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MESTRADO

TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS

# Competitive exclusion of toxic cyanobacterial species by an allelopathic strain of *Phormidium*

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2020

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M.ICBAS 2020

SEDE ADMINISTRATIVA

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FACULDADE DE CIÊNCIAS

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# Competitive exclusion of toxic cyanobacterial species by an allelopathic strain of *Phormidium*

Dissertation for the master degree in Environmental Toxicology and Contamination submitted to the Abel Salazar Biomedical Sciences Institute from the Porto University

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## Acknowledgments

My appreciations go out to all the people who intervened directly or indirectly in carrying out this work, especially to my place of work and to carry out this dissertation.

To my supervisor, Dr. Aldo Barreiro for his full-time availability, for the continuous support at all stages and for the teaching transmitted during this work. For the internal course on the statistical R program, for the passed-on knowledge and for the patience with which he helped me in the doubts and errors that appeared during the work.

To the people of the LEGE team, in the person of Professor Doctor Vítor Vasconcelos, for the welcome, the availability of help, advice, shared moments and the good working environment provided. Also to my co-worker and under the guidance, José Quaresma from Universidad Pablo de Olavide in Sevilla, Spain.

To my lunch and conversation colleagues at CIIMAR, Daniela Ferreira, João Sousa, Murilo Mourão, Rita Biltes and Sara Gonçalves for their friendship and the relaxed and comical conversations we had and the good times we spent together at this stage of our lives.

To my friends, for accompanying me in good and bad times and to my master degree colleagues, especially João Silva and Luís Silva, who accompanied me since graduation and now finish another stage with me.

To my family, especially my girlfriend, who accompanied me throughout this Master's year closely and helped me in what I could and even in what I could not and always encouraged me to do my best.

To the NOVELMAR project - Novel marine products with biotechnological applications (NORTE-01-0145-FEDER-000035) for supporting this work, funded by FCT (Foundation for Science and Technology, Portugal).

## Abstract

Blooms of freshwater toxic cyanobacteria species are a growing environmental health problem, induced by anthropogenic eutrophication and climate change. The availability of nitrogen and/or phosphorus is one of the most important conditions that leads to blooming, as well as a variety of environmental conditions (temperature, light, agitation) that influence its development in fresh and marine waters. A variety of techniques have been tested for its remediation, from physical methods using artificial mixing or flocculation, to chemical methods using synthetic or natural compounds.

In the present work, we carried out a microcosm scale evaluation of the utility of the allelochemicals released by a filamentous cyanobacteria, *Phormidium* sp., for the bioremediation of the proliferation of four toxic freshwater cyanobacteria species (genera *Cylindrospermopsis*, *Chrysosporum*, *Anabaena* and *Nodularia*). Portoamides, small peptides, are the only allelochemical compounds detected in this *Phormidium* strain. Their effect was tested in bioassays using cell-free filtrate, resulting that all four strains were sensitive. In addition, we performed phosphate-limited long-term competition experiments in continuous cultures, in which *Phormidium* was co-cultured with each of the toxic cyanobacterial strains. The purpose of these later experiments was to employ higher population abundances and also to demonstrate that allelopathy was the real responsible for the ecological exclusion of toxic cyanobacterial strains. For this reason, we previously tested the competitive ability of each species for the limiting resource employed in these experiments (phosphate). Previous work showed that, if there is a trade-off between allelopathic activity and affinity for the limiting resource, the winner of the competition will be determined by the initial relative abundance of competing species. *Phormidium* sp. resulted to be better competitor for phosphate than three of the strains used, and worse than one of them. Hence, in this later case, we could demonstrate that the ecological exclusion of toxic cyanobacteria was indeed caused by allelopathy. An interspecific competition model including only phosphate competition and an allelopathic interaction was able to accurately describe the patterns of population dynamics observed in our experiments, supporting our conclusions regarding competition for phosphate and allelochemical interactions.

## Resumo

O desenvolvimento excessivo de espécies de cianobactérias tóxicas de água doce é um problema crescente de saúde ambiental, induzido pela eutrofização antropogénica e mudanças climáticas. A disponibilidade de azoto e/ou de fósforo são uma das mais importantes condições que leva ao seu crescimento em massa, assim como uma variedade de condições ambientais (temperatura, luz, turbulência) que influenciam o seu desenvolvimento em águas doces e marinhas. Uma diversidade de técnicas foi testada para sua remediação, desde métodos físicos usando mistura artificial ou floculação, até métodos químicos empregando compostos sintéticos ou naturais.

No presente trabalho, realizamos uma avaliação em escala de microcosmos da utilidade dos aleloquímicos produzidos por uma estirpe da cianobactéria filamentosa *Phormidium* sp., para a biorremediação da proliferação de quatro espécies de cianobactérias de água doce tóxicas (géneros *Cylindrospermopsis*, *Chrysosporum*, *Anabaena* e *Nodularia*). As portoamidas, pequenos peptídeos, são os únicos compostos aleloquímicos detetados nesta estirpe de *Phormidium*. O seu efeito foi testado em bioensaios usando o filtrado livre de células, resultando que todas as quatro estirpes eram sensíveis. Além disso, realizamos ensaios de competição de longo prazo limitados por fosfato em culturas contínuas, nas quais *Phormidium* foi co-cultivado com cada uma das cianobactérias tóxicas. O objetivo destes ensaios posteriores foi empregar maiores abundâncias populacionais e também demonstrar que a alelopatia foi a real responsável pela exclusão ecológica das espécies de cianobactérias tóxicas. Por esse motivo, testamