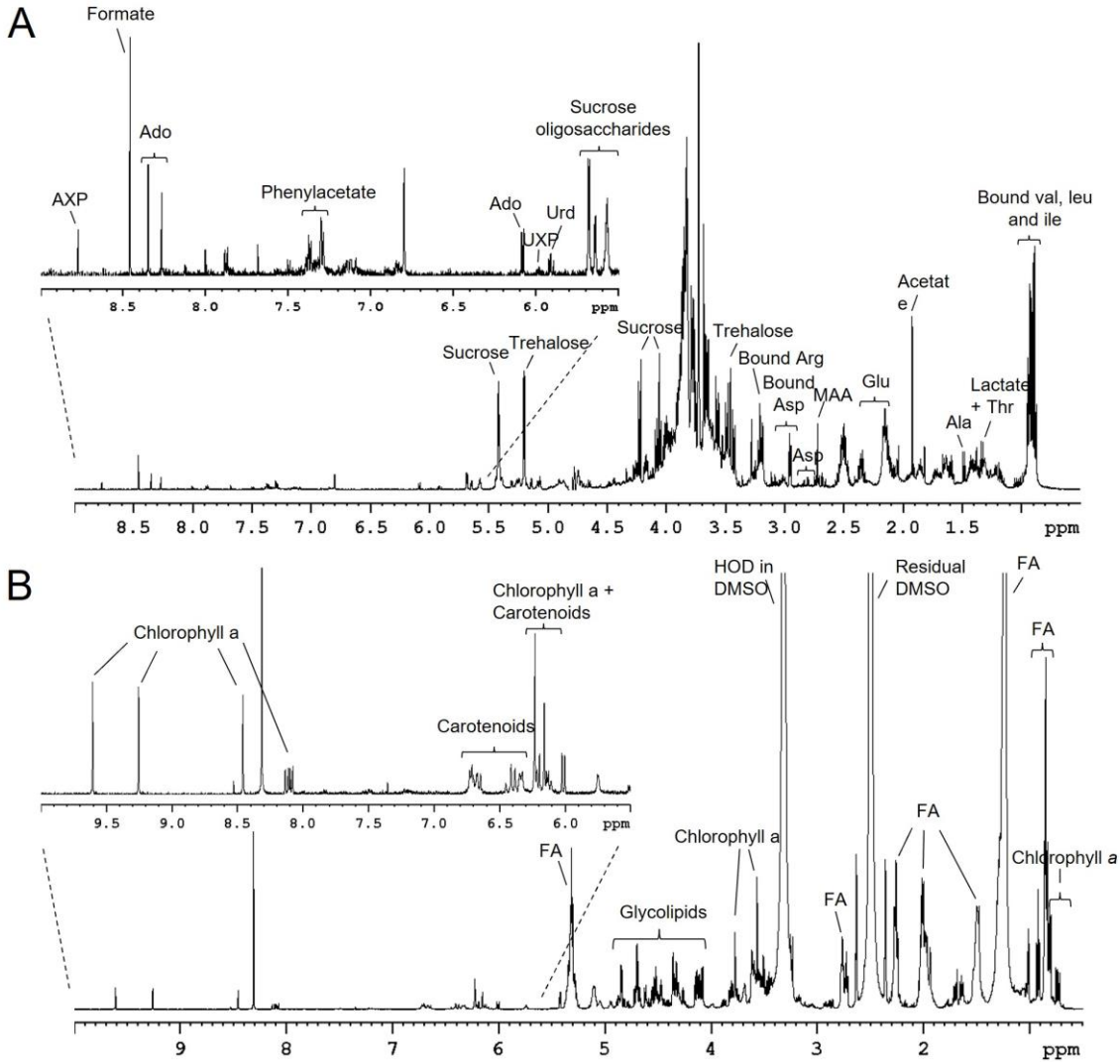

Figure V-2. Representative 500 MHz ^{1}H NMR spectra of the aqueous (A) and organic phase (B) of *N. muscorum* culture at day 70. Some assignments are indicated, while a full list of identified compounds is provided in Supplementary Table V-S1. Three-letter code used for amino acids; MAA, mycosporine-like amino acid; Ado, adenosine; AXP, adenosine mono/di/tri-phosphate; UXP, uridine mono/di/tri-phosphate; FA, fatty acyl chains in lipids.

As a first approach to identify grouping trends within the samples, unsupervised PCA was applied to signal areas (representative of the different metabolites identified). The scores scatter plot relating PC1 and PC3 (Figure V-3A) showed a clear time-related gradient

distribution of samples along PC1, which explained 54.3 % of the data variance. This is particularly evident up to day 56, after which the samples from different time points were less well separated, indicating higher similarity between their metabolic profiles. This observation was further corroborated by the dendrogram obtained by HCA (Figure V-3B), where samples from days 84 and 112 were clustered closely together and included in a larger cluster also comprising the later time points (from day 56 onwards), which separated from another branch comprised of days 14, 28 and 43.

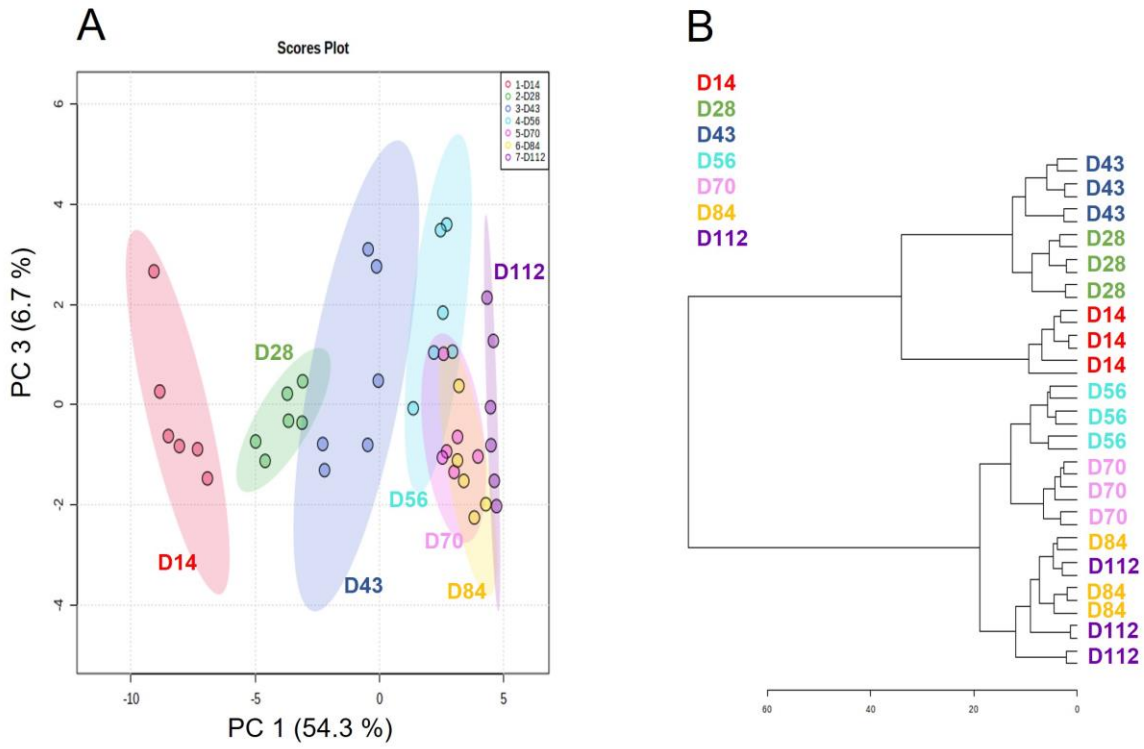

Figure V-3. A) Principal component analysis (PCA) scores scatter plot and B) Hierarchical cluster analysis (HCA) dendrogram (Euclidean distance, Ward clustering algorithm), evidencing the changes in the metabolome of *N. muscorum* through a 112-days monitoring period. Samples are color-coded by sampling timepoint (days 14, 28, 43, 56, 70, 84 and 112). In the PC1 *vs* PC3 scores scatter plot (best representing the separation between sample groups), the shadowed areas represent 95 % confidence regions.

The heatmap representation of the 36 signal areas measured for all samples (Figure V-4) also showed two main metabolic signatures corresponding to the first and second half of the growth period. In days 14, 28 and 43, cyanobacteria were characterized by higher relative abundances of malto-oligosaccharides, amino acids, nucleotides, chlorophyll *a*, and

some lipids, mainly polyunsaturated fatty acids and glycolipids. In the second half of the growth period (from day 56 onwards), the levels of these compounds were contrastingly lower, while the levels of sucrose-derived oligosaccharides, nucleosides and peptides, among others, were relatively higher. Furthermore, metabolites such as carotenoids, valine, isoleucine and trehalose did not follow any of the patterns described above, being produced heterogeneously during the growth of *N. muscorum*.

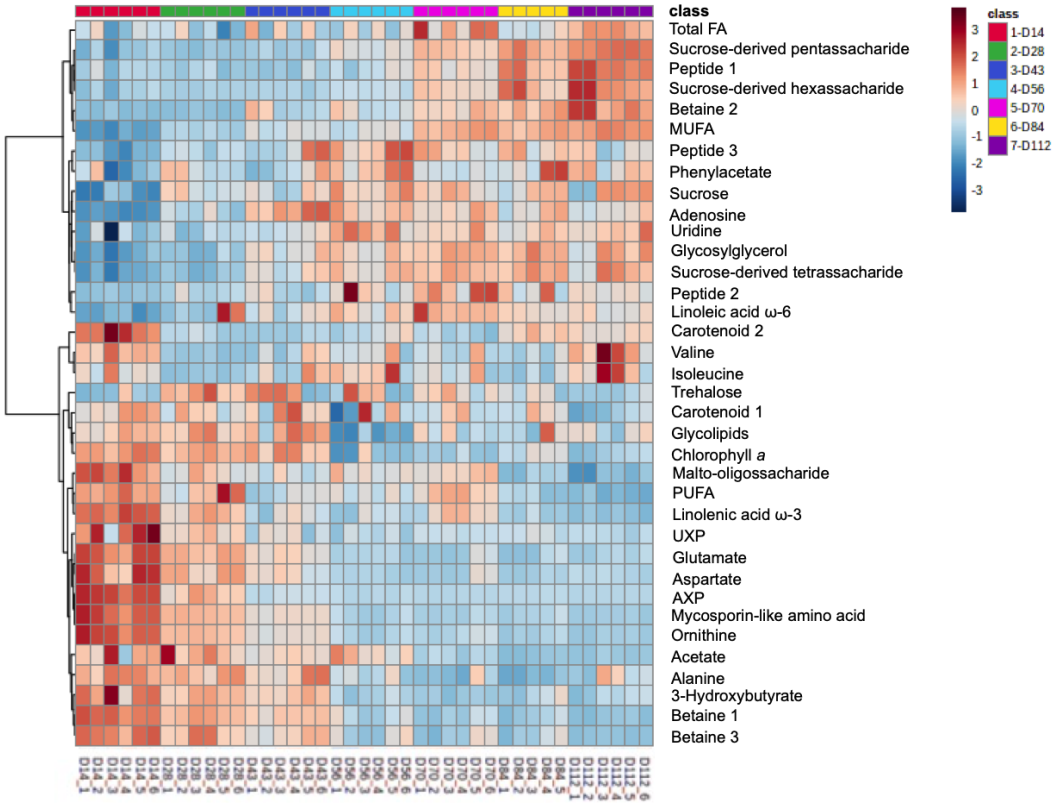

Figure V-4. Heatmap representation of relative abundances (from low, in blue, to high, in red) of the 36 metabolites (or metabolite groups) detected in *N. muscorum* extracts at different culture time points. The left-hand cladogram evidences the similarities between compound production patterns through time.

To determine the compounds with greater importance for time-related sample separation, PLS-DA was applied to the 36-variables matrix. The resulting model discriminated the sample groups with high robustness (Q^2 0.96 for 2 components, Figure V-5A) and showed a trajectory along the first latent variable (LV) according to culture time.

The compounds contributing the most to the observed LV1 scores distribution (VIP ≥ 1) are highlighted in Figure V-5B. These results were further confirmed by correlation analysis, since all the compounds with VIP ≥ 1 were also significantly correlated with time ($|r| > 0.6$; $p < 0.05$), as shown in Figure V-5C.

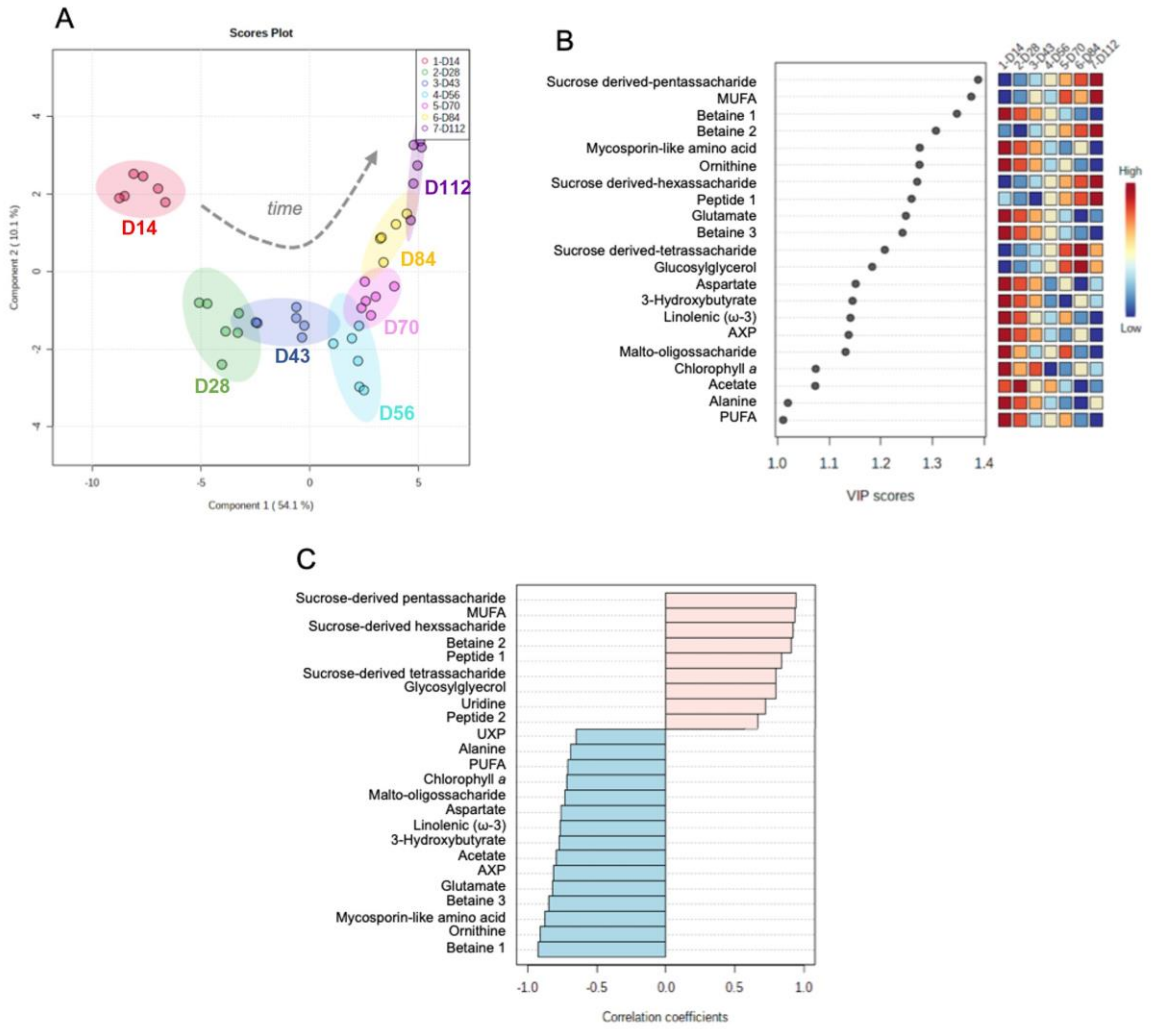

Figure V-5. A) Partial Least Squares Regression with Discriminant Analysis (PLS-DA) scores scatter plot and B) the variables (metabolites) most important (VIP ≥ 1) for sample distribution along the first PLS-DA component; C) top 24 metabolites correlated with time, from day 14 to day 112 (Spearman rank correlation).

The relative levels of compounds with VIP ≥ 1 and with significant correlation coefficients, at different culture times, are displayed in Figure V-6. A number of amino acids, namely glutamate, aspartate, ornithine, alanine and a mycosporin-like amino acid, decreased

consistently over time, especially from 14 to 56 days. On the other hand, the levels of peptides increased, especially in the second half of the growth period. There were also marked changes in carbohydrates. Maltose-derived oligosaccharides showed a decreasing trend through time, whereas sucrose-derived oligosaccharides were very low in early time points and steadily accumulated over time. As for compatible solutes, glucosylglycerol and one betaine increased through culture growth, whereas two other betaines decreased. Lipid-related compounds also displayed diverse variation patterns. While polyunsaturated fatty acids (PUFA), including linolenic acid, decreased with time, those with a single unsaturation (MUFA) were accumulated. As for the levels of total fatty acids (first row in the heatmap of Figure V-4), there was greater heterogeneity between samples, although an increasing trend could be seen towards the end of the growth period. Moreover, the ratio of linoleic (ω-6) to linolenic (ω-3) acid increased significantly from early to late time points (Figure V-6). Other compounds with VIP ≥ 1 and/or $|r| > 0.6$ included 3-hydroxybutyrate, acetate, nucleotides, and chlorophyll *a*. They all had higher relative levels in younger cultures and decreased over the second half of the growth period.

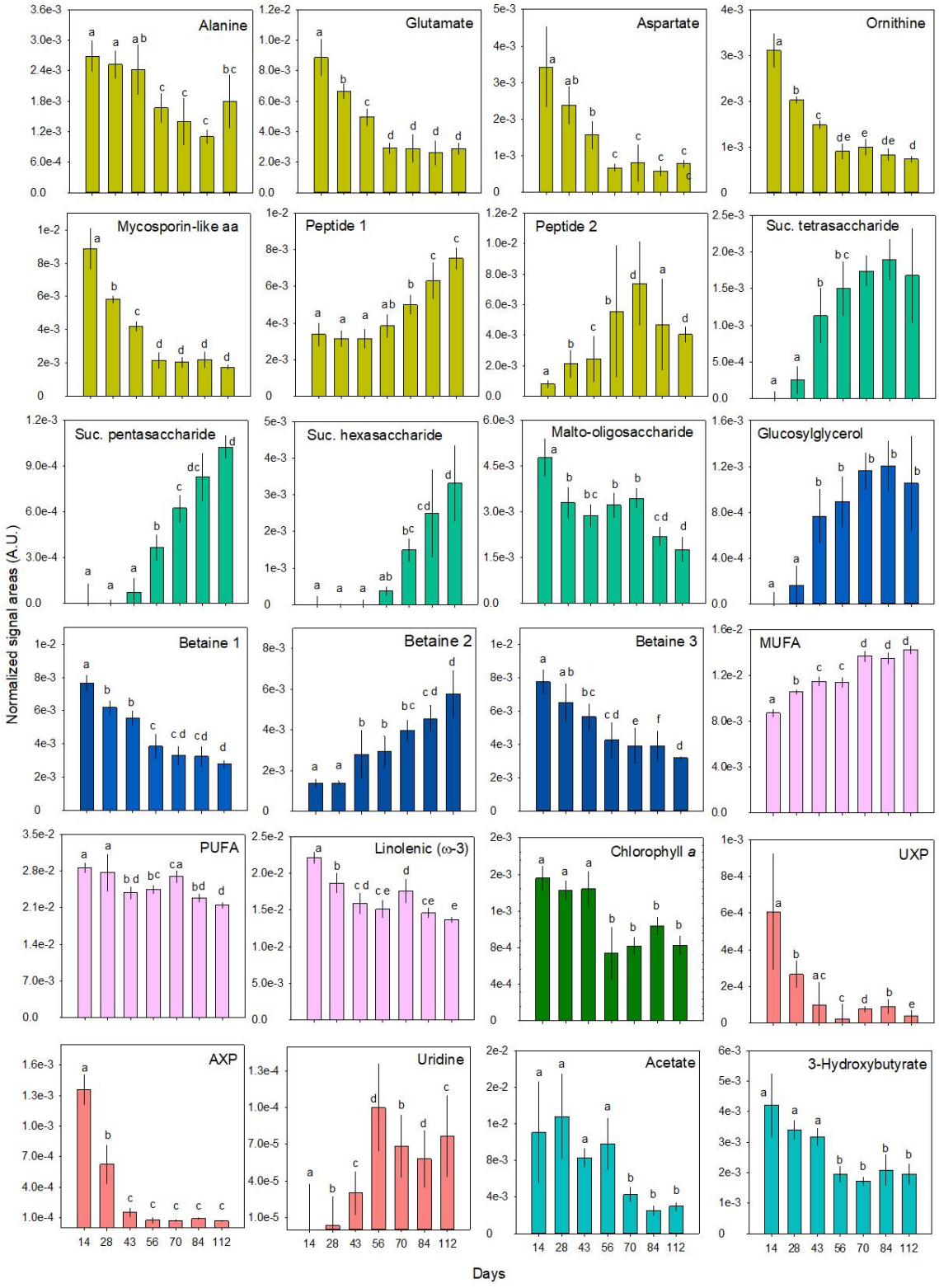

Figure V-6. Relative levels of compounds with VIP ≥ 1 and significant correlation with time, at different culture time points. Results are expressed as the mean (bars) ± standard deviation (error bars) of six replicates. Different letters indicate statistically significant differences among sampling time-points (Tukey test; $p < 0.05$ following one-way ANOVA - statistical summary in supplemental

Table V-S2, Annex III). Graphic colors represent different classes of metabolites: olive green for amino acids and peptides, green for carbohydrates, dark blue for compatible solutes, purple for lipids, dark green for chlorophyll *a*, light red for nucleotides/nucleosides, and light blue for others.

V-4. Discussion

Cyanobacteria are currently regarded as a promising natural source of valuable compounds. Among the most explored species, those from the *Arthrospira* genus are doubtless the most known and studied. However, the characterization of the metabolic profiles of other species is of critical importance, especially given that a large interspecific variation in ecophysiological features is a known attribute of cyanobacteria (e.g. Cerasino et al. 2017). Such variability enlarges the scope for an efficient exploitation of the production of specific compounds of biotechnological interest, including novel compounds. Like *Arthrospira*, *Nostoc* is a genus known for its edibility and it has been used as a food source for many years (Gao 1998; Johnson et al. 2008), although it is far less studied. One of the advantages of *Arthrospira* species is that they are easy to culture within an industrial context. The present study demonstrates that the use of *N. muscorum* is also not limitative in this regard. Indeed, the growth of *N. muscorum* assessed in the present study was impressive, since it was stable for more than 100 days without any additional nutrient supplementation. This suggests particularly low requirements of *N. muscorum* cultures, hence allowing long-term maintenance costs to be reduced, which is key to sustainable industrial exploitation.

The use of NMR spectroscopy for the screening of cyanobacteria metabolites has already been proven useful, although it has only been applied to single or to a limited number of time points (e.g. Aikawa et al. 2019; des Aulnois et al. 2019; Iglesias et al. 2020). To the best of our knowledge, this is the first study where this technique, or, in fact, any metabolic profiling technique, was applied to monitor the variations in the cyanobacteria metabolome in the long term. Such knowledge not only improves our understanding of these organisms' physiology, as it is useful from an applied perspective, namely regarding the identification of the most appropriate growth stages to explore a specific metabolite or group of metabolites. Along with assuring that the stock cultures are growing at an optimal and stable pace in the long term, this is key for industrial exploitation of target compounds, so that harvesting can be scheduled to the growth phase where their maximal production occurs.

The NMR profiling carried out in the present study proved that important metabolites with several biotechnological applications are naturally produced by *N. muscorum*, hence

confirming our first hypothesis. Also, the metabolic profile of *N. muscorum* over the growth period was not homogeneous in the long term, and some key metabolites were preferentially produced in specific phases of the culture growth. Thus, our second hypothesis was verified, evidencing that it is possible to optimise the best harvesting period for the extraction of a desired metabolite. The analysis of the production patterns suggests a pronounced shift in metabolism at approximately half term of the studied period (from day 43 to 56). This could possibly reflect some age-related metabolic adaptations, as the number of older cells, relative to total cell numbers, naturally increases with time. Moreover, as the culture medium was not supplemented during the growth period, consumption of certain nutrients and release of metabolic waste products is also expected to influence the cells metabolic activity. In future studies, a parallel analysis of extracellular medium is warranted to verify this hypothesis.

Several compounds such as amino acids showed higher production during earlier stages of the growth period (up to 43 days). Within the amino acids identified in *N. muscorum*, mycosporin-like amino acids (MAAs) are particularly interesting. So far, more than 30 MAAs were identified (Kultschar and Llewellyn 2018), and are present in several biological groups, such as corals, micro and macroalgae, dinoflagellates, fungi, lichens and cyanobacteria (Chrapusta et al. 2017). Specifically for *Nostoc* species, several MAAs were reported (e.g. MAA-312, MAA-330, shinorine, glycosylated MAAs, porphyra-334 and extracellular MAAs) (Jain et al. 2017). The biosynthesis of MAAs is thought to include intermediates from different metabolic pathways, namely the shikimate pathway, used for the biosynthesis of aromatic amino acids, and the pentose-phosphate pathway (Geraldes and Pinto 2021). Moreover, MAA biosynthesis is known to be non-species-specific and to depend on abiotic conditions, especially the spectral distribution and intensity of radiation (Rastogi et al. 2010). In cyanobacteria, UV-B is the most important factor constraining the accumulation of MAAs, but nitrogen availability also constrains its composition (Singh et al. 2008). In our experiment, no radiation stress was induced, suggesting that MAAs production is naturally high in the *Nostoc* species studied, and has the potential to be even further stimulated by specific conditions. There are several applications for these compounds related to their roles in photoprotection, antioxidant protection, osmotic regulation, reproduction control, and as nitrogen reservoirs (Shick and Dunlap 2002). The most studied feature of MAAs is photoprotection (Shick and Dunlap 2002), and as anti-photoaging agents in wrinkle reduction (Kageyama and Waditee-Sirisattha 2019), wound healing agents (Choi

et al. 2015), and antitumor agents (e.g. B16-F1 murin skin melanoma; Yuan et al., 2009; and HeLa human adenocarcinoma; Athukorala et al., 2016).

While the production of amino acids decreased within the growth, the production of peptides increased, although it was not possible to identify the peptides found. Some of these compounds are very attractive for the pharmaceutical industry because they are considered a promising starting point for the development of novel drugs (Shah et al. 2017). Peptides are a class of pharmaceutical compounds that are different from small molecules and proteins, both molecularly, biochemically, and therapeutically. They bind selectively to cellular targets, which reduces the risk of side effects (Lau and Dunn 2018). Also, peptides can present biological activity (e.g. antitumor and antifungal; Shah et al., 2017). While some peptides are common to several genera, like the toxin microcystin, others have only been identified in species of the genus *Nostoc* (e.g. nostocyclopeptides, cryptophycins, nostopeptolides) (Fidor et al. 2019).

Sugars and other metabolites are produced by cyanobacteria, and by most microorganisms, as compatible solutes with functions related to the response against osmotic stress (Hagemann 2011). Sucrose, trehalose, glycosylglycerol (identified in the present study), glucosylglycerate, and glycine betaine are typical in cyanobacteria (Frigaard 2018). Freshwater cyanobacteria species with low halotolerance mostly accumulate sucrose and/or trehalose, while moderately halotolerant (marine) species mostly use glucosylcerol as the main compatible solute and glucosylglycerate as a secondary compatible solute; glycine-betaine or glutamate betaine are normally used by halophilic species (Hagemann 2011). The *Nostoc* strain used in this study was collected in a rice field. However, along with sucrose and trehalose, gluycosylglycerol was also produced by the species, which suggests a higher halotolerance than expected for a freshwater species. In fact, some *Nostoc* species are known to be tolerant to desiccation and to accumulate trehalose (Yoshida and Sakamoto 2009). Glucosylglycerol was also reported for other freshwater species (Reed et al. 1984), indicating higher salt resistance than the sucrose- and/or trehalose-accumulating species (Hagemann et al. 1999). The production of sucrose and glucosylglycerol, mostly at later culture phases, may indicate a disturbance in the osmotic balance caused by changes on medium composition. The consumption of nutrients and the excretion of metabolites inherent to the cyanobacterium growth are consistent with such a scenario. To tackle this challenge, most organisms use a “salt-out” strategy, i.e. they produce these compatible

solutes to lower internal water potential in the presence of high external salinity, allowing osmotic regulation (Bremer 2000). Photoautotrophic cyanobacteria normally use *de novo* synthesis instead of taking up compatible solutes, which are normally not available in their environment (Hagemann 2011).

Contrarily to several carbohydrates, lipids like linolenic acid (ω-3), 3-hydroxybutyrate, and polyunsaturated fatty acids (PUFA) slightly decreased through time. Lipids are essential molecules in the primary metabolism, especially in light reactions of the photosynthesis (Kern et al. 2009). Lipids from cyanobacteria are different from algae, plant or animal lipids, being mostly composed of diacylglycerols (Sheng et al. 2011), and are mostly located in the thylakoid membranes (Gombos et al. 1996). In photosynthesis, lipids can play different functions, such as the mediation of protein-protein interactions, the oligomerization or protein-cofactor interactions, and as providers for lipophilic regions within protein complexes (Kern et al. 2009). Thus, the observed decrease in the production of some lipids is consistent with the reduction in the production of chlorophyll *a*. This relationship was also described for *Synechocystis* sp. PCC 6803, where an increase in lipid content was reported during the phases of faster cell division and concomitant higher photosynthetic demand (Hewelt-Belka et al. 2020). While the production of total MUFA showed a slight increase with time, total PUFA decreased slightly. In cyanobacteria, and also in higher plants, changes in the unsaturation of fatty acids could affect the membrane fluidity and the photochemical and electron-transport reactions that occur in thylakoid and other membranes (Allakhverdiev et al. 2009). Lipid composition is also dependent on growth conditions (Hewelt-Belka et al. 2020), thus medium changes caused by nutrients depletion and/or by the excretion of metabolites can support our results. This is also a class of compounds with high market value. Among the most important PUFA are eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), γ-linolenic acid (GLA) and arachidonic acid (ARA), which can be used in pharmaceutical and therapeutic applications (Crawford et al. 1997; Holub 2009; Ytrestøyl et al. 2015; Echeverría et al. 2017; Newell et al. 2017). Linolenic acid, an ω-3 PUFA, and linoleic acid (ω-6) are amongst the essential fatty acids taken up by human cells to support key functions, including defence mechanisms against microorganisms or cell damage, wound healing and tissue repair (Kapoor and Huang 2006). Inside the human body, linolenic acid is easily converted to arachidonic acid and then into prostaglandin E2 (Euler and Eliassen 1967), having an important role in the anti-

inflammatory response (Fan and Chapkin 1998). It has also been argued to have a positive effect in pathologies like atherosclerosis, sciatic endoneurial blood flow, atopic eczema, Parkinson's disease, premenstrual syndrome, multiple sclerosis, coronary and heart diseases, and arthritis (Choopani et al. 2016). The ratio of ω-3 to ω-6 is species-dependent (Abdo et al. 2015), and several plants, including algae, and cyanobacteria are known producers of linolenic acid (Choopani et al. 2016). *Arthrospira* is the cyanobacteria species most referred to as a good linolenic acid producer, being able to accumulate 1% of this fatty acid in dry cell mass (Qiang et al. 1997), but both the present study and others (Vargas et al. 1998) report the production of linolenic acid in *Nostoc* species as well.

Other lipids like glycolipids also bear a range of interesting bioactivities with applications in biomedical, pharmaceutical and cosmetic sectors (Varvaresou and Iakovou 2015) as their amphiphilic nature provides good surfactant capacity. In fact, microbial glycolipids perform better compared to petroleum-derived or plant-based synthetic surfactants by bearing higher surface activity, higher emulsifying power, lower critical micelle concentrations, higher biodegradability (compared to petroleum-derived surfactants), and lower ecotoxicity (Poremba et al. 1991). The major constraint to a wider exploitation of microbial glycolipids is their low yield and high production cost, especially for pharmaceutical applications, where high purity is required (Abdel-Mawgoud and Stephanopoulos 2018). In this context, cyanobacteria represent a so far under looked alternative, as glycolipids are the main constituents of their membranes, e.g. representing approximately 90 mol% of the total lipids in the thylakoid membranes (Apdila and Awai 2017). According to our results, *N. muscorum* is a promising candidate for glycolipids production.

Metabolites related to energy production (i.e. UXP and AXP), and the pigment chlorophyll *a* tends to be less produced through time. This suggests growth deceleration, which is not consistent with the optical density monitoring showing constant biomass increase although with high variability during the last period. However, it is important to recognize that spectrophotometry is blind to whether the cells are alive or dead, and that the increased turbidity caused by high cell concentration, as well as cell morphology (i.e. presence of filaments), can interfere with these measurements. The decrease in the chlorophyll *a* content may have been caused by nutrient deficiencies, which are known modulators of photosynthetic activity. For instance, iron and molybdenum play a major role

in cyanobacterial growth and physiology, being involved in photosynthesis and in nitrogen fixation (Gonzáles et al. 2018). The biosynthesis of chlorophyll and phycobilin is also iron-dependent, although neither contains iron (Rueter and Petersen 1987). Iron limitation causes the decreasing on the synthesis of pigments, hence possibly explaining the lower levels of chlorophyll *a* found after the 43th day. Photosynthesis is not the single process affected by limitations in specific nutrients. The metabolism involved in nitrogen fixation also requires the synthesis of iron and molybdenum subunits. Iron limitation decreases the levels of nitrite reductase (Storch and Dunham 1986), which is essential for the reduction of nitrate from di-nitrogen fixation or from an external source. In the case of diazotrophic cyanobacteria, as *N. muscorum.*, the iron demand is higher due to the abundance of iron-containing enzymes necessary to nitrogen-fixation (Gonzáles et al. 2018). Molybdenum is also involved in nitrogen metabolism, working as a cofactor in nitrate reductase and nitrogenase (Glass et al. 2012). Therefore, its scarcity leads to lower nitrogen fixation rates (Glass et al. 2012), to the increase in number of heterocysts, and to a decrease in pigment content (Fay and de Vasconcelos 1974).

Pigments are among the compounds that are currently extracted from cyanobacteria with the highest commercial value, in particular carotenoids and phycobilins (Koller et al. 2014). These natural antioxidants protect the organisms from reactive oxygen species (ROS) produced during photosynthesis and respiration. Common carotenoids in cyanobacteria include β-carotene, myxoxanthophyl, nostoxanthin, caloxanthin, zeaxanthin, canthaxanthin, and echinenone (Schagerl and Donabaum 2003; Llewellyn et al. 2020). The examined *Nostoc* culture produced two different detectable carotenoids, one of which (carotenoid 1) likely being β-carotene. Carotenoids cannot be synthesized by humans, who have to acquire them through diet or direct absorption, hence these pigments have great biotechnological relevance in the food, nutraceutical and cosmetic industries (Novoveská et al. 2019). Carotenoids are used in several products often with a dual function, i.e. profiting from their colour properties (Markets and Markets 2015) and concomitantly from their biological activity. For instance, their antioxidant activity is an important feature of cosmetics, applicable to creams and lotions (e.g. Eclaé Cosmetics, (n.d.)). There are also studies highlighting other benefits of carotenoids for human health, namely the reduction of the risk of inflammation, heart disease, cancer, type 2 diabetes, chronic eye and macular diseases, obesity, Alzheimer's and Parkinson's disease, amyotrophic lateral sclerosis, as well as some

mental diseases (Novoveská et al. 2019). Although most carotenoids with cosmetic/pharmaceutical applications are produced synthetically (Koller et al. 2014), the increasing consumers' demand for natural products raises the relative value of natural carotenoids (Novoveská et al. 2019). Their market size reached more than USD 200 million in 2015 and is likely to exceed USD 300 million by 2024 (https://www.gminsights.com, assessed on May 17th 2021). Among the available carotenoids, β-carotene, lycopene, astaxanthin, zeaxanthin, and lutein are the most demanded in this context (Balić and Mokos 2019).

V-5. Conclusion

N. muscorum was proven to be a very interesting cyanobacteria species, worth to be further explored. It was able to grow for 112 days, without medium replacement or supplementation, suggesting low nutritional requirements, which has a positive implication for its industrial exploitation and long-term maintenance. NMR successfully monitored 36 compounds (or compound groups), accumulated in the cyanobacterium over an extended growth period. Several compounds with interesting bioactivities and potential biotechnological applications were identified, including mycosporin-like amino acids, carotenoids, sugars, and important lipids (e.g. ω-3 and ω-6 fatty acids and glycolipids). A detailed analysis of the metabolic profile through the growth period of the cyanobacterium allowed identifying the patterns of production of these compounds. Several compounds showed higher production during earlier stages of the growth period (up to 43 days), including malto-oligosaccharides, some amino acids, polyunsaturated fatty acids (namely linolenic acid), glycolipids, nucleotides and chlorophyll *a*. On the other hand, cells from later time points (from day 56 onwards) were relatively richer in sucrose and sucrose-derived oligosaccharides, glucosylglycerol, peptides, fatty acids (including linoleic acid), and nucleosides. The production of compounds like carotenoids and trehalose did not follow clear time-dependent patterns. The metabolic profile changed through the growth period, and this knowledge is critical to define the most appropriate harvesting moments, thus allowing the biotechnological exploitation of different molecules of interest to be optimized. This study demonstrates that understanding the metabolic dynamics of cultures to optimize culturing conditions and harvesting schedules is an efficient path towards sustainable exploitation protocols.

References

Abdel-Mawgoud AM, Stephanopoulos G (2018) Simple glycolipids of microbes: Chemistry, biological activity and metabolic engineering. Synth Syst Biotechnol 3:3–19. doi: 10.1016/j.synbio.2017.12.001

Abdo SM, Ali G, El-baz FK (2015) Potential production of omega fatty acids from microalgae. Int J Pharm Sci Res 35:210–215.

Aikawa S, Nishida A, Hasunuma T, et al. (2019) Short-term temporal metabolic behavior in halophilic cyanobacterium *Synechococcus* sp. Strain PCC 7002 after salt shock. Metabolites 9:297. doi: 10.3390/metabo9120297

Allakhverdiev SI, Los DA, Murata N (2009) Regulatory roles in photosynthesis of unsaturated fatty acids in membrane lipids. In: Wada H, Murata N (eds) Adv. Photosynth. Respir. Springer Science + Business Media, pp 373–388

Apdila ET, Awai K (2017) Configuration of the sugar head of glycolipids in thylakoid membranes. Genes Genet Syst 92:235–242. doi: 10.1266/ggs.17-00047

Arora N, Dubey D, Sharma M, et al. (2018) NMR-based betabolomic approach to elucidate the differential cellular responses during mitigation of arsenic (III, V) in a green microalga. ACS Omega 3:11847–11856. doi: 10.1021/acsomega.8b01692

Athukorala Y, Trang S, Kwok C, Yuan Y V. (2016) Antiproliferative and antioxidant activities and mycosporine-like amino acid profiles of wild-harvested and cultivated edible canadian marine red macroalgae. Molecules 21:119. doi: 10.3390/molecules21010119

des Aulnois MG, Roux P, Caruana A, et al. (2019) Physiological and metabolic responses of freshwater and brackish-water strains of *Microcystis aeruginosa* acclimated to a salinity gradient: insight into salt tolerance. Appl Env Microbiol 85:01614–19.

Balić A, Mokos M (2019) Do we utilize our knowledge of the skin protective effects of carotenoids enough? MDPI 8:259. doi: 10.3390/antiox8080259

Borowitzka MA (2018) Biology of microalgae. In: Levine IA, Fleurance J (eds) Microalgae Heal. Dis. Prev. Elsevier Inc., pp 23–72

Bremer E (2000) Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes in bacteria. Bact Stress responses 79–97. doi: 10.1016/S1095-6433(00)80031-8

Burja AM, Banaigs B, Abou-Mansour E, et al. (2001) Marine cyanobacteria: a profilic

source of natural products. Tetrahedron 57:9347–9377. doi: 10.1016/S0040-4020(01)00931-0

Cerasino L, Capelli C, Salmaso N (2017) A comparative study of the metabolic profiles of common nuisance cyanobacteria in southern perialpine lakes. Adv Oceanogr Limnol 8:22–32. doi: 10.4081/aiol.2017.6381

Choi YH, Yang DJ, Kulkarni A, et al. (2015) Mycosporine-like amino acids promote wound healing through focal adhesion kinase (FAK) and mitogen-activated protein kinases (MAP kinases) signaling pathway in keratinocytes. Mar Drugs 13:7055–7066. doi: 10.3390/md13127056

Choopani A, Poorsoltan M, Fazilati M, et al. (2016) *Spirulina*: A source of gamma-linoleic acid and its applications. J Appl Biotechnol Reports 3:483–488.

Chrapusta E, Kaminski A, Duchnik K, et al. (2017) Mycosporine-like amino acids: potential health and beauty ingredients. Mar Drugs 15:1–29. doi: 10.3390/md15100326

Crawford MA, Costeloe K, Ghebremeskel K, et al. (1997) Are deficits of arachidonic and docosahexaenoic acids and vascular complications of responsible for the neural preterm babies? Am J Clin Nutr 66:1032–1041. doi: 10.1093/ajcn/66.4.1032S

Dona AC, Kyriakides M, Scott F, et al. (2016) A guide to the identification of metabolites in NMR-based metabonomics/metabolomics experiments. Comput Struct Biotechnol J 14:135–153. doi: 10.1016/j.csbj.2016.02.005

Echeverría F, Valenzuela R, Hernandez-Rodas MC, Valenzuela A (2017) Docosahexaenoic acid (DHA), a fundamental fatty acid for the brain: New dietary sources. Prostaglandins Leukot Essent Fat Acids 124:1–10. doi: 10.1016/j.plefa.2017.08.001

Eclaé Cosmetics No Title. https://www.eclae.com/fr/#dunaliellasalina. Accessed 15 Apr 2021

Euler US, Eliassen R (1967) Prostaglandins. Academic Press, New York

Fan TWM, Lane AN (2008) Structure-based profiling of metabolites and isotopomers by NMR. Prog Nucl Magn Reson Spectrosc 52:69–117. doi: 10.1016/j.pnmrs.2007.03.002

Fan YY, Chapkin RS (1998) Importance of dietary gamma-linolenic acid in human health and nutrition. J Nutr 128:1411–1414. doi: 10.1093/jn/128.9.1411

Fay P, de Vasconcelos L (1974) Nitrogen metabolism and ultrastructure in *Anabaena cylindrica* - II. The effect of molybdenum and vanadium. Arch Microbiol 99:221–230. doi: 10.1007/BF00696236

Fidor A, Konkel R, Mazur-Marzec H (2019) Bioactive peptides produced by cyanobacteria of the genus *Nostoc*: A review. Mar Drugs 17:1–16. doi: 10.3390/md17100561

Frigaard NU (2018) Sugar and sugar alcohol production in genetically modified cyanobacteria. In: Holban AM, Mihai A (eds) Genet. Eng. Foods. Academic Press, pp 31–47

Gao K (1998) Chinese studies on the edible blue-green alga, *Nostoc flagelliforme*: A review. J Appl Phycol 10:37–49. doi: 10.1023/A:1008014424247

Geraldes V, Pinto E (2021) Mycosporine-like amino acids (MAAs): Biology, chemistry and identification features. Pharmaceuticals 14:1–17. doi: 10.3390/ph14010063

Glass JB, Axler RP, Chandra S, Goldman CR (2012) Molybdenum limitation of microbial nitrogen assimilation in aquatic ecosystems and pure cultures. Front Microbiol 3:1–11. doi: 10.3389/fmicb.2012.00331

Gombos Z, Wada H, Varkonyi Z, et al. (1996) Characterization of the Fad 12 mutant of *Synechocystis* that is defective in Δ12 acyl-lipid desaturase activity. Biochim Biophys Acta - Lipids Lipid Metab 1299:117–123. doi: 10.1016/0005-2760(95)00204-9

Gonzáles A, Fillat MF, Bes MT, et al. (2018) The challange of iron stress in Cyanobacteria. In: Tiwari A (ed) Cyanobacteria. IntechOpen, pp 137–144

Gupta V, Thakur RS, Reddy CRK, Jha B (2013) Central metabolic processes of marine macrophytic algae revealed from NMR based metabolome analysis. RSC Adv 3:7037–7047. doi: 10.1039/c3ra23017a

Hagemann M (2011) Molecular biology of cyanobacterial salt acclimation. FEMS Microbiol Rev 35:87–123. doi: 10.1111/j.1574-6976.2010.00234.x

Hagemann M, Schoor A, Uta E, et al. (1999) The biochemistry and genetics of the glucosylglycerol synthesis in *Synechocystis* sp. PCC 6803. In: Peschek GA, Löffelhardt W, Schmetterer G (eds) The Phototrophic Prokaryotes, 1st ed. Springer, Boston, MA, pp 583–587

Haque F, Banayan S, Yee J, Chiang YW (2017) Extraction and applications of cyanotoxins and other cyanobacterial secondary metabolites. Chemosphere 183:164–175. doi: 10.1016/j.chemosphere.2017.05.106

Hauksson JB, Bergqvist MHJ, Rilfors L (1995) Structure of digalactosyldiacylglycerol from oats. Chem Phys Lipids 78:97–102. doi: 10.1016/0009-3084(95)02483-Y

Hewelt-Belka W, Kot-Wasik A, Tamagnini P, Oliveira P (2020) Untargeted lipidomics

analysis of the cyanobacterium *Synechocystis* sp. PCC 6803: Lipid composition variation in response to alternative cultivation eetups and to gene deletion. Int J Mol Sci 21:8883. doi: 10.3390/ijms21238883

Holub BJ (2009) Docosahexaenoic acid (DHA) and cardiovascular disease risk factors. Prostaglandins Leukot Essent Fat Acids 81:199–204. doi: 10.1016/j.plefa.2009.05.016

Huang IS, Zimba P V. (2019) Cyanobacterial bioactive metabolites - a review of their chemistry and biology. Harmful Algae 83:42–94. doi: 10.1016/j.hal.2018.11.008

Iglesias MJ, Soengas R, Martins CB, et al. (2020) Chemotaxonomic profiling through NMR. J Phycol 56:521–539. doi: 10.1111/jpy.12959

Ito T, Tanaka M, Shinkawa H, et al. (2013) Metabolic and morphological changes of an oil accumulating trebouxiophycean alga in nitrogen-deficient conditions. Metabolomics 9:178–187. doi: 10.1007/s11306-012-0463-z

Jain S, Prajapat G, Abrar M, et al. (2017) Cyanobacteria as efficient producers of mycosporine-like amino acids. J Basic Microbiol 57:715–727. doi: 10.1002/jobm.201700044

Johnson HE, King SR, Banack SA, et al. (2008) Cyanobacteria (*Nostoc commune*) used as a dietary item in the Peruvian highlands produce the neurotoxic amino acid BMAA. J Ethnopharmacol 118:159–165. doi: 10.1016/j.jep.2008.04.008

Kageyama H, Waditee-Sirisattha R (2019) Antioxidative, anti-inflammatory, and anti-aging properties of mycosporine-like amino acids: Molecular and cellular mechanisms in the protection of skin-aging. Mar Drugs. doi: 10.3390/md17040222

Kapoor R, Huang Y-S (2006) Gamma linolenic acid: an antiinflammatory omega-6 fatty acid. Curr Pharm Biotechnol 7:531–534. doi: 10.2174/138920106779116874

Kern J, Zouni A, Guskov A, Krauß N (2009) Lipids in the structure of Photosystem I, Photosystem II and the cytochrome b6f complex. In: Wada H, Murata N (eds) Adv. Photosynth. Respir. Springer Science + Business Media, pp 203–242

Kim SW, Ban SH, Ahn CY, et al. (2006) Taxonomic discrimination of cyanobacteria by metabolic fingerprinting using proton nuclear magnetic resonance spectra and multivariate statistical analysis. J Plant Biol 49:271–275. doi: 10.1007/BF03031154

Kobayashi M, Akutsu S, Fujinuma D, et al. (2013) Physicochemical properties of chlorophylls in oxygenic photosynthesis - succession of co-factors from anoxygenic to oxygenic photosynthesis. Intech. doi: 10.5772/55460

Koller M, Muhr A, Braunegg G (2014) Microalgae as versatile cellular factories for valued products. Algal Res 6:52–63. doi: 10.1016/j.algal.2014.09.002

Kultschar B, Llewellyn C (2018) Secondary metabolites in cyanobacteria. In: Vijayakumar R, Raja SSS (eds) Second. Metab. - Sources Appl. pp 23–36

Kumar D, Singh B, Bauddh K, Korstad J (2015) Bio-oil and biodiesel as biofuels derived from microalgal oil and their characterization by using instrumental techniques. Algae Environ Sustain. doi: 10.1007/978-81-322-2641-3_7

Lafountain AM, Pacheco C, Prum RO, Frank HA (2013) Nuclear magnetic resonance analysis of carotenoids from the burgundy plumage of the Pompadour Cotinga (*Xipholena punicea*). Arch Biochem Biophys 539:133–141. doi: 10.1016/j.abb.2013.08.012

Lau JL, Dunn MK (2018) Therapeutic peptides: Historical perspectives, current development trends, and future directions. Bioorganic Med Chem 26:2700–2707. doi: 10.1016/j.bmc.2017.06.052

Llewellyn CA, Airs RL, Farnham G, Greig C (2020) Synthesis, regulation and degradation of carotenoids under low level UV-B radiation in the filamentous cyanobacterium *Chlorogloeopsis fritschii* PCC 6912. Front Microbiol 11:1–13. doi: 10.3389/fmicb.2020.00163

Macário IPE, Ventura SPM, Gonçalves FJM, et al. (2021) The "bright side" of cyanobacteria: revising the nuisance potential and prospecting innovative biotechnology-based solutions to integrate water management programs. ACS Sustain Chem Eng 9:7182–7197. doi: 10.1021/acssuschemeng.1c00458

Marcolongo G, De Appolonia F, Venzo A, et al. (2006) Diacylglycerolipids isolated from a thermophile cyanobacterium from the Euganean hot springs. Nat Prod Res 20:766–774. doi: 10.1080/14786410500176393

Markets and Markets (2015) Carotenoids market by type (Astaxanthin, Beta-Carotene, Lutein, Lycopene, Canthaxanthin, and Zeaxanthin), application (feed, food & beverages, dietary supplements, cosmetics, and pharmaceuticals), source, formulation, and region - Global Forecast to 2026. 1–172.

Martínez-Francés E, Escudero-Oñate C (2018) Cyanobacteria and Microalgae in the production of valuable bioactive compounds. Microalgal Biotechnol. IntechOpen, pp 105–128

Newell M, Baker K, Postovit LM, Field CJ (2017) A critical review on the effect of docosahexaenoic acid (DHA) on cancer cell cycle progression. Int J Mol Sci 18:1784. doi: 10.3390/ijms18081784

Nichols HW (1973) Growth media-freshwater. In: Stein JR (ed) Handb. Phycol. methods Cult. methods growth Meas. Cambridge University Press, Cambridge, UK, pp 16–17

Novoveská L, Ross ME, Stanley MS, et al. (2019) Microalgal carotenoids: A review of production, current markets, regulations, and future direction. Mar Drugs 17:1–21. doi: 10.3390/md17110640

Nylund GM, Weinberger F, Rempt M, Pohnert G (2011) Metabolomic assessment of induced and activated chemical defence in the invasive red alga *Gracilaria vermiculophylla*. PLoS One. doi: 10.1371/journal.pone.0029359

Pontis HG, Vargas WA, Salerno GL (2007) Structural characterization of the members of a polymer series, compatible solutes in *Anabaena* cells exposed to salt stress. Plant Sci 172:29–35. doi: 10.1016/j.plantsci.2006.07.007

Poremba K, Gunkel W, Lang S, Wagner F (1991) Marine biosurfactants, III. Toxicity testing with marine microorganisms and comparison with synthetic surfactants. Zeitschrift fur Naturforsch - Sect C J Biosci 46:210–216. doi: 10.1515/znc-1991-3-409

Potts M (2002) *Nostoc*. In: Whitton BA, Potts M (eds) Ecol. Cyanobacteria. Their Divers. Time Sp., 1st ed. Springer Netherlands, pp 465–504

Qiang H, Zheungu H, Cohen Z, Richond A (1997) Enhancement of eicosapentaenoic acid (EPA) and γ-linolenic acid (GLA) production by manipulating algal density of outdoor cultures of *Monodus subterraneus* (Eustigmatophyta) and *Spirulina platensis* (Cyanobacteria). Eur J Phycol 32:81–86. doi: 10.1080/09541449710001719395

Raheem A, Prinsen P, Vuppaladadiyam AK, et al. (2018) A review on sustainable microalgae based biofuel and bioenergy production: Recent developments. J Clean Prod 181:42–59. doi: 10.1016/j.jclepro.2018.01.125

Rastogi RP, Richa, Sinha RP, et al. (2010) Photoprotective compounds from marine organisms. J Ind Microbiol Biotechnol 37:537–558. doi: 10.1007/s10295-010-0718-5

Reed RH, Richardson DL, Warr SRC, Stewart WDP (1984) Carbohydrate accumulation and osmotic stress in cyanobacteria. J Gen Microbiol 130:1–4. doi: 10.1099/00221287-130-1-1

Rueter JG, Petersen RR (1987) Micronutrient effects on cyanobacterial growth and

physiology. New Zeal J Mar Freshw Res 21:435–445. doi: 10.1080/00288330.1987.9516239

Sallans L, Giner JL, Kiemle DJ, et al. (2013) Structural identities of four glycosylated lipids in the oral bacterium *Streptococcus* mutans UA159. Biochim Biophys Acta 1831:1239–1249. doi: 10.1016/j.bbalip.2013.03.012

Schagerl M, Donabaum K (2003) Patterns of major photosynthetic pigments in freshwater algae. 1. Cyanoprokaryota, Rhodophyta and Cryptophyta. Ann Limnol 39:35–47. doi: 10.1051/limn/2003003

Schwarz D, Orf I, Kopka J, Hagemann M (2013) Recent applications of metabolomics toward cyanobacteria. Metabolites 3:72–100. doi: 10.3390/metabo3010072

Shah SAA, Akhter N, Auckloo BN, et al. (2017) Structural diversity, biological properties and applications of natural products from cyanobacteria. A review. Mar Drugs 15:1–20. doi: 10.3390/md15110354

Sheng J, Vannela R, Rittmann BE (2011) Evaluation of methods to extract and quantify lipids from *Synechocystis* PCC 6803. Bioresour Technol 102:1697–1703. doi: 10.1016/j.biortech.2010.08.007

Shick JM, Dunlap WC (2002) Mycosporine-like amino acids and related gadusols: Biosynthesis, accumulation, and UV-protective functions in aquatic organisms. Annu Rev Physiol 64:223–262. doi: 10.1146/annurev.physiol.64.081501.155802

Singh JS, Kumar A, Rai AN, Singh DP (2016) Cyanobacteria: A precious bio-resource in agriculture, ecosystem, and environmental sustainability. Front Microbiol 7:1–19. doi: 10.3389/fmicb.2016.00529

Singh S, Kate BN, Banecjee UC (2005) Bioactive compounds from cyanobacteria and microalgae: An overview. Crit Rev Biotechnol 25:73–95. doi: 10.1080/07388550500248498

Singh SP, Klisch M, Sinha RP, Häder D-P (2008) Effects of abiotic stressors on synthesis of the mycosporine-like amino acid shinorine in the Cyacobacterium *Anabaena variabilis* PCC 7937. Photochem Photobiol 84:1500–1505. doi: 10.1111/j.1751-1097.2008.00376.x

Smith GD, Thanh Doan N (1999) Cyanobacterial metabolites with bioactivity against photosynthesis in cyanobacteria, algae and higher plants. J Appl Phycol 11:337–344. doi: 10.1023/A:1008115818348

Storch TA, Dunham VL (1986) Iron-mediated changes in the growth of Lake Erie phytoplankton and axenoc algal cultures. J Phycol 22:109–117.

Vargas MA, Rodríguez H, Moreno J, et al. (1998) Biochemical composition and fatty acid content of filamentous nitrogen-fixing cyanobacteria. J Phycol 34:812–817. doi: 10.1046/j.1529-8817.1998.340812.x

Varvaresou A, Iakovou K (2015) Biosurfactants in cosmetics and biopharmaceuticals. Lett Appl Microbiol 61:214–223. doi: 10.1111/lam.12440

Wishart DS, Feunang YD, Marcu A, et al. (2018) HMDB 4.0: the human metabolome database for 2018. Nucleic Acids Res 46:D608–D617. doi: 10.1093/nar/gkx1089

Yoshida T, Sakamoto T (2009) Water-stress induced trehalose accumulation and control of trehalase in the cyanobacterium *Nostoc punctiforme* IAM M-15. J Gen Appl Microbiol 55:135–145. doi: 10.2323/jgam.55.135

Ytrestøyl T, Aas TS, Åsgård T (2015) Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. Aquaculture 448:365–374. doi: 10.1016/j.aquaculture.2015.06.023

Yuan YV, Westcott ND, Hu C, Kitts DD (2009) Mycosporine-like amino acid composition of the edible red alga, *Palmaria palmata* (dulse) harvested from the west and east coasts of Grand Manan Island, New Brunswick. Food Chem 112:321–328. doi: 10.1016/j.foodchem.2008.05.066

Obando CZ, Linossier I, Kervarec N, et al. (2016) Rapid identification of osmolytes in tropical microalgae and cyanobacteria by ^{1}H HR-MAS NMR spectroscopy. Talanta 153:372–380. doi: 10.1016/j.talanta.2016.02.024

CHAPTER VI

Cyanobacteria as candidate to support Mars colonization: growth and biofertilization evaluation using Mars regolith

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Contributions: I. P. E. M. wrote the manuscript; I. P. E. M., T. V., and S. F. acquired the experimental data; I. P. E. M. performed the data analysis; H. P. analysed the composition of the Mars extract; C. S. performed the FTIR-ATR analysis; J. S., F. J. M. G., S. P. V., and J. L. P. supervised

Abstract: Cyanobacteria are pointed out as possible Mars colonizers, to contribute to change the atmosphere composition in a similar way that they did on Earth, billions of years ago. The goal of this work was to evaluate the ability of three species of cyanobacteria (*Anabaena cylindrica, Nostoc muscorum*, and *Arthrospira platensis*) and a green microalgae (*Chlorella vulgaris*) to growth using only resources existing in Mars (i.e. water and Martian regolith (MGS-1)). A Martian regolith extract was produced and used as culture medium to these species. Their growth was assessed during 25 days, using optical density and fluorometric parameters. After this period the possible contribution of end-of-life cyanobacteria/ microalgae as biofertilizing agents was also assessed, using the macrophyte *Lemna minor*, as a vegetal model. Among these species, *N. muscorum* was the one that showed the best growth performance, and hence this should be the target of future studies. *A. platensis* and the microalgae also showed good abilities as biofertilizing agents, i.e. they stimulate biomass (i.e. dry weight) production levels comparable to the plants that grew on standard synthetic medium. The highest yield was reached with *A. platensis*, while the lowest was achieved using the mediums with *N. muscorum.* These results lead to the analysis of the nutritional composition of these plants, and FTIR-ATR (Fourier transform infrared with attenuated total reflectance) spectroscopy showed that the differences between plants grown on mediums with or without Martian regolith seems to be related mainly with polysaccharides.

Keywords: cyanobacteria, microalgae, MGS-1, *Lemna minor*, biofertilization

VI-1. Introduction

Every day, the dream to colonize Mars becomes closer to become a reality. However, the challenges for human survival in Mars are huge and one of the key issues is ensuring low-to-no dependence on Earth resources. Therefore, several bioregenerative life-support systems for recycling food, water, and gases are under development (e.g. Nelson et al. 2010; Häder 2020), and one pursued solution is the use of Martian resources to support live. Indeed, Mars can possibly provide water, solar energy, carbon, nitrogen, and other nutrients (Cockell 2014).

In this context, cyanobacteria are pointed out as potentially suitable organisms for use in basic life-support systems (Arai et al. 2008; Arai 2009; Kimura et al. 2015; Verseux et al. 2016; Verseux et al. 2021). This is because cyanobacteria are simple (i.e. prokaryotic) organisms, easy to culture with low nutritional requirements, and able to live nearly anywhere on Earth (e.g. from freshwater to salt and brackish waters, rainforests, deserts and other terrestrial habitats, and even in the air), including under extreme environmental conditions (Gaysina et al. 2018). Furthermore, cyanobacteria are photosynthetic bacteria, hence they produce oxygen, which is and will be a remarkably limited resource in Mars. Indeed, this critical element to support human survival only represents 0.13 % of Mars atmosphere, it represents 21 % of the Earth atmosphere (Verseux et al. 2016). On Mars, oxygen is expected to be produced on site by photosynthetic organisms and/or through physicochemical methods, such as the electrolysis of the regolith brine (Gayen et al. 2020).

Compared to plants, photosynthetic microorganisms are better at capturing solar energy on a volume-to-output basis, and therefore are very efficient oxygen producers. While trees release *ca.* 2.5 – 11 tonnes of O_2 ha^{-1} yr^{-1}, cyanobacteria (i.e. *Arthrospira* in industrial cultivation in open ponds) can release up to 16.8 tonnes of O_2 ha^{-1} yr^{-1} (Verseux et al. 2016). Moreover, plants are more demanding as to environmental tolerance ranges and require more space, resources, thus they are more costly than cyanobacteria, particularly when Mars culturing is equated. Besides oxygen, these organisms are able to produce a wide range of other interesting compounds with several applications, e.g. amino acids, pigments, lipids, polysaccharides, or proteins, with applications in the food and pharmaceutical industries (Eriksen 2008; Sekar and Chandramohan 2008; Macário et al. 2021). Different species can also be used directly as food sources (e.g. *Arthrospira* and *Nostoc* sp.; Gao, 1998; Johnson et al., 2008), to produce biofuels (Raheem et al. 2018), in bioremediation (Fawzy

and Mohamed 2017), and as biofertilizers (Grzesik and Kalaji 2017). These features render cyanobacteria highly promising for culturing and exploitation in harsh environments under high resources scarcity conditions, such as in Mars.

Indeed, cyanobacteria were already proven to be able to survive in space, namely on the outer surface of the International Space Station, for 16 months, supporting extreme temperature fluctuation, the vacuum condition, and high UV and cosmic radiation (Fraunhofer-Gesellschaft). In addition, besides the production of oxygen, their biomass can be used to supply different commodities, contributing to the development of sustainable and less Earth-dependent life support systems. This was the motto for the present study, where it was evaluated the ability of several species of cyanobacteria (*Anabaena cylindrica*, *Nostoc muscorum*, and *Arthrospira platensis*) to growth using only resources existing in Mars (i.e. water and Martian regolith) was evaluated. For performance comparison with the cyanobacteria, we also tested the green microalgae *Chlorella vulgaris*, an eukaryote that can be also used for most of the applications previously mentioned (Mostafa 2012) hence being an alternative candidate to support Mars colonization. Behind the growth parameter, this work also studied the possible contribution of end-of-life cyanobacteria as biofertilizers for vegetables cultivation, considering that plants are an essential component of long-term, bioregenerative life support systems in Mars, according to The National Aeronautics and Space Administration's (NASA) and the European Space Agency's (ESA) (Heinse et al. 2007). For the purpose, we tested cyanobacteria/ microalgae cultures grown on Mars regolith extract as a biofertilizer using the macrophyte *Lemna minor* as a preliminary model.

VI-2. Material and Methods

VI-2.1. Cyanobacteria and microalgae cultures and culturing conditions

Three species of filamentous cyanobacteria (*Anabaena cylindrica* PCC 7122, *Nostoc muscorum* UTAD_N213., and *Arthrospira platensis* UTEX LB 2340), and one species of microalgae (*Chlorella vulgaris*), were used. Cultures were previously established in Woods Hole MBL synthetic medium (Nichols 1973) or in Spirulina medium (SAG 2008) (for the culturing of *A. platensis*). All species were progressively acclimated for more than 6 months to minimum nutrient supply (17 % of synthetic media and 83 % of ultrapure water). The cultures were kept in 100 mL Erlenmeyer vessels in an incubation chamber at (20 ± 2) °C,

with 16 h light:8 h dark photoperiod (7 μmol photons m^{-2} s^{-1}; Quantum meter MQ-200, Apogee Instruments, Logan, Utah, USA) provided by cool white fluorescent tubes.

VI-2.2. Mars regolith extract preparation and characterisation

Mars Global Simulant (MGS-1) was obtained from the Center of Lunar and Asteroid Surface Science (Orlando, Florida, USA). This simulant was used to prepare an aqueous extract serving as the growth medium for cyanobacteria and microalgae (see section VI-2.3.1. below). Suspensions of the regolith in ultrapure water were prepared following a 4:1 (v:w) ratio, then autoclaved at 121 ºC for 1 h. The autoclaved suspension was left for the settling of particulate material at room temperature, and then centrifuged (4111 ×g, 14 ºC, 5 min) for the final collection of the overlying fraction. This aqueous extract was autoclaved again (121 ºC, 30 min) to ensure sterilization.

The composition of the extract was determined by total X-ray fluorescence (TXRF) using a benchtop Picofox S2 (Bruker Nano) spectrometer with a molybdenum X-ray source. Both quartz glass and acrylic sample carriers were used. Sample carriers were pretreated with 10 μL of a solution of silicon in isopropanol and dried in a heat plate at 80 °C. Then, 10 μL of Y standard solution was added to 1 g of aqueous extract sample. A total of 10 μL of this mixture was added to treated sample carriers, which were dried under vacuum for 1 h and analyzed in the TXRF spectrometer for 300 s.

VI-2.3. Experimental design

VI-2.3.1. Cyanobacteria and microalgae growth experiment

The four test species were cultured for 25 days in triplicate, under three treatments: ultrapure water, Mars regolith extract, and 17 % MBL/Spirulina medium. The species were cultured in 100 mL Erlenmeyer vessels with 75 mL of the respective treatment plus 5 mL of a 1-month old inoculum, under incubation conditions as described for bulk cultures. Twice a week, 2 mL samples were collected in a sterilized environment for the measurement of optical density and fluorometric parameters (see section VI-2.4).

VI-2.3.2. Lemna minor *growth experiment*

L. minor was used as a vegetable model because of its small size, low requirements for culturing in terms of space and volume of culture media, as well as considering the

availability of standard guidelines for accurately evaluate growth performance. Following the growth experiment (section VI-2.3.1), the cells cultures which grew in Mars regolith extract were tested as a growing medium for the macrophyte *L. minor*, to assess their potential application as biofertilizers. Five growth media were established: (i) Steinberg medium (standard medium for *Lemna* sp. growth; OECD 2006); (ii) ultrapure water; (iii) Mars regolith extract prepared as detailed in section VI-2.2; (iv) 25-days old cultures in Mars regolith extract filtered through 0.45-µm mesh size cellulose acetate membranes; (v) 25-days old cultures in Mars regolith extract after sonication at 17 W (Vibra Cell, Sonics) until homogenization. These last two treatments (iv and v) addressed two hypotheses. The treatment (iv) supports the hypotheses that cyanobacteria/ microalgae released metabolites responsible for the biofertilization; while the treatment (v) tested the biofertilization abilities of sonicated cells, with the consequent liberation of their cellular content.

The growth assay with *L. minor* was performed following the appropriate OECD guidelines (OECD 2006) adapted to the use of 6-well plates (Kaza et al. 2007). Each treatment was tested in triplicate in wells filled with 10 mL of test solution. The test was initiated by adding three macrophyte colonies of three fronds each, harvested from a weekly renewed laboratory culture in Steinberg medium held at 23 ± 2 ºC under continuous illumination (OECD 2006). The test plates were incubated for 7 days under the same culturing conditions as used for the *L. minor* culture. Fluorometric parameters F_v/F_m were analysed at day 0, 2, 4, and 7, according to the methodology described in section VI-2.4. At the end of the test, the fronds present in each well were counted and oven-dried (at least 24 h at 60 °C) for dry weight records. The effects of the treatments were assessed regarding the yields considering frond number and dry weight (the initial dry weight was measured in eight additional groups composed of 3 colonies with 3 fronds each) records. The dried fronds were then used for the FTIR-ATR (Fourier transform infrared with attenuated total reflectance) analysis.

VI-2.4. Spectroscopic and fluorometric measurements

The growth of cyanobacteria/ microalgae cultures was monitored two times a week by measuring the optical density (OD) records at 440 nm (Shimadzu UV 1800, Shimadzu Coorporation; Kyoto, Japan). Furthermore, an imaging chlorophyll fluorometer (Open FluorCAM 800-O/1010, Photon Systems Instruments; Brno, Czech Republic) was used to

capture the maximum photosynthetic quantum yield of Photosystem II (F_v/F_m) after 15 min of dark adaptation. The excitation light peaks at 621 nm with a 40 nm band width with a saturating pulse intensity of about 7000 µmol quanta m^{-2} s^{-1} and the duration set to 0.8 s. Chlorophyll *a* fluorescence was captured by a 2/300 CCD camera (CCD381) with a F1.2 (2.8–6 mm) objective resulting in images with 512 3 512 pixels and a spectral range of 695–780 nm. Images were processed using the FluorCam7 software (Photon Systems Instruments; Brno, Czech Republic). Fluorometric measurements were made on each culture of cyanobacteria/ microalgae, in triplicate. For the purpose, aliquots of 2 mL of each culture were transferred into 6-well plates. In the case of *L. minor*, the measurements were made directly in the 6-well plates of the test. The maximum quantum yield of PSII, or maximal PSII efficiency (F_v/F_m), was calculated through Eq. VI-1 (Ogawa et al. 2017).

$$\frac{F_v}{F_m} = \frac{F_m - F_o}{F_m} \qquad \textbf{Eq. VI-1}$$

Where F_o is the minimum fluorescence in dark adapted samples, and F_m the maximal fluorescence after exposure to a saturating light pulse.

VI-2.5. Infrared spectra acquisition

The mid-infrared spectra of lyophilized and grinded *Lemna* plants were obtained on a Fourier transform PerkinElmer Spectrum BX FTIR System spectrophotometer (USA) with a DTGS detector. Spectra were acquired in diffuse reflectance mode through a PIKE Technologies Gladi attenuated total reflectance (ATR) accessory within the wavenumber interval of 4000 to 600 cm^{-1}, with a resolution of 4 cm^{-1}. Each spectrum resulted from 32 scan co-additions. For each sample, a small portion was transferred for the ATR crystal and a constant pressure was applied. The ATR crystal was cleaned and a background was acquired between each sample. For each *L. minor* replicate, three spectra were acquired (instrumental replicates).

VI-2.6. Data analysis

Data regarding the monitoring of the cultures growth (optical density and F_v/F_m) were graphically expressed as the mean ± standard deviation (SD) of three replicates. The effect of the different culture compositions on *L. minor* yield was statistically addressed

using a one-way ANOVA approach followed by the post-hoc Tukey test to distinguish differences among the groups. An alpha level of 0.05 was considered in these analyses. FTIR-ATR spectra were processed with standard normal variate (SNV) (Næs et al. 2002) followed by the application of a Savitzky-Golay filter (15 smoothing points, 2nd order polynomial and first derivative) (Savitzky and Golay 1964). Spectra were additionally mean-centered and analysed by Principal Component Analysis (PCA) (Jolliffe 1986). All chemometric models were performed in Matlab version 9.5 Release 2018b (MathWorks) and PLS Toolbox version 8.7 (2019) for Matlab (Eigenvector Research, Manson, WA).

VI-3. Results

VI-3.1. Analysis on Mars regolith extract

The composition of the Mars regolith simulant (MGS-1) was known as given by the manufacturer (Table VI-S1, Annex IV). However, the composition of the regolith extract as tested was unknown and thus it was analysed by TXRF. This analysis revealed that the extract was rich in S, K, and Ca and contained smaller concentrations of Mn, Fe, and Sr, and trace concentrations of Cu, Zn, Br, and Rb (Table VI-1).

Table VI-1. Elements identified in the Mars regolith extract, by TXRF.

Element	**[M] / (mg kg^{-1})**	**± σ / (mg kg^{-1})**
S	338	2
K	5.4	0.1
Ca	90.7	0.4
Mn	0.228	0.007
Fe	0.235	0.006
Cu	0.009	0.002
Zn	0.030	0.002
Br	0.025	0.001
Rb	0.025	0.001
Sr	0.247	0.003

VI-3.2. Cyanobacteria and microalgae growth experiment

The growth of cyanobacteria and microalgae were monitored during 25 days through optical density. Additionally, fluorometry was used to assess the variation in PSII efficiency (F_v/F_m), thus to address the photosynthetic efficiency of the species through the growth experiment. The highest optical density was recorded when species were cultured in diluted synthetic media, except for *A. platensis*, while the lower growth was observed when species were cultured in the treatment with ultrapure water (Figure VI-1). *N. muscorum* and *A. cylindrica* were able to grow on Mars regolith extract, while *A. platensis* and *C. vulgaris* showed only mild growth in this treatment. It must be noted that optical density measurements are highly biased by the morphology of cyanobacteria cultures. Indeed, the photographic documentation of the cultures (Figure VI-1; Table VI-S2, Annex IV) often denotes that relevant growth occurred in Mars regolith regardless of the outcome of the optical density measurements.

N. muscorum was the species showing the most consistent growth, as well as the highest optical density records, both in diluted synthetic medium and in Mars regolith extract (Figure VI-1, left-hand panel). Regarding the maximal PSII efficiency (F_v/F_m) *N. muscorum* recorded always above 0.40 values through the experiment in these two treatments, although with a decreasing tendency through time (Figure VI-1b). Interestingly, *N. muscorum* showed higher values of F_v/F_m in Mars regolith extract than in MBL. *A. cylindrica* recorded stable F_v/F_m values when growing in Mars regolith extract, but photosynthetic efficiency remarkably decreased in cultures growing in the regolith extract from day 10 onwards (Figure VI-1d). Indeed, after the 3rd week of culturing in the regolith extract, the biomass of *A. cylindrica* became brownish, which was reflected in a low F_v/F_m compared to the cultures in ultrapure water and diluted synthetic medium (see Figure VI-1f). The growth of both *N. muscorum* and *A. cylindrica* was so limited in ultrapure water that the fluorescence signal of the samples was not detectable, preventing F_v/F_m measurements. Conversely, *A. platensis* growing in ultrapure water showed F_v/F_m values higher than in regolith extract (Figure VI-1f). However, this outcome should be carefully interpreted as the colonies of this species clogged strongly (Table VI-S2, Annex IV), preventing reliable measurements of optical density primarily, but also fluorescence parameters. Despite this constraint, the direct observation of these cultures allowed confirming mild growth, specifically in diluted synthetic medium. In the case of the microalgae *C. vulgaris*, only the culture in diluted MBL

produced sufficient biomass to support fluorescence measurements (Figure VI-1h). F_v/F_m showed a decreasing tendency throughout the growth period. However, the visual observation of the cultures suggests that minimal growth of *C. vulgaris* occurred in Mars regolith extract and in ultrapure water.

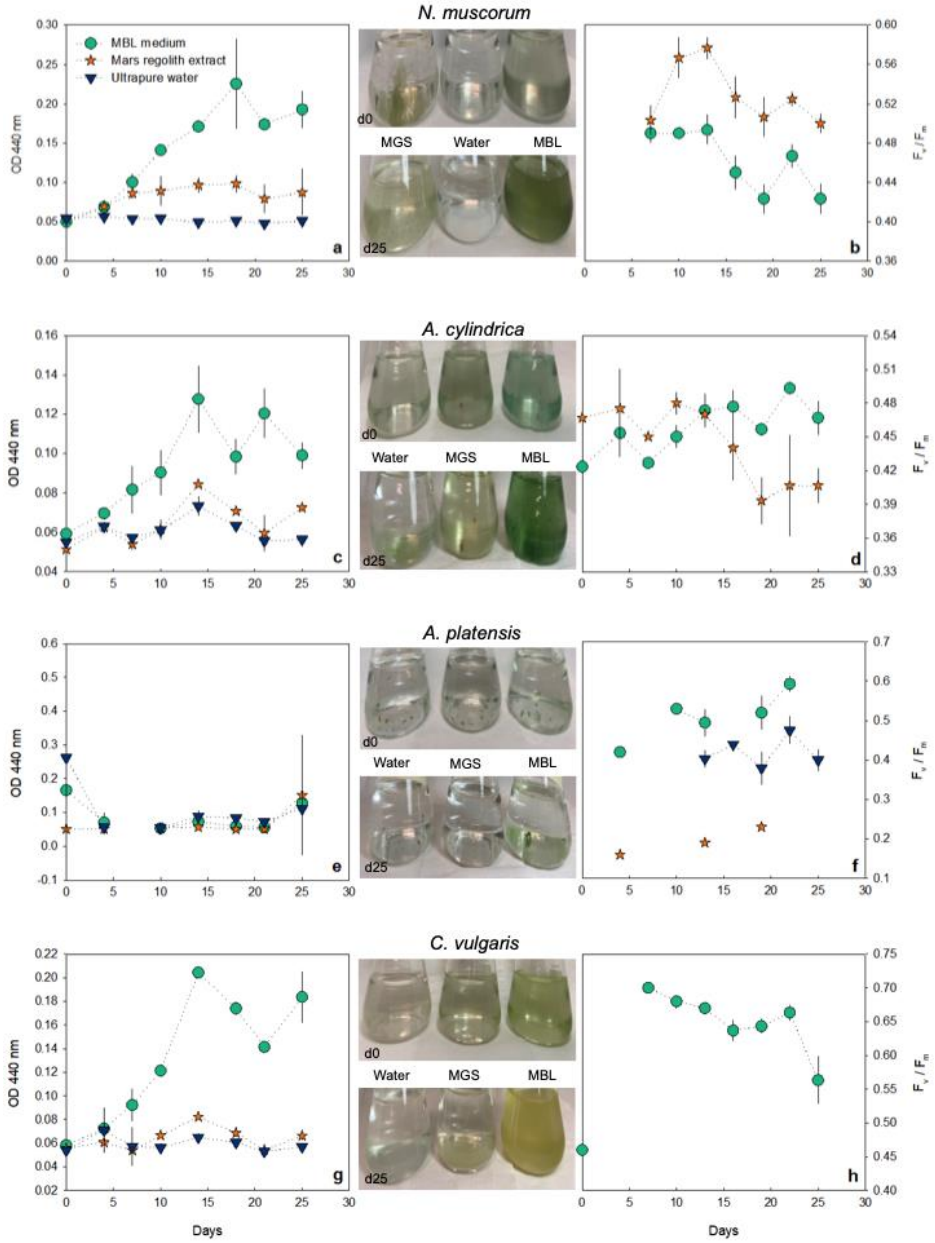


Figure VI-1: Profiling of *N. muscorum*, *A. cylindrica*, *A. platensis*, and *C. vulgaris* throughout a monitoring period of 25 days, regarding OD measurements at 440 nm (a, c, e, f), and PSII efficiency (F_v/F_m) (b, d, f, h) (only data with $F_0 > 100$ are depicted). In all graphics, marks represent the mean

and the error bars represent the standard deviation (three replicates were used, except in some cases where F_0 values < 100 were detected). A line was added joining the marks within each series for clarity purposes, not reflecting any fitted model. In the central panel, a summary of the photographic documentation of the cultures growth is provided (full documentation is available in Table VI-S2, Annex IV), with MGS indicated the treatment with Mars regolith extract, water the treatment with ultrapure water, and MBL the treatment with diluted synthetic media.

VI-3.3. L. minor *growth experiment*

L. minor was used herein to test for the potential of cyanobacteria and microalgae cultures as biofertilizers. As expected, *L. minor* tested in Steinberg medium recorded the best yields regarding both the number of fronds and dry weight (Figure VI-2). They further grew better in Mars regolith extract than in ultrapure water although with no statistical differences. *L. minor* grown in cyanobacteria/microalgae cultures showed frond number yields close to those obtained with regolith extract or ultrapure water depending on the species involved (Figure VI-2). However, the dry weight yield of the treatments with *A. platensis* and *C. vulgaris* are notably closer to the control with Steinberg medium. Among *L. minor* cultures in cyanobacteria/microalgae, the highest biomass was reached with *L. minor* grown in filtered *A. platensis* medium (28.7 fronds), which did not differ significantly from equivalent records from cultures in ultrapure water. The same treatment reached an average dry weight yield of 3.66 mg, which did not significantly differ from the dry weight reached in plants cultured in Steinberg medium (3.71 mg), as evidenced in Figure IV-2. An even higher dry weight yield was reached with sonicated *A. platensis* (3.87 mg), also not significantly differing from the records for the Steinberg control. The lowest *L. minor* growth was recorded with *N. muscorum* (21.3 fronds and 2.72 mg of dry weight). No significant differences were found between filtered and sonicated cultures of the same species in promoting the growth of *L. minor*.

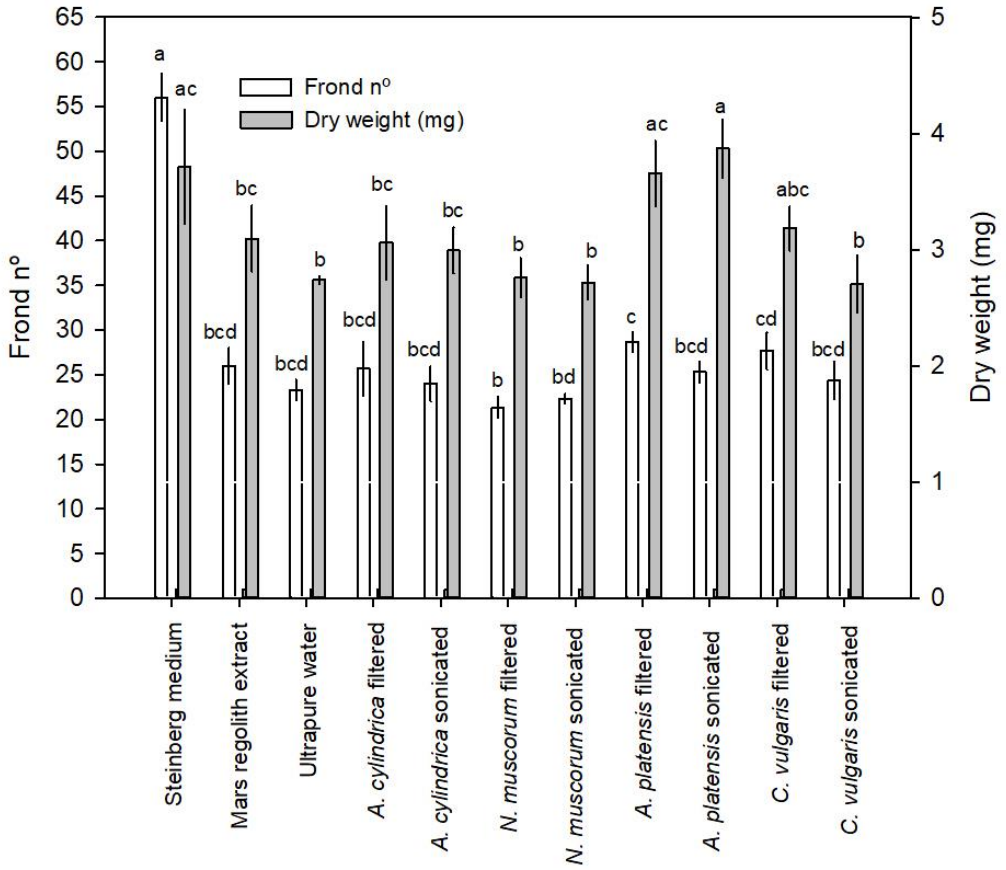


Figure VI-2. *L. minor* biomass yield (in frond number or dry weight) after 7 days of culturing in Steinberg medium, Mars regolith extract, ultrapure water, and cyanobacteria/microalgae grown in Mars regolith extract, either filtered or sonicated. Bars represent the mean of three replicates and the error bars represent the standard deviation. Different letters indicate significant differences among treatments as retrieved through the Tukey test ($p < 0.05$) preceded by one-way ANOVA for dry weight ($F_{10, 32} = 79.190$; $p < 0.001$) or frond number ($F_{10, 32} = 8.040$; $p < 0.001$).

Highest PSII yield was generally recorded for *L. minor* growing in Steinberg medium, showing a decrease after 2 days. A similar decreasing pattern was also recorded for plants growing in ultrapure water (Figure VI-3a). Interestingly, F_v/F_m values recorded for *L. minor* growing in the regolith extract initial decreased along the first four days before it increased, almost reaching initial levels by day 7. There are no apparent differences in the F_v/F_m profiles of *L. minor* growing in filtered or sonicated cultures of the same cyanobacteria/microalgae species (Figure VI-3). *L. minor* grown in *N. muscorum*, *A. cylindrica*, and *A. platensis* recorded F_v/F_m profiles similar to plants grown in regolith extract

only, with a decrease in F_v/F_m values until day 4, followed by a recovery to values closer to the initial levels by day 7. Differently, *L. minor* grown in *C. vulgaris* showed a decrease in F_v/F_m values until day 4, but from then onwards the records stabilized. At the end of the test period, all plants growing in cyanobacteria reached F_v/F_m ranges (0.72 – 0.74) higher than that recorded for plants grown in Steinberg medium (0.66) and ultrapure water (0.68).

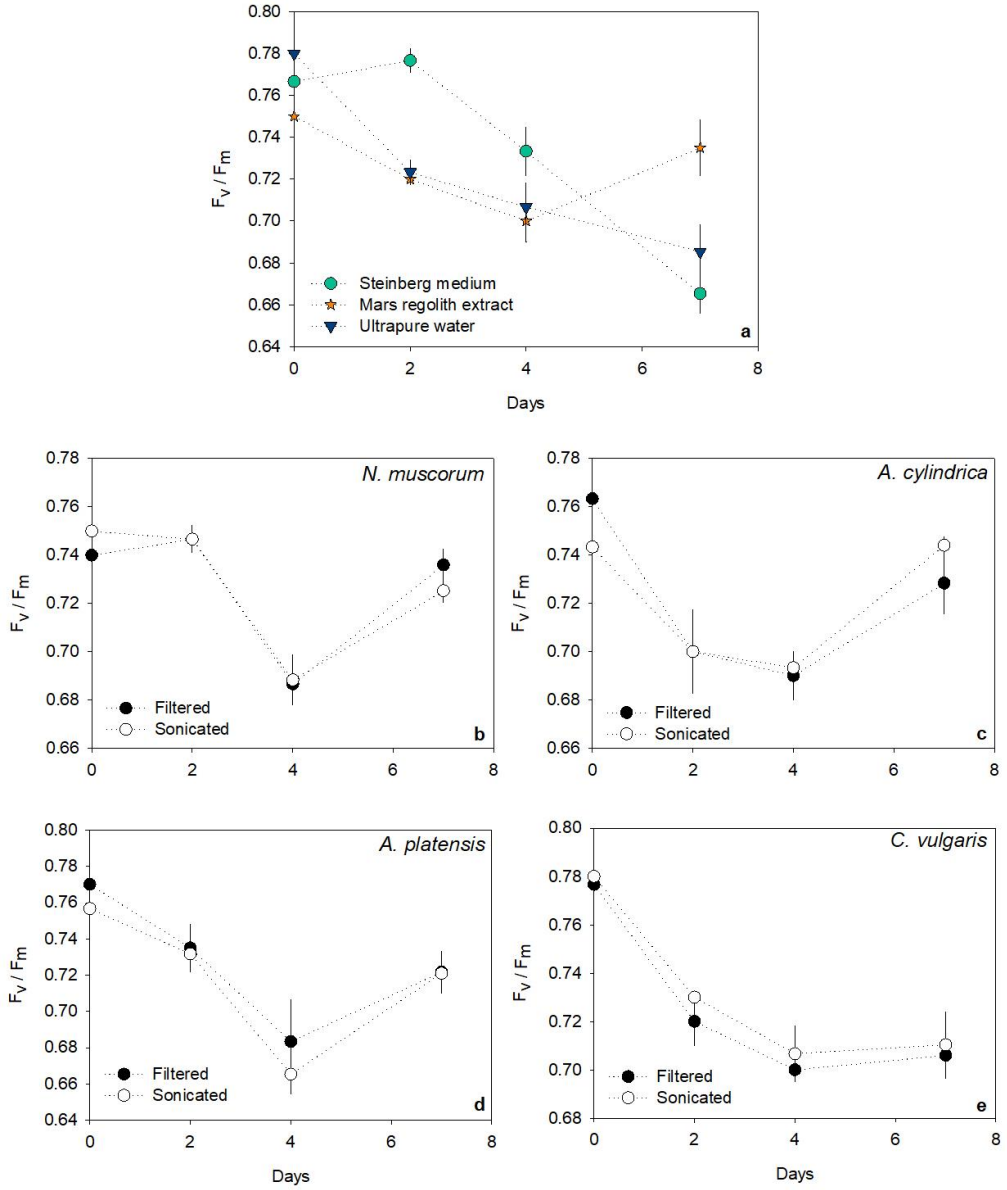


Figure VI-3: PSII efficiency (F_v/F_m) over time for *L. minor* cultured in Steinberg medium, Mars regolith extract, ultrapure water, and cyanobacteria/microalgae cultured in Mars regolith extract after filtration or sonication. Marks represent the mean of three replicates and the error bars represent the standard deviation. A line was added joining the marks within each series for clarity purposes, not reflecting any fitted model.

Analysis on the nutritional composition of *L. minor* grown in different treatments was assessed by FTIR-ATR, in order to understand if profiles were different depending on treatment. The infrared spectrum of *L. minor* presented typical bands which can be attributed to some major classes of compounds (Figure VI-4). Region I (between 3000-2800 cm^{-1}) is dominated by $–CH_3$ and $–CH_2$ stretching vibrations. Region II (between 1800-1500 cm^{-1}) is characterized by the C=O stretching vibration at 1738 cm^{-1}, indicating the presence of ester containing compounds from cell wall and membrane lipids; at 1656 and 1563 cm^{-1} vibrations of amide I and amide II, respectively; and at 1513 cm^{-1} vibrations of aromatic ring like lignin derivates. In region III (between 1500-1200 cm^{-1}) it is possible to identify $–CH_3$ and $–CH_2$ bending (1460 and 1400 cm^{-1}). Finally, in the region IV (between 1200-900 cm^{-1}) the intense infrared absorption indicates a high content in polysaccharides (C–H bending and/or C–O and C–C stretching of cellulose and hemicellulose).

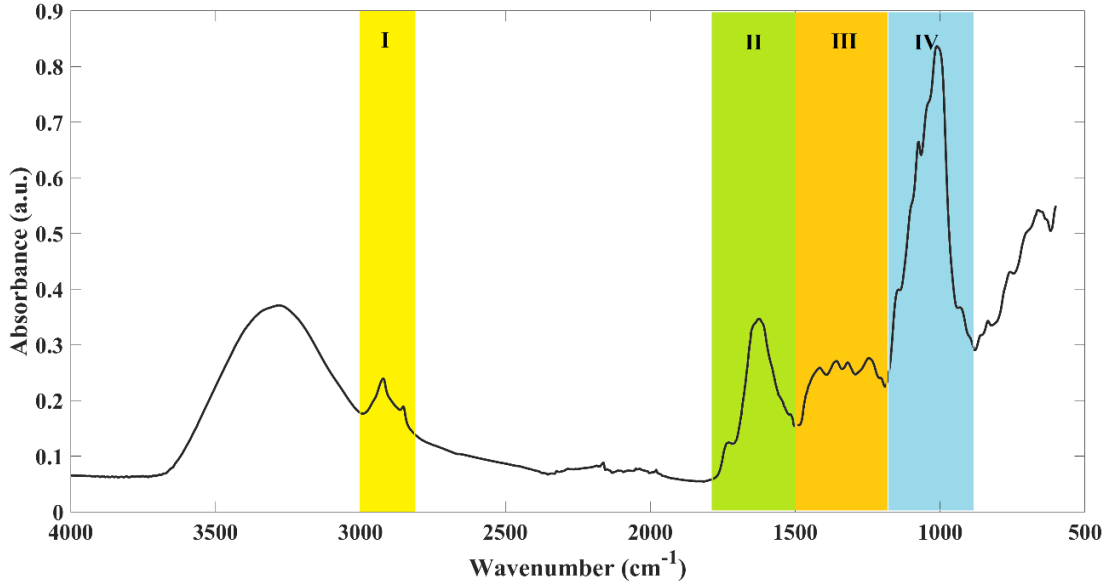


Figure VI-4. Example of an infrared spectrum of *L. minor*. Four major regions (I-IV) were identified, representing major classes of compounds.

High spectral similarity was found among samples, however two main groups were clearly identified and separated through the PC1, which encompasses for around 65 % of the spectral variability (Figure VI-5A). One cluster (negative part of PC1) was composed by samples grown in ultrapure water and in Steinberg medium, while the second cluster (positive part of PC1) was composed by samples that grown in Mars regolith extract and in

cyanobacteria/microalgae cultured in Mars regolith extract. Within this second group, there was no resolution in the analysis to discriminate among the different cyanobacteria/microalgae species. Indeed, the infrared spectra of this group were randomly widespread across the second PC (PC2) (Figure VI-5A). The PCA model loadings on the PC1 (where the discrimination between the presence/absence of Mars soil simulant occurs) (Figure VI-5B), denotes that the spectral region mainly accounting for the referred discrimination is the region between 1200-900 cm^{-1} (with the higher loading values). It is known that this spectral region is dominated by polysaccharides vibrations pointing that this class of compounds is the one that mostly differs in *Lemna* plants grown in the presence/absence of Mars regolith extract.

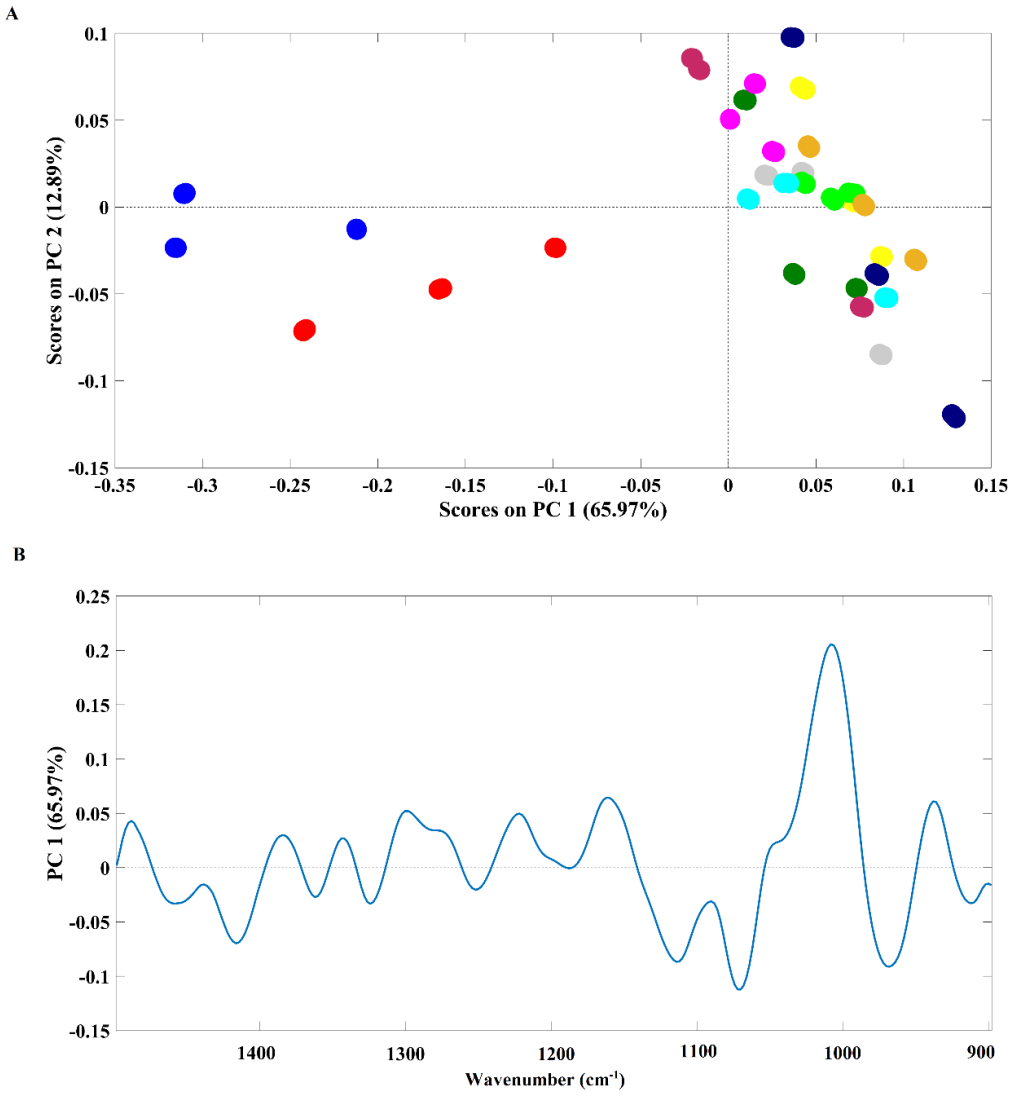


Figure VI-5. A) Principal component analysis (PCA) scatter plot based on PC1 and PC2 scores (better representing the separation between sample groups); B) loading plot of the PCA model,

evidencing changes in the chemical composition of *L. minor* after the growth period in 11 different media. Samples were color-coded by growth medium: ● ultrapure water; ● Mars regolith extract; ● Steinberg medium; ● *A. cylindrica* filtered; ● *A. cylindrica* sonicated; ● *N. muscorum* filtered; ● *N. muscorum* sonicated; ● *A. platensis* filtered; ● *A. platensis* sonicated; ● *C. vulgaris* filtered; ● *C. vulgaris* sonicated.

VI-4. Discussion

Forecasts highlight the decade of 2030 as the years of the launch of a human Space flight mission to Mars (2017). However, Martian conditions challenge human survival, and cyanobacteria can thrive in this context as Mars colonizers for oxygen production, as well as feedstock for other applications (e.g. food supplements, biofertilizers, and biofuel production). Several works already proved that these organisms are able to live with Mars resources, namely in water and Martian regolith (Arai et al. 2008; Arai 2009; Kimura et al. 2015; Verseux et al. 2021). In Mars, water can be found in the form of ice in the northern polar region (Shotwell 2005) as well as in regolithic brines (Martínez and Renno 2013; Lauro et al. 2021). Although neither liquid water nor liquid brines are currently stable in the surface, they can occur temporarily in shallow subsurfaces (Martínez and Renno 2013). Therefore, water can be "mined" and extracted from atmospheric water vapor, ground water, absorbed water, hydrated minerals, and ice (Hoffman et al. 2016). While having water is the first step, the following could be the use of Martian soil. The composition of Mars soil is known, and simulants of Martian regolith are available for experiments (e.g. MGS-1).

These two components (i.e. water and Mars regolith) were proven herein to be enough to support cyanobacteria growth, on the basis of optical density and the fluorimetric parameter F_v/F_m. However, not all tested species were able to grow well in Mars regolith extract. *A. platensis* and *C. vulgaris* showed a very limited growth, while *N. muscorum* and *A. cylindrica* thrived in Mars regolith extract, with the records of F_v/F_m for *N. muscorum* being higher in Mars regolith extract than in synthetic medium. In this way, considering the main perspective of using cyanobacteria for oxygen production in Mars, *N. muscorum* can be highlighted among the tested species as a stronger candidate for further studies. These are fundamental to understand the suitability of the species regarding other critical factors constraining their use in Mars, e.g. (i) their ability to survive the journey and hold very low pressures and vacuum conditions (Arai et al. 2008); (ii) their capacity to thrive under different atmospheres (see the studies of Verseux et. (2021) with the cyanobacterium *A.*

cylindrica). It is worth mentioning in this context that specific devices are being developed to create a controlled and Earth-like environment, e.g. the A'MED (Arai´s Mars Eco-systems Dome), which is a closed dome where temperature, humidity and light can be controlled and protection is ensured against ultraviolet radiation, cosmic rays, and low pressures (Arai et al. 2008).

Concerning the perspective of the maximizing of the use of Martian resources, only organisms with low nutritional requirements should be considered given the poor composition of the Mars regolith. Indeed, *C. vulgaris* and *A. platensis* did not grow expressively in Mars regolith extract, suggesting that nutritional requirements for this microalgae are higher than for the three cyanobacteria. Our results show that, despite long-term survival and growth is unknown, poor nutrition as provided by Mars regolith extract supports cyanobacterial growth in the short term (i.e. one month). Carbon, nitrogen, phosphorus, sulfur, potassium, iron, are the critical nutrients for cyanobacteria growth (Markou et al. 2014).

Carbon and nitrogen can be obtained by cyanobacteria autonomously from atmospheric CO_2 and from atmospheric N fixation, respectively (Markou et al. 2014). However, only diazotrophic species such as *A. cylindrica* and *N. muscorum* are able to fix atmospheric nitrogen through specialized structures (Flores and Herrero 2014), which can explain the lack of growth observed for *S. platensis* in Mars regolith extract. No traces of phosphorus were found in the Mars regolith extract analysis, but both cyanobacteria and microalgae can accumulate phosphorus reserves as polyphosphate granules, and these reserves can be used in cases of phosphorus shortage (Markou et al. 2014). As the cultures were established from laboratory stocks maintained under sub-optimal growth conditions, reserves mobilization should have been the mechanism supporting appropriate phosphorous supply for cyanobacteria growing in Mars regolith extract.

Although phosphorus was not present in the tested Mars regolith extract, other important elements such as sulphur, calcium, potassium, and manganese were available. Potassium is, along with nitrogen and phosphorus, a main macronutrient for the growth of photosynthetic organisms (Markou et al. 2014). Cyanobacteria thylakoids bear ion channels where potassium flows to allow a maximal efficiency of the photosynthesis (Checchetto et al. 2012). Potassium also plays significant roles as an activator in several enzymes involved

in photosynthesis and respiration, on protein and carbohydrate synthesis, and in the regulation of the osmotic potential inside the cells (Markou et al. 2014).

While manganese is required as a micronutrient, it can be toxic in high (micromolar range) concentrations to cyanobacteria, negatively affecting photosynthesis, growth rates, and nitrogen fixation (Rueter and Petersen 1987). Manganese is more toxic to cyanobacteria than to microalgae (Rueter and Petersen 1987), thus it is unlikely that this element can explain the limited growth of *C. vulgaris* in Mars regolith extract. Calcium has a central role as secondary messenger and signalling molecule in all living organisms (Clapham 2007). It is also involved in atmospheric nitrogen fixation (Allen and Arnon 1955), and its concentration has influence in the biomass and proteins building (Walter et al. 2016). Sulphur is important for protein synthesis in general and in the biosynthesis of coenzymes (Schmidt 1988), but contrarily to animals for example, cyanobacteria are not dependent on the external supply of reduced sulphur compounds, since they can autonomously reduce sulphate for protein and coenzyme synthesis. In these organisms, sulphur is needed for sulfolipid biosynthesis, found in photosynthetic membranes (Schmidt 1988). Although surprising, the presence of strontium in Mars regolith extract was also detected in similar circumstances in other works (e.g. Olsson-Francis and Cockell 2010), which suggests that this element is a common contaminant in MGS-1. The presence of this element is not beneficial to cyanobacteria as it negatively affects their growth rates and chlorophyll *a* content (Foster et al. 2020). Although the composition of Mars regolith extract allowed cyanobacterial growth in the present study, it is important to remark that extracts can be prepared under different protocols (e.g. Verseux et al. 2021), possibly allowing optimization of bioavailability of some elements and rendering it more suitable as a culture medium for defined species. In addition, there are several types of Mars regolith with different compositions that can perform better for cyanobacteria/ microalgae growth (https://sciences.ucf.edu/class/exolithlab/ assessed on October 19th, 2021).

Besides the main task of producing oxygen, a claimed application for cyanobacteria is as biofertilizers (Chittora et al. 2020). Diazotrophic cyanobacteria (e.g. *Anabaena* sp. and *Nostoc* sp.) are particularly pointed out as eco-friendly biofertilizers due to their ability to fix atmospheric nitrogen, delivering it to plants (Rashid et al. 2016). Furthermore, cyanobacteria improve soil porosity and water holding capacity, produce adhesive substances and excrete phytohormones, vitamins and amino acids (Chittora et al. 2020).

Indeed, their successful application as biofertilizers was already reported for several vegetable cultures (e.g. barley, oat, tomato, radish, cotton, sugarcane, maize, chilli and lettuce) (Chittora et al. 2020). The nutrients necessary to grow plants are present is Mars (Verseux et al. 2016), and several crop cultures (e.g. tomato, rye, carrot, garden cress) were already proven to grow on Mars regolith, developing at similar rates on Martian and on low-nutrient Earth soil, due to the larger water holding capacity of the Martian soil simulants (Wamelink et al. 2014). Although these are exciting results, there are few organic molecules on Mars (e.g. Heinz and Schulze-Makuch 2020) that can support soils overall fertility in the long-term, and plants cannot fix atmospheric N, which turns the enrichment with N, as that operated by diazotrophic cyanobacteria, very important (Verseux et al. 2016). Arai et al. (2008) demonstrated that a terrestrial *Nostoc* sp. HK-01 can successfully grow in Mars regolith, releasing polysaccharides that contributed to convert regolith into an organic soil. The ability of *A. cylindrica* to release K, Mg, Na, Ca, Fe, Mn, Ni, Sr, Cu, Li, and Zn from a Mars basalt analogue was also shown (Olsson-Francis and Cockell 2010), and this feature can increase the odds for successful plants growth on Martian regolith. Despite these encouraging evidences, it is necessary to mention that the culturing conditions influence the composition of cyanobacteria, such as the carbohydrates composition (Depraetere et al. 2015). The way this can constrain the biofertilizer role of cyanobacteria should be considered for future research.

In the present work, *L. minor* was used as a vegetable model however typical crop species should be tested in the future to confirm the outcomes. Our results show that *L. minor* grew better in Mars regolith extract than in ultrapure water, and that photosynthetic efficiency, in some cases, was higher in Mars regolith extract than in the standard culture medium. This suggests that, although *L. minor* grows at a smaller pace in Mars regolith extract than in optimal media, its healthy growth can last for longer towards better overall biomass production. The treatment made to the cyanobacteria/ microalgae cultures provided as biofertilizers (filtration or sonication), did not affect *L. minor* photosynthetic efficiency. As well, it is worth noting that *L. minor* photosynthetic efficiency did not differ among treatments with cyanobacteria and sole Mars regolith extract culturing, while treatments where the microalgae were supplied as a biofertilized harmed the photosynthetic efficiency of the plant. Actually, despite *C. vulgaris* being one of the most studied species for space

life support, some of its by-products were already proven to be toxic to higher plant crops (Terskov et al. 1979).

Interestingly, the parameters assessing on *L. minor* biomass yield confirm the outcomes of photosynthetic efficiency analysis. Briefly, while the number of fronds increased more throughout the experiment in the control with synthetic medium than in any of the other treatments, the dry weight yield recorded for plants growing in Mars regolith extract supplemented with cyanobacteria and microalgae is similar to that recorded in the control. Thus, in spite of a higher reproduction rate, the plant culture in synthetic medium does not necessarily produce higher biomass than the cultures in Mars regolith supplemented with cyanobacteria and microalgae, and certainly questions arise regarding the differential nutritional value of the cultured plants. A preliminary analysis of our samples by FTIR-ATR separated the nutritional profile (polysaccharides in particular) of the plants grown in regolith and supplemented regolith from those grown in synthetic media and ultrapure water. Plants are important sources of carbohydrates, responsible for providing energy to humans. These carbohydrates can be divided in energy storage carbohydrates like starch, oligosaccharides and sugars, and in structural cell wall polysaccharides (Lovegrove et al. 2017), which differ regarding for example their digestibility. Given the importance of these features when considering crop production in Mars to support colonization attempts with high healthy supplies, differences in the nutritional profiles of different experimental treatments such as that noticed herein are a critical object for future research. Namely, in-depth analysis of major nutritional groups (i.e. proteins, carbohydrates, lipids, minerals, fibers) is required to gain a robust insight on the nutritional value of vegetable species grown in Martian soil.

VI-5. Conclusion

Are cyanobacteria able to growth using only water and Martian regolith? This work showed that they could. Within the tested species, *N. muscorum* showed to be one that best performs under these conditions, and should be the target of future studies. Not only these organisms could be pointed out as possible oxygen producers in Mars, as could also be used as biofertilizers. This work showed that *L. minor* was able to grow in Mars regolith extract and eventually reached higher biomass density, comparing to the control group (i.e.

Steinberg medium). The good performance of *L. minor* that grew on supplemented regolith concerning the dry weight also gave some insights on the nutritional composition of these plants, which is marked by changes in the polysaccharide group. In the future, analysis on the nutritional composition of species that grown using these resources would be an essential step, prior the implementation of these systems.

References

Allen MB, Arnon DI (1955) Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. Plant Physiol 30:366–372. doi: 10.1039/an9487300121

Arai M (2009) Cyanobacteria for Space agriculture on Mars. Biol Sci Sp 23:203–210. doi: 10.2187/bss.23.203

Arai M, Tomita-Yokotani K, Sato S, et al. (2008) Growth of terrestrial cyanobacterium, *Nostoc* sp., on Martian Regolith Simulant and its vacuum tolerance. Biol Sci Sp 22:8–17. doi: 10.2187/bss.22.8

Borowitzka MA (2018) Biology of microalgae. In: Levine IA, Fleurance J (eds) Microalgae Heal. Dis. Prev. Elsevier Inc., pp 23–72

Checchetto V, Segalla A, Allorent G, et al. (2012) Thylakoid potassium channel is required for efficient photosynthesis in cyanobacteria. Proc Natl Acad Sci U S A 109:11043–11048. doi: 10.1073/pnas.1205960109

Chittora D, Meena M, Barupal T, Swapnil P (2020) Cyanobacteria as a source of biofertilizers for sustainable agriculture. Biochem Biophys Reports 22:100737. doi: 10.1016/j.bbrep.2020.100737

Clapham DE (2007) Calcium signaling. Cell 131:1047–1058. doi: 10.1016/j.cell.2007.11.028

Cockell CS (2014) Trajectories of martian habitability. Astrobiology 14:182–203. doi: 10.1089/ast.2013.1106

Depraetere O, Pierre G, Deschoenmaeker F, et al. (2015) Harvesting carbohydrate-rich *Arthrospira platensis* by spontaneous settling. Bioresour Technol 180:16–21. doi: 10.1016/j.biortech.2014.12.084

Eriksen NT (2008) Production of phycocyanin - a pigment with applications in biology, biotechnology, foods and medicine. Appl Microbiol Biotechnol 80:1–14. doi: 10.1007/s00253-008-1542-y

Fawzy MA, Mohamed AKSH (2017) Bioremediation of heavy metals from municipal sewage by cyanobacteria and its effects on growth and some metabolites of *Beta vulgaris*. J Plant Nutr 40:2550–2561. doi: 10.1080/01904167.2017.1380822

Flores E, Herrero A (2014) The cell biology of cyanobacteria. Caister Academic Press, Norfolk, UK

Foster L, Morris K, Cleary A, et al. (2020) Biomineralization of Sr by the Cyanobacterium *Pseudanabaena catenata* under alkaline conditions. Front Earth Sci 8:1–10. doi: 10.3389/feart.2020.556244

Fraunhofer-Gesellschaft Algae survive heat, cold and cosmic radiation. In: ScienceDaily. www.sciencedaily.com/releases/2017/02/170207104231.htm. Accessed 24 Sep 2021

Gao K (1998) Chinese studies on the edible blue-green alga, *Nostoc flagelliforme*: A review. J Appl Phycol 10:37–49. doi: 10.1023/A:1008014424247

Gayen P, Sankarasubramanian S, Ramani VK (2020) Fuel and oxygen harvesting from Martian regolithic brine. Proc Natl Acad Sci U S A 117:31685–31689. doi: 10.1073/pnas.2008613117

Gaysina LA, Saraf A, Singh P (2018) Cyanobacteria in Diverse Habitats. Cyanobacteria From Basic Sci to Appl. doi: 10.1016/B978-0-12-814667-5.00001-5

Gonzáles A, Fillat MF, Bes MT, et al. (2018) The challange of iron stress in Cyanobacteria. In: Tiwari A (ed) Cyanobacteria. IntechOpen, pp 137–144

Grzesik M, Kalaji HM (2017) Effectiveness of cyanobacteria and green algae in enhancing the photosynthetic performance and growth of willow (*Salix viminalis* L.) plants under limited synthetic fertilizers application. Photosynthetica 55:510–521. doi: 10.1007/s11099-017-0716-1

Häder D (2020) On the way to Mars - Flagellated algae in bioregenerative life support systems under microgravity conditions. Front Plant Sci 10:1–5. doi: 10.3389/fpls.2019.01621

Heinse R, Jones SB, Steinberg SL, et al. (2007) Measurements and modeling of variable gravity effects on water distribution and flow in unsaturated porous media. Vadose Zo J 6:713–724. doi: 10.2136/vzj2006.0105

Heinz J, Schulze-Makuch D (2020) Thiophenes on Mars: Biotic or abiotic origin? Astrobiology 20:552–561. doi: 10.1089/ast.2019.2139

Hoffman S, Andrews A, Watts K (2016) "Mining" water ice on Mars. An assessment of ISRU option in support of future human missions.

Johnson HE, King SR, Banack SA, et al. (2008) Cyanobacteria (*Nostoc commune*) used as a dietary item in the Peruvian highlands produce the neurotoxic amino acid BMAA. J Ethnopharmacol 118:159–165. doi: 10.1016/j.jep.2008.04.008

Jolliffe IT (1986) Principal Component Analysis. Berlin

Kaza M, Mankiewicz-Boczek J, Izydorczyk K, Sawicki J (2007) Toxicity assessment of water samples from rivers in central Poland using a battery of microbiotests - A pilot study. Polish J Environ Stud 16:81–89.

Kimura Y, Kimura S, Sato S, et al. (2015) Evaluation of a Cyanobacterium, *Nostoc* sp. HK-01, as food material for Space agriculture on Mars. Biol Sci Sp 29:24–31. doi: 10.2187/bss.29.24

Lauro SE, Pettinelli E, Caprarelli G, et al. (2021) Multiple subglacial water bodies below the south pole of Mars unveiled by new MARSIS data. Nat Astron 5:63–70. doi: 10.1038/s41550-020-1200-6

Lovegrove A, Edwards CH, De Noni I, et al. (2017) Role of polysaccharides in food, digestion, and health. Crit Rev Food Sci Nutr 57:237–253. doi: 10.1080/10408398.2014.939263

Macário IPE, Ventura SPM, Gonçalves FJM, et al. (2021) The "bright side" of cyanobacteria: revising the nuisance potential and prospecting innovative biotechnology-based solutions to integrate water management programs. ACS Sustain Chem Eng 9:7182–7197. doi: 10.1021/acssuschemeng.1c00458

Markou G, Vandamme D, Muylaert K (2014) Microalgal and cyanobacterial cultivation: The supply of nutrients. Water Res 65:186–202. doi: 10.1016/j.watres.2014.07.025

Martínez GM, Renno NO (2013) Water and brines on Mars: Current evidence and implications for MSL. Space Sci Rev 175:29–51. doi: 10.1007/s11214-012-9956-3

Mostafa SSM (2012) Microalgal biotechnology: Prospects and applications. In: Dhal NK, Sahu SC (eds) Plant Sci. IntechOpen, pp 275–314

Næs T, Isaksson T, Fearn T, Davis T (2002) A user-friendly guide to multivariate calibration and classification. doi: 10.1255/978-1-906715-25-0

Nelson M, Pechurkin NS, Allen JP, et al. (2010) Closed ecological systems, space life support and biospherics. In: Wang LK, Ivanov V, Tay J-H, Hung Y-T (eds) Handb. Environ. Eng. Vol. 10 Environ. Biotechnol. Humana Press, New York, pp 517–565

Nichols HW (1973) Growth media-freshwater. In: Stein JR (ed) Handb. Phycol. methods Cult. methods growth Meas. Cambridge University Press, Cambridge, UK, pp 16–17

OECD (2006) Test No. 221: *Lemna* sp. Growth Inhibition Test. Guidel Test Chem 1–26.

Ogawa T, Misumi M, Sonoike K (2017) Estimation of photosynthesis in cyanobacteria by pulse-amplitude modulation chlorophyll fluorescence: problems and solutions. Photosynth Res 133:63–73. doi: 10.1007/s11120-017-0367-x

Olsson-Francis K, Cockell CS (2010) Use of cyanobacteria for in-situ resource use in space applications. Planet Space Sci 58:1279–1285. doi: 10.1016/j.pss.2010.05.005

Raheem A, Prinsen P, Vuppaladadiyam AK, et al. (2018) A review on sustainable microalgae based biofuel and bioenergy production: Recent developments. J Clean Prod 181:42–59. doi: 10.1016/j.jclepro.2018.01.125

Rashid A, Mir MR, Hakeem KE (2016) Biofertilizer use for sustainable agricultural production. In: Hakeem K, Akhtar M, Abdullah S (eds) Plant, Soil Microbes. Springer, pp 163–180

Rueter JG, Petersen RR (1987) Micronutrient effects on cyanobacterial growth and physiology. New Zeal J Mar Freshw Res 21:435–445. doi: 10.1080/00288330.1987.9516239

SAG (2008) Maintenance of Cultures - Spirulina Medium Recipe-Vers. 10. 2008. Sammlung von Algenkulturen Göttingen Cult. Collect. Algae

Savitzky A, Golay MJE (1964) Smoothing and differentiation of data by simplified least squares procesures. Anal Chem 36:1627–1639.

Schmidt ABT-M in E (1988) Sulfur metabolism in cyanobacteria. Cyanobacteria. Academic Press, pp 572–583

Sekar S, Chandramohan M (2008) Phycobiliproteins as a commodity: Trends in applied research, patents and commercialization. J Appl Phycol 20:113–136. doi: 10.1007/s10811-007-9188-1

Shotwell R (2005) Phoenix - The first Mars Scout mission. Acta Astronaut 57:121–134. doi: 10.1016/j.actaastro.2005.03.038

Terskov IA, Gitelson JI, Kovrov BG (1979) Closed system: Man-higher plants (four month

experiment), translation of Nauka Press, Siberian Branch, NASA-TM76452, Washington, D.C.

Verseux C, Baqué M, Lehto K, et al. (2016) Sustainable life support on Mars - The potential roles of cyanobacteria. Int J Astrobiol 15:65–92. doi: 10.1017/S147355041500021X

Verseux C, Heinicke C, Ramalho TP, et al. (2021) A low-pressure, N_2/CO_2 atmosphere is suitable for cyanobacterium-based life-support systems on Mars. Front Microbiol 12:611798. doi: 10.3389/fmicb.2021.611798

Walter J, Lynch F, Battchikova N, et al. (2016) Calcium impacts carbon and nitrogen balance in the filamentous cyanobacterium *Anabaena* sp. PCC 7120. J Exp Bot 67:3997–4008. doi: 10.1093/jxb/erw112

Wamelink GWW, Frissel JY, Krijnen WHJ, et al. (2014) Can plants grow on Mars and the moon: A growth experiment on mars and moon soil simulants. PLoS One. doi: 10.1371/journal.pone.0103138

(2017) 115th Congress - National Aeronautics and Space Administration Transition Authorization Act of 2017.

CHAPTER VII

Final remarks and future perspectives

In a world with a growing human population and a consequent increased resources scarcity, the need to increase the range of feedstocks and biomasses available to explore arose. Cyanobacteria are one of those microorganism groups whose exploitation can help to fulfil some of these requests, while contributing to achieve several of the Goals proposed by the United Nations (SDG) towards a sustainable development, both concerning environmental protection and sustainable production/consumption. In addition, worldwide consumers are becoming more aware of resources scarcity, on the one hand, and of the improved biological compatibility of natural products over synthetic counterparts, on the other hand, which greatly supports any endeavours aiming at filling gaps in these two fields. This overall context highlights the role of Cyanobacteria as an important and sustainable player in global economy nowadays and in the future, featuring high impact for related research results.

The major goal of this Thesis was to apply to concept of biorefinery to the valorization of cyanobacterial blooms, which are a current and growing problematic worldwide that will likely aggravate the climate change consequences. To characterise the fields and boundaries of this overarching aim, as well as properly kick-off and contextualize the options made for the experimental work, a critical review (Chapter II) was elaborated on the 'dark side' of cyanoHABs, followed by a conceptual proposal of integrating their 'bright side' as a new axis to improve the efficiency of nuisance management frameworks. A framework with four sequential steps is presented, following the principles of the circular economy and driven by the idea of using the economic income generated with the valorization of cyanobacteria biomass removed from waterbodies affected by blooms in further actions regarding environmental protection and restoration. This is not a traditional or straightforward approach, neither is free of controversy, but new times demand new solutions as that suggested towards the building of win-win relationships between environmental sustainability and economic income. The application of such a framework presents several constrains and would require strong regulation prior to implementation. For example, this type of approach could be applied in developing countries, as a mechanism to improve the economic income of disfavoured communities and promote environmental restoration (and health safety in the case of harmful algal blooms) in parallel. However, the risks of considering blooms as assets that are worth the efforts for inducing them in natural waterbodies are high, especially if no strict preventive regulation is in force. For fully

established industries in high-income countries and more comprehensive regulation frameworks, the risk of misinterpretation of the idea would be lower (note that there are already companies at least in the United States that collect blooms biomass to feed exploitation pipelines; Levi 2018), but the sustainability of the strategy can be hampered by the seasonality of the blooms depending on the geography. This shortcoming can be overcome if the exploitation of blooms is faced as a complementary strategy to own cultivation, with the additional benefits of filling environmental sustainability quotes that meet, for example, the climate change mitigation worldwide.

Following the elaboration of the broader vision and the main philosophy of the thesis (Chapter II), the experimental work plan was developed underneath, exploring the created umbrella, by developing on several "bright sides" of cyanobacteria under the biorefinery principles. If assuming the focus on sustainable industry development, the logical path was indeed the investigation of several routes for the valorization of cyanobacteria that could potentially become exploitation axes. Because natural blooms are complex and variable assemblages bearing often multiple species/strains of cyanobacteria and microalgae, and considering that much is still to be done to fully understand the array of valuable cyanobacteria metabolites, their bioactivity and their suitability for different biotechnological applications, the option to proceed with the work plan using single strains was logical. Despite the increase research efforts in this field, a remarkable example exposing the scarcity of reliable evidence on the biological activities of C-phycocyanin extracts can be found in Chapter IV. In addition, the exploitation of natural blooms requires, necessarily, toxin quantification (see Chapter II). If anchoring the experimental work plan on natural blooms, developments in the current technologies for toxin detection (easier targeting and cheaper methods) would have to be incorporated, and the most recent trends in this context would require investment in new fields of expertise (e.g. biosensors field, especially graphene-based; Bertani and Lu 2021) that were not easily reachable within the timeline.

In the industrial context, *Arthrospira* is one of the most explored species, not only due to its rich nutritional profile but also because it is a non-toxic and a very well-known species. However, considering the high number of known cyanobacteria species and the scarcity of broad characterisation of their growth dynamics and metabolite production, it is highly unlikely that *Arthrospira* would be the best species to use for all purposes. This was

the reasoning for the research exposed in Chapter III, where *Arthrospira platensis* was used to compare to other two, less-explored cyanobacteria species, *Anabaena cylindrica* and *N. muscorum*, regarding growth dynamics and pigment production patterns. All of these species showed an extraordinary longevity, and *A. platensis* was indeed shown to be the best raw biomass producer. However, *A. platensis* was not the best pigment producer, confirming the initial hypothesis of the study. In general, *N. muscorum* was proven to be the most interesting species for industrial exploitation concerning pigments, since it presents high growth rates, as well as high productions of chlorophyll *a* and C-phycocyanin. However, it should be recognized that different culturing conditions might lead to different outcomes, hence the critical relevance of optimization stages in industrial settings exploring cyanobacteria metabolites. This study essentially highlighted on how meaningful species selection can be to leverage exploitation outcomes, reinforcing the need for previous systematic knowledge on the growth dynamics of different species/strains. To address this aspect, different methods were tested in parallel (spectrophotometry and fluorimetry) as to their capacity to be used as proxies of pigment production that can serve monitoring purposes in exploitation settings. None was proved flawless, which again reinforces the importance of previous small-scale studies to optimise on species/strain selection and growth conditions towards better exploitation efficiency levels.

Since pigments are among the extractable compounds with higher market value, emphasis was put on the specific analysis of the yield and claimed biological activity of the blue pigment C-phycocyanin in Chapter IV. However, the analyzed C-phycocyanin extracts from *A. cylindrica* presented mild or unrecognizable biological activities, unlike what is commonly described in literature (Basha et al. 2008; Mohite et al. 2015; Shanmugam et al. 2017; Jiang et al. 2018; Renugadevi et al. 2018; Jang and Kim 2021). These disappointing results inspired a follow-up work under the sound bite that *being blue is not enough*. The results of such a second-stage, developed with C-phycocyanin extracts from several species (produced with different solvents and extraction steps), showed that not only the species is a determinant of biological activity, but also that the choice of the solvents and the extraction protocols are critical aspects to consider in this regard. Overall, the work exposed in Chapter IV notices that either the biological activities claimed by the literature are the probable outcome of synergic effects with components other than C-phycocyanin, or reflect that C-phycocyanin biological activities are species/strain-specific. The observation of a better

performance of raw extracts compared to purified extracts (i.e. higher antioxidant activity, better biocompatibility, and even antitumor activity in some concentrations) corroborates these perspectives. Future efforts to characterize these extracts are critical to deeply understand what are the compounds and/or mixtures truly responsible for the biological activity.

Although *A. cylindrica* was not recognized as the best species for C-phycocyanin extraction, other added-value compounds beside pigments can be exploited in cyanobacteria as addressed in Chapter V. Actually, when the metabolome of *N. muscorum* was inspected, it was possible to observe the production of interesting compounds bearing also a relevant market value (e.g. ω-3 and ω-6 fatty acids, and mycosporin-like amino acids). Therefore, the dynamics of the metabolome of *N. muscorum* (and of other cyanobacteria species) is a meaningful future research target, with particular emphasis on mycosporin-like amino acids due to their biotechnological potential (Geraldes and Pinto 2021) and because these metabolites are naturally produced during the first stages of *N. muscorum* growth. In addition, this study highlighted that compositional changes of the metabolome occur throughout culture growth, which remarks that it is possible to optimise the best harvesting period for the extraction of a desired metabolite. Particularly for *N. muscorum*, a significant shift in the composition of the metabolome occurs around day 43 to 56 (Figure V-4). Consistently, a brief plateau in the growth curves of three cyanobacteria species was identified within this period, especially recognizable in *N. muscorum* and *A. platensis* (Figures III-1-3c). It is therefore reasonable to hypothesize that cells hold investment in duplication possibly to face nutrient depletion and/or osmotic balance disturbance, then adapting to the harsher conditions with the consequent shifting in metabolites production. Given that some cyanobacteria can onset processes of metabolite excretion (Carrieri et al. 2012), a future research direction should be focused on the parallel analysis of the extracellular medium for a broader mechanistic understanding of this potential adaptation stage followed by the noticed metabolic shift. This type of analysis would help to understand what are the specific triggers responsible for such metabolic changes. This would play major importance in industrial culturing since it would allow promoting the production of specific compounds.

One of the most interesting findings through the course of the experimental work was the fact that *A. cylindrica*, *N. muscorum* and *A. platensis* were able to grow for more

than 112 days without any medium supplementation. This incredible longevity raised several questions and opened research perspectives. Within the context of the present Thesis, the application of cyanobacteria as resources for Space exploration was pursued among these perspectives. Their longevity, along with their low nutritional requirements and the ability to produce oxygen, are extremely attractive and supportive of the promotion of cyanobacteria as valuable assets in the establishment of life support systems in planets other than Earth. *A. platensis, A. cylindrica,* and *N. muscorum* (plus the microalgae *C. vulgaris* as a reference) were used to address this research avenue (Chapter VI). The study suggests that not all species are able to thrive under limited resources as simulated (culturing in water supplemented with Martian regolith), the diazotrophic species being the best performers among the tested organisms; note that neither green microalgae nor the widely used cyanobacteria *A. platensis* in the context of Space exploration research are diazotrophic. While cyanobacteria are primarily thought to be used as oxygen producers within life support systems in Space, the study also highlighted the potential for the downstream application of aged biomass batches as biofertilization agents. Indeed, high levels of vegetal biomass were achieved using media supplemented with cyanobacteria grown under limited nutrient supply. Although this work was a brief incursion in the field bearing several limitations, it corroborated previous claims on the suitability of cyanobacteria as Mars colonizers (Arai et al. 2008; Arai 2009; Olsson-Francis and Cockell 2010; Verseux et al. 2016; Verseux et al. 2021) and provided new evidence highlighting that not all cyanobacteria species should be selected for this mission. Follow-up studies are necessary to clarify different aspects and consolidate the promotion of cyanobacteria in the Space exploration arena. For example, oxygen production levels should be measured for a realistic appraisal on how the benefits scale compared to higher plants culturing settings (in solid substrates or hydroponics); the yield of the cyanobacteria cultures should be tested under more realistic conditions, e.g. using an atmospheric composition more similar to the one found on Mars; the nutritional composition of plants fertilized with cyanobacteria biomass should be scrutinized to broadly appraise the potential benefits of this downstream recycling strategy.

Finally, it is worth noticing that many arenas where cyanobacteria have remarkable application potential remained unexplored in this Thesis, despite their compliance several United Nations priorities (i.e. SDG 2 - Zero Hunger; SDG 3 - Good Health and Well-Being; SDG 6 - Clean Water and Sanitation; SDG 7 - Affordable and Clean Energy; and SDG 13 -

Climate Action), and a few examples follow. In this context, efforts should be directed to the systematic search for species that can be more/or as nutritionally rich as *Arthrospira*, to more efficiently tackle malnutrition problems arising worldwide in developing and developed countries. The search for new biologically active compounds produced by cyanobacteria should continue to assist the development of alternative therapeutic approaches to existing and eventually new health fragilities. The abilities of cyanobacteria as bioremediation agents should be better explored as a meaningful contribution to mitigate water pollution, especially considering their suitability for easy genome edition, which is amongst the most recent tools to efficiently tackle the degradation of recalcitrant pollutants in wastewater treatment processes (El-Sheekh et al. 2021). The use of cyanobacteria as a feedstock for biofuel and biopolymers production could be helpful as additional assets towards the reduction of the humanity's dependency on fossil fuels, yet this exploitation line still requires technological advances in cultivation systems and extraction techniques to render the process economically sustainable and ultimately attractive for wide investment (Slade and Bauen 2013; Ciebiada et al. 2020). The incorporation of cyanobacteria/microalgae in building façades was already proposed to support the mission and commitment of cities worldwide towards the neutralization of CO_2 emissions in urban environments (Biloria and Thakkar 2020).

A sustainable future is a greener future, and despite their rather bluish tone, cyanobacteria have the potential to be part of it.

References

Arai M (2009) Cyanobacteria for Space agriculture on Mars. Biol Sci Sp 23:203–210. doi: 10.2187/bss.23.203

Arai M, Tomita-Yokotani K, Sato S, et al. (2008) Growth of terrestrial cyanobacterium, *Nostoc* sp., on Martian Regolith Simulant and its vacuum tolerance. Biol Sci Sp 22:8–17. doi: 10.2187/bss.22.8

Basha OM, Hafez RA, El-Ayouty YM, et al. (2008) C-Phycocyanin inhibits cell proliferation and may induce apoptosis in human HepG2 cells. Egypt J Immunol 15:161–167. doi: 10.1016/j.algal.2013.01.004

Bertani P, Lu W (2021) Cyanobacterial toxin biosensors for environmental monitoring and protection. Med Nov Technol Devices 10:100059. doi: 10.1016/j.medntd.2021.100059

Biloria N, Thakkar Y (2020) Integrating algae building technology in the built environment: A cost and benefit perspective. Front Archit Res 9:370–384. doi: 10.1016/j.foar.2019.12.004

Carrieri D, Paddock T, Maness PC, et al. (2012) Photo-catalytic conversion of carbon dioxide to organic acids by a recombinant cyanobacterium incapable of glycogen storage. Energy Environ Sci 5:9457–9461. doi: 10.1039/c2ee23181f

Ciebiada M, Kubiak K, Daroch M (2020) Modifying the cyanobacterial metabolism as a key to efficient biopolymer production in photosynthetic microorganisms. Int J Mol Sci 21:1–24. doi: 10.3390/ijms21197204

El-Sheekh M, El-Dalatony MM, Thakur N, et al. (2021) Role of microalgae and cyanobacteria in wastewater treatment: genetic engineering and omics approaches. Int J Environ Sci Technol. doi: 10.1007/s13762-021-03270-w

Geraldes V, Pinto E (2021) Mycosporine-like amino acids (MAAs): Biology, chemistry and identification features. Pharmaceuticals 14:1–17. doi: 10.3390/ph14010063

Jang YA, Kim BA (2021) Protective effect of *Spirulina*-derived C-phycocyanin against ultraviolet B-induced damage in HaCaT cells. Medicina (B Aires) 57:1–12.

Jiang L, Wang Y, Liu G, et al. (2018) C-Phycocyanin exerts anti-cancer effects via the MAPK signaling pathway in MDA-MB-231 cells. Cancer Cell Int 18:1–14. doi: 10.1186/s12935-018-0511-5

Levi D (2018) Adressing an ecological crisis. https://aecom.com/blog/addressing-an-ecological-crisis. Accessed 23 Oct 2020

Mohite YS, Shrivastava ND, Sahu DG (2015) Antimicrobial activity of C-phycocyanin from *Arthrospira platensis* isolated from extreme haloalkaline environment of Lonar Lake. IOSR J Environ Sci Toxicol Food Technol 1:40–45.

Olsson-Francis K, Cockell CS (2010) Use of cyanobacteria for in-situ resource use in space applications. Planet Space Sci 58:1279–1285. doi: 10.1016/j.pss.2010.05.005

Renugadevi K, Nachiyar CV, Sowmiya P, Sunkar S (2018) Antioxidant activity of phycocyanin pigment extracted from marine filamentous cyanobacteria *Geitlerinema* sp. TRV57. Biocatal Agric Biotechnol 16:237–242. doi: 10.1016/j.bcab.2018.08.009

Shanmugam A, Sigamani S, Venkatachalam H, et al. (2017) Antibacterial activity of

extracted phycocyanin from *Oscillatoria* sp. J Appl Pharm Sci 7:62–67. doi: 10.7324/JAPS.2017.70310

Slade R, Bauen A (2013) Micro-algae cultivation for biofuels: Cost, energy balance, environmental impacts and future prospects. Biomass and Bioenergy 53:29–38. doi: 10.1016/j.biombioe.2012.12.019

Verseux C, Baqué M, Lehto K, et al. (2016) Sustainable life support on Mars - The potential roles of cyanobacteria. Int J Astrobiol 15:65–92. doi: 10.1017/S147355041500021X

Verseux C, Heinicke C, Ramalho TP, et al. (2021) A low-pressure, N_2/CO_2 atmosphere is suitable for cyanobacterium-based life-support systems on mars. Front Microbiol 12:611798. doi: 10.3389/fmicb.2021.611798

ANNEXES

Annex I

Chapter III: Are Cyanobacteria a nearly immortal sources of high market value compounds?

Table III-S1. MBL - Woods Hole medium composition (Nichols 1973).

$CaCl_2 \cdot 2H_2O$	36.76 $mg.L^{-1}$
$MgSO_4 \cdot 7H_2O$	36.97 $mg.L^{-1}$
$NaHCO_3$	12.60 $mg.L^{-1}$
K_2HPO_4	8.71 $mg.L^{-1}$
$NaNO_3$	85.01 $mg.L^{-1}$
$Na_2SiO_3 \cdot 9H_2O$	28.42 $mg.L^{-1}$
$Na_2 \cdot EDTA$	4.36 $g.L^{-1}$
$FeCl_3 \cdot 6H_2O$	3.15 $g.L^{-1}$
$CuSO_4 \cdot 5H_2O$	0.01 $g.L^{-1}$
$ZnSO_4 \cdot 7H_2O$	0.022 $g.L^{-1}$
$CoCl_2 \cdot 6H_2O$	0.01 $g.L^{-1}$
$MnCl_2 \cdot 4H_2O$	0.18 $g.L^{-1}$
$Na_2MoO_4 \cdot 2H_2O$	0.006 $g.L^{-1}$
Tris(hydroxymethyl)-aminomethane)	250 $g.L^{-1}$

Table III-S2. Spirulina medium (SAG 2008).

Solution I		**Solution II**		**Micronutrient solution**	
$NaHCO_3$	27.22 $g.L^{-1}$	$NaNO_3$	5.0 $g.L^{-1}$	$ZnSO_4 \cdot 7H_2O$	1 $mg.L^{-1}$
$NaCO_3$	8.06 $g.L^{-1}$	K_2SO_4	2.0 $g.L^{-1}$	$MnSO_4 \cdot 4H_2O$	2 $mg.L^{-1}$
K_2HPO_4	1.0 $g.L^{-1}$	NaCl	2.0 $g.L^{-1}$	H_3BO_3	10 $mg.L^{-1}$
		$MgSO_4 \cdot 7H_2O$	0.4 $g.L^{-1}$	$Co(NO_3)2 \cdot 6H_2O$	1 $mg.L^{-1}$
		$CaCl_2 \cdot 2H_2O$	0.08 $g.L^{-1}$	$Na_2MoO_4 \cdot 2H_2O$	1 $mg.L^{-1}$
		$FeSO_4 \cdot 7H_2O$	0.02 $g.L^{-1}$	$CuSO_4 \cdot 5H_2O$	0.005
		EDTA (Titriplex III)	0.16 $g.L^{-1}$	$FeSO_4 \cdot 7H_2O$	$mg.L^{-1}$
		Micronutrient solution	10 mL. L^{-1}	EDTA (Titriplex III)	0.7 $g.L^{-1}$
					0.8 $g.L^{-1}$

Table III-S3. Pearson and Spearman correlation between optical density at 750 nm and F_o for *A. cylindrica*, *N. muscorum*, and *A. platensis*.

		Pearson		Spearman	
Species	N	Correlation coefficient	*p-value*	Correlation coefficient	*p-value*
A. cylindrica	40	0.924	< 0.005	0.971	< 0.005
N. muscorum	39	0.982	< 0.005	0.990	< 0.005
A. platensis	39	0.933	< 0.005	0.995	< 0.005

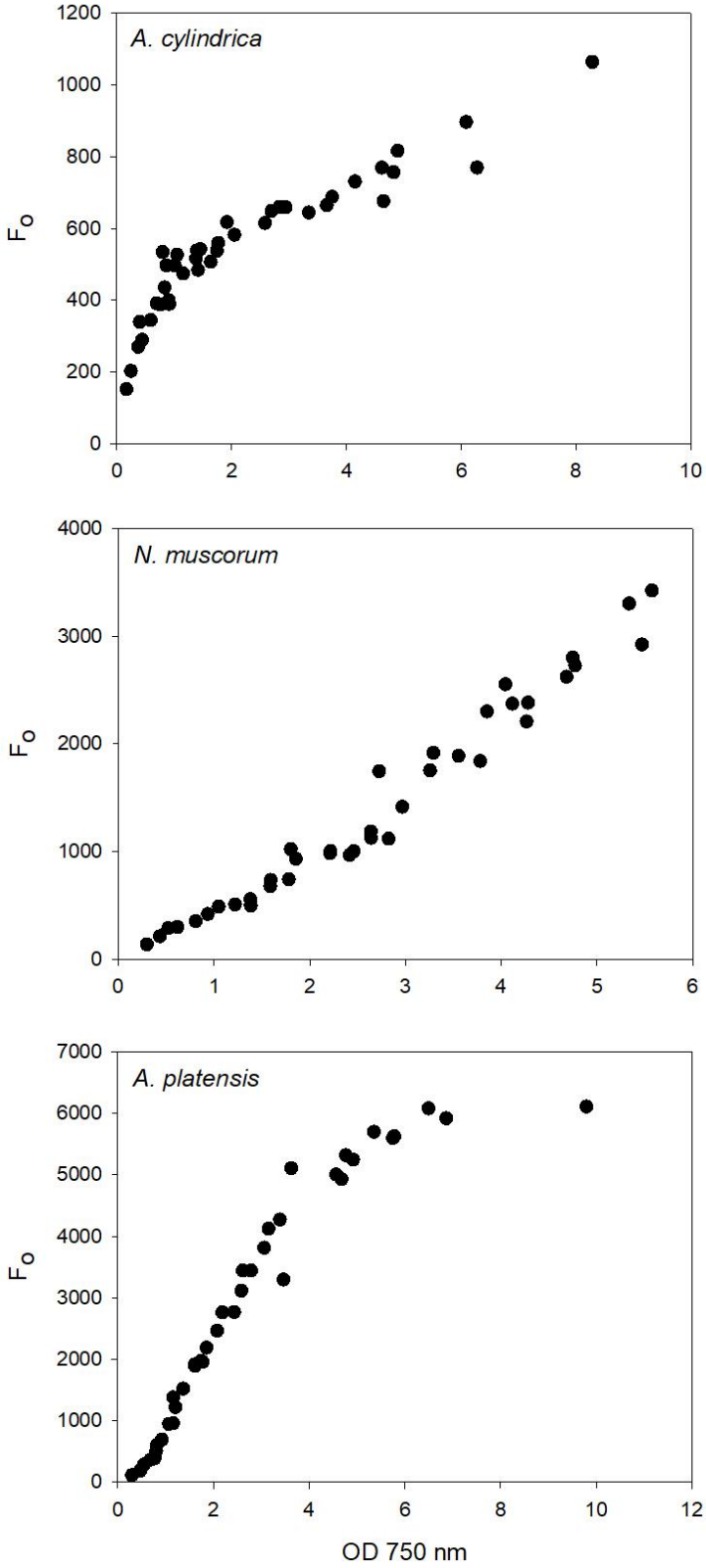


Figure III-S1. Relationship between optical density at 750 nm and F_o for *A. cylindrica, N. muscorum,* and *A. platensis.*

Annex II

Chapter IV: Blue is not enough: biological activities of C-phycocyanin extracts from *Anabaena cylindrica*

Table IV-S1. Percentage of scavenging activity of the tested extracts measured through the ABTS method. Ascorbic acid was used as a positive control.

		C-PC concentration $\mu g.mL^{-1}$	Scavenging activity (%) 10 min	Stdev	Scavenging activity (%) 30 min	Stdev	Scavenging activity (%) 60 min	Stdev
Purified	Fresh	500	4.39	2.84	3.69	2.64	0.00	5.19
	Lyophilize	500	3.12	0.16	3.02	0.62	3.28	0.90
	Fresh	2500	15.5	0.54	19.2	0.68	20.2	4.02
	Lyophilized	2500	12.2	0.94	15.6	1.09	17.4	1.08
Raw	Fresh	500	2.11	1.62	8.36	2.12	11.3	3.87
	Lyophilized	500	8.62	0.85	9.82	0.87	10.5	0.91
	Fresh	2500	13.1	2.33	28.9	1.68	44.9	5.09
	Lyophilized	2500	20.9	3.81	38.6	3.81	46.5	4.29
	Ascorbic acid	5	1.88	0.27	1.54	0.67	1.97	1.12
	Ascorbic acid	50	12.4	0.48	11.0	0.25	11.3	1.12
	Ascorbic acid	100	28.7	0.89	22.9	1.09	22.2	1.15
	Ascorbic acid	200	48.6	4.97	45.3	1.37	45.4	1.12
	Ascorbic acid	500	98.0	1.51	99.2	0.33	99.5	0.00

Table IV-S2: Results of the antimicrobial test with 11 pathogenic bacteria, against the purified and the raw extract. Two antibiotics were also tested: Chloramphenicol and Penicillin.

Pathogenic tested	Inhibition with purified extract	Inhibition with raw extract	Inhibition with antibiotic	Picture of the test
Micrococcus luteus ATCC 4698	No	No	Chloramphenicol	
E. coli ATCC 25922	No	No	Chloramphenicol	
Pseudomonas aeruginosa ATCC 27853	No	No	Penicillin	
Bacillus cereus ATCC 14579	No	No	Chloramphenicol	
Staphylococcus aureus ATCC 25923	No	No	Chloramphenicol	
Salmonella enterica ATCC 14028	No	No	Chloramphenicol	

Klebsiella pneumoniae ATCC 13883	No	No	Chloramphenicol	
Aeromonas sp.	No	No	Penicillin	
Proteus mirabilis ATCC 43071	No	No	Chloramphenicol	
Enterococcus faecalis ATCC 29212	No	No	Chloramphenicol	No image available
Bacillus sphaericus ATCC 12488	No	No	Chloramphenicol	No image available

Table IV-S3. ANOVA on ranks summary table for viability assays conducted for each control. Test (*H*) statistics and degrees of freedom are shown.

Type of control	**Cell line**	**Degrees of freedom**	**Test statistic (H)**	***p***
Ultrapure water	HaCaT	6	42.729	< 0.001
Ultrapure water	MNT-1	6	36.717	< 0.001
Sodium phosphate buffer	HaCaT	6	45.264	< 0.001
Sodium phosphate buffer	MNT-1	6	30.383	< 0.001

Table IV-S4. ANOVA summary table for viability assays conducted for each extract. Test (*F*) statistics and degrees of freedom are shown. MS stands for the mean squares (= variance) of the error term in the ANOVA.

Type of extract	**Cell line**	**Source of variation**	**Degrees of freedom**	**Adjusted MS**	**Test statistic (F)**	***p***
Raw extract fresh	HaCaT	Treatment	5	1599.864	46.723	< 0.001
		Error	12	34.241		
	MNT-1	Treatment	5	190.012	4.969	< 0.05
		Error	12	38.239		
Raw extract lyophilized	HaCaT	Treatment	5	611.470	6.004	< 0.05
		Error	12	101.838		
	MNT-1	Treatment	5	57.576	1.427	0.284
		Error	12	40.360		
Purified extract fresh	HaCaT	Treatment	5	283.746	37.827	< 0.001
		Error	12	7.501		
	MNT-1	Treatment	5	158.887	0.997	0.460
		Error	12	159.419		
Purified extract lyophilized	HaCaT	Treatment	5	500.951	61.278	< 0.001
		Error	12	8.175		
	MNT-1	Treatment	5	27.144	0.318	0.893
		Error	12	85.391		

Table IV-S5. Percentage of scavenging activity, measured through the ABTS method, of the C-PC extracts of four different species (*Anabaena cylindrica*, *Nostoc* sp., *Arthrospira platensis*, and a dried powder of *Arthrospira* from a commercial source). Water and sodium phosphate buffer (PBS) with sodium azide were used as solvents to produce the extracts, according to the procedure described in the methodology.

Species	Solvent	C-PC μg.mL^{-1}	Scavenging activity (%) 10 min	Stdev	Scavenging activity (%) 30 min	Stdev	Scavenging activity (%) 60 min	Stdev
Anabaena cylindrica	water	500	4.56	0.16	4.49	3.84	5.28	3.55
	PBS	500	4.89	0.47	8.77	1.98	10.21	2.23
	PBS	161	3.22	0.31	3.16	0.33	4.25	0.00
***Nostoc* sp.**	PBS	500	12.3	0.00	15.2	0.49	16.9	3.09
Arthrospira platensis	water	183	25.8	0.63	30.5	1.17	33.5	1.59
	PBS	500	26.1	0.31	31.3	0.16	34.5	2.58
	PBS	118	8.12	0.31	10.9	0.99	14.6	2.23
***Arthrospira* sp. commercial**	water	500	9.67	1.09	11.4	2.07	15.3	4.29
	water	500	5.78	1.26	13.7	8.51	6.53	2.84
	PBS	500	10.9	0.79	12.4	0.83	15.9	1.03
	PBS	500	7.45	0.31	8.30	0.99	10.6	2.75

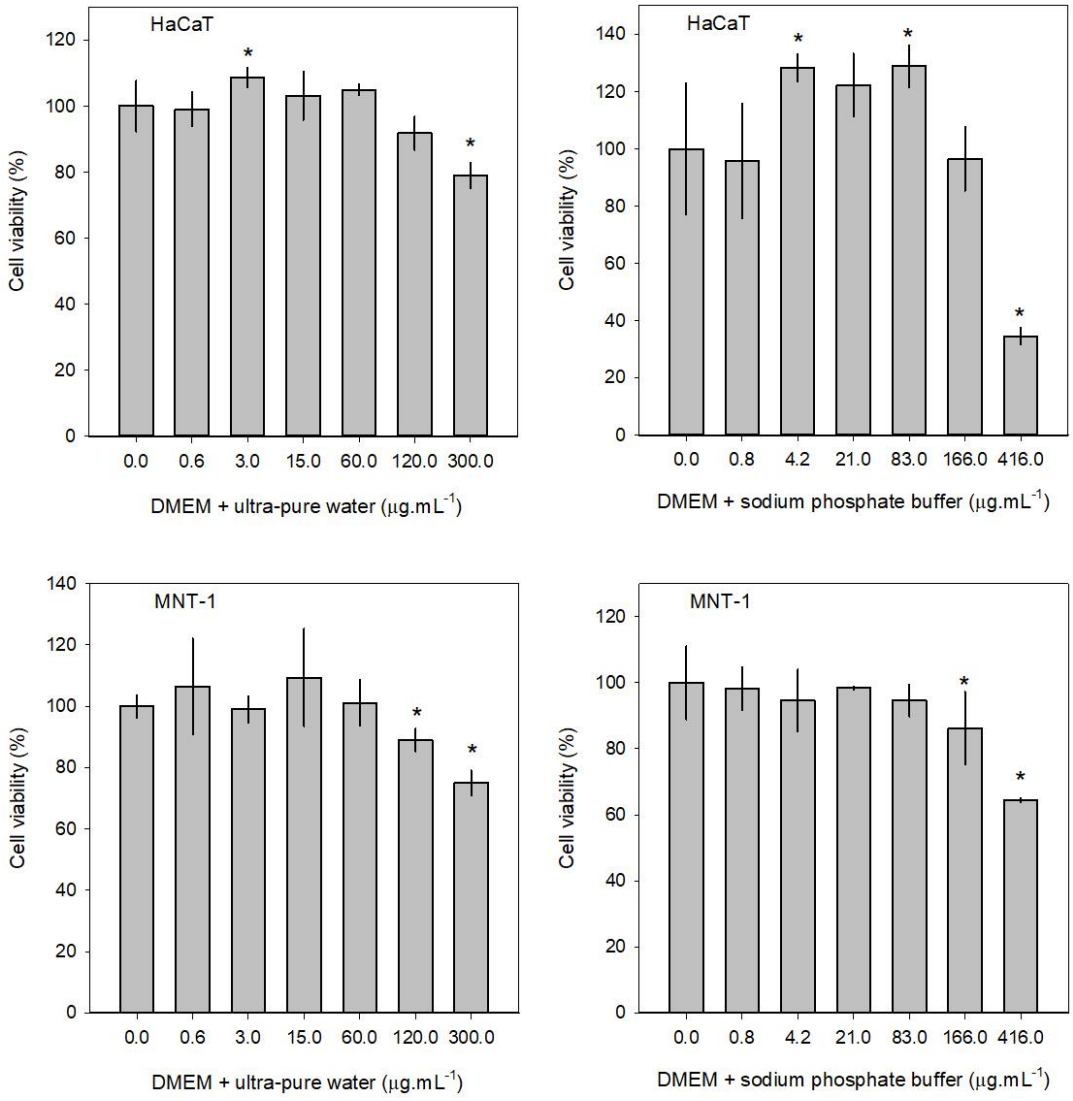


Figure IV-S1: Cell viability of HaCaT and MNT-1 cells with the respective controls, after 72 h of exposure. Ultrapure water was used for the controls with the purified fresh extracts, while sodium phosphate buffer was used for the controls with the raw fresh extracts. The results are expressed as the mean (bars) ± standard deviation (error bars) of nine replicates. The asterisks denote statistically significant differences between the control made with only DMEM, and between the other controls where ultrapure water/ sodium phosphate buffer where also added in the same concentrations as in the tested extracts (Dunnet test; p <0.05).

Annex III

Chapter V: Metabolic composition of the cyanobacterium *Nostoc muscorum* as a function of culture time: a ^{1}H NMR metabolomics study

Table V-S1. Metabolites detected in the ^{1}H-NMR spectra of aqueous (AQ) and organic (ORG) extracts from *N. muscorum*, and their respective chemical shifts (in ppm). The ^{1}H chemical shifts in bold identify the signal that was integrated for quantitative comparisons. BCAA: branched chain amino acids. Multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets.

Compound	δ ^{1}H (multiplicity) | δ ^{13}C	Extract
Acetate	1.92 (s) | 26.20	AQ
Adenosine	3.86, 4.30, 4.44, 4.75, 6.08 (d), 8.27 (s), **8.35** (s)	AQ
Alanine	**1.48** (d), 3.78 (q)	AQ
Aspartate	**2.68** (dd), 2.82 (dd), 3.89 (dd)	AQ
Aspartate - Peptide 2	2.91, **2.95**, 4.00	AQ
AXP	4.53, 6.15 (d), **8.28** (s), 8.61 (s)	AQ
BCAA - Peptide 3	**0.90** | 20.00, 0.94 | 21.60, 4.08 | 63.70 (bound valine) **0.89** | 23.60, 0.93 | 25.30, 1.59, 4.35 (bound leucine) **0.88** | 13.70, 0.92 | 18.20, 1.18, 1.84, 4.08 | 63.10 (bound isoleucine)	AQ
Betaine 1	**3.18** (s) | 47.40	AQ
Betaine 2	**3.19** (s) | 54.8, 3.94 (s) | 63.50	AQ
Betaine 3	**3.28** (s) | 55.2	AQ
Carotenoid 1	**6.16** (s), 6.33, 6.40, 6.71	ORG
Carotenoid 2	**6.67**, 6.97	ORG
Chlorophyll *a*	0.71 (d), 0.74 (d), 0.80 (d), 1.70 (d), 3.57 (s), 3.78 (s), 6.01 | 119.60, 6.20 | 119.60, 6.22 (s) | 64.60, 8.11 (dd), 8.46 (s) | 92.60, 9.25 (s) | 99.20, **9.61** (s) | 107.10	ORG
Fatty acyl (FA) chains	0.78-0.88 | 13.60-15.90, **1.16-1.35** | 21.80-33.50 (total FA), 1.44-1.54 | 24.20-28.10, **1.90-1.99** | 26.30 (MUFA), 1.99-2.06 | 26.30, 2.22-2.33 | 33.30, **2.71-2.80** | 24.90 (PUFA), 5.25-5.38 | 126.80-131.00	ORG

Glucosylglycerol	3.57, 3.75, **5.14** (d)	AQ
Glutamate	**2.07** (m) | 29.80, 2.15 (m) | 29.80, 2.35 (m) | 36.40, 3.76 (t) | 57.20	AQ
Glutamate - Peptide 1	2.01, 2.29, **4.30**	AQ
Glycolipids – galactosyl moieties	3.25, 3.59, 4.09, 4.63	ORG
Glycolipids – glucosyl moieties	3.22, 3.24, 3.53. 3.56, 4.81, 4.87	ORG
Glycolipids – glyceryl moieties	3.62, 3.81, 4.12, 4.32, **5.10**	ORG
3-Hydroxybutyrate	**1.20** (d), 2.32 (dd), 2.40 (dd), 4.17 (m)	AQ
Isoleucine	0.95 (t), **1.02** (d), 1.26, 1.98, 3.68 (d)	AQ
Linoleic acid	1.28, 2.01, **2.73**, 5.32	ORG
Linolenic acid	0.92 (t), 1.28, 2.02, **2.76**, 5.33	ORG
Malto-oligosaccharides	3.28, **3.44**, 3.58, 3.67 | 79.70, 3.78 | 63.20, 3.97 | 76.50, 4.66 (d) | 99.10, 5.24 (d), 5.41 (d) | 102.60	AQ
Mycosporin-like AA	**2.72** (s) | 35.50, 3.42 (s) | 61.80, 3.52 (s) | 62.20	AQ
Ornithine	1.73, 1.85, 1.94, **3.07**, 3.78	AQ
Phenylacetate	3.52 (s), **7.29** (m), 7.36 (m)	AQ
Sucrose	3.48 (t) | 72.10, 3.57 (dd) | 73.90, 3.68 (s) | 64.20, 3.77 (t) | 75.60, 3.83 (m) |63.10, 3.83 (m) | 65.20, 3.85 (m) | 75.20, 3.90 (m) | 84.30, 4.06 (t) | 77.00, **4.23** (d) | 79.20, 5.42 (d) | 95.10	AQ
Sucrose-derived hexasaccharide	3.57, 3.90, **5.57**	AQ
Sucrose-derived pentasaccharide	3.52, 3.86, **5.64** (d)	AQ
Sucrose-derived tetrasaccharide	3.61, 3.89, **5.68** (d)	AQ
Trehalose	3.46 (t) | 72.60, 3.65 (dd) | 74.10, 3.77 (dd) | 63.30, 3.79 | 75.40, 3.82 | 75.10, 3.86 (m) | 63.30, **5.20** (d) | 96.20	AQ
Uridine	3.81, 3.89, 4.22, 4.35, **5.92**, 7.88 (d)	AQ
UXP	4.05, 4.28, 4.38, **5.98**, 7.98	AQ
Valine	1.00 (d), **1.05** (d), 3.62 (d)	AQ

Table V-S2. ANOVA summary table for the metabolites with a VIP ≥ 1. Test (*F*) value and corresponding P-value, as well as corresponding degrees of freedom and mean squares of the error (MSresidual) are shown.

Source of variation	df	MSresidual	F	P
3-Hydroxybutyrate	6, 43	0.0284	24.692	< 0.001
Alanine	6, 34	0.000000148	14.172	< 0.001
Acetate	6, 34	0.100	23.233	< 0.001
Glutamate	6, 34	0.000000566	60.630	< 0.001
Aspartate	6, 34	0.100	27.592	< 0.001
Mycosporin-like AA	6, 34	0.0204	113.807	< 0.001
Ornithine	6, 34	0.0173	97.269	< 0.001
Betaine 1	6, 34	0.000000279	70.470	< 0.001
Betaine 2	6, 34	0.000000565	27.582	< 0.001
Betaine 3	6, 34	0.000000803	20.403	< 0.001
Malto-oligosaccharides	6, 34	0.000000190	28.941	< 0.001
Peptide 1 (Thr/Glu/Gl)	6, 34	0.000000410	42.589	< 0.001
Glucosylglycerol	6, 34	0.000000063	25.152	< 0.001
Sucrose- derived hexasaccharides	6, 34	0.000000387	29.974	< 0.001
Sucrose- derived pentasaccharides	6, 34	0.0000000134	81.423	< 0.001
Sucrose- derived tetrasaccharides	6, 34	0.000000137	27.912	< 0.001
AXP 8.27	6, 34	0.00000000912	155.952	< 0.001
PUFA	6, 34	0.00000269	15.660	< 0.001
MUFA	6, 34	0.000000169	139.232	< 0.001
Linolenic (ω-3)	6, 34	0.00000138	36.317	< 0.001
Peptide 2 (Aspartate)	6, 34	0.000034	5.585	< 0.001
Uridine	6, 34	0.0000000130	9.162	< 0.001
UXP	6, 34	4.226	10.914	< 0.001
Chlorophyll *a*	6, 34	0.0000000256	28.182	< 0.001

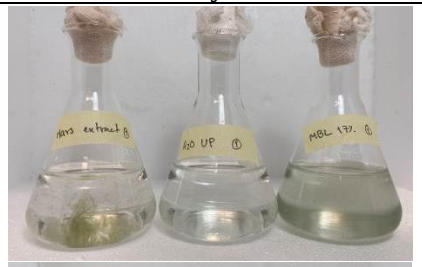
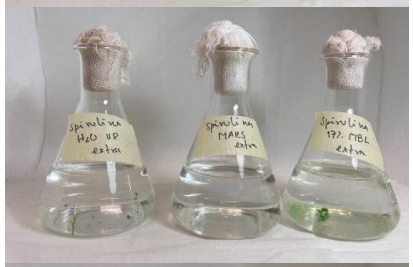
Annex IV

Chapter VI: Cyanobacteria as candidate to support Mars colonization: growth and biofertilization evaluation using Mars regolith

Table VI-S1. MGS-1 Mars Global Simulant bulk chemistry, according to the manufacturer (Exolith Lab).

Compound	Weight percent measured by TXRF
SiO_2	45.6
TiO_2	0.3
Al_2O_3	9.4
Cr_2O_3	0.1
FeO_T	16.9
MnO	0.1
MgO	16.5
CaO	4.0
Na_2O	3.7
K_2O	0.4
P_2O_5	0.4
SO_3	2.6

Table VI-S2: Pictures of the cyanobacteria/microalgae cultures growing during the experiment.

Species	Day 7	Day 14	Day 21	Day 25
Nostoc muscorum				
Anabaena cylindrica				
Arthrospira platensis				
Chlorella vulgaris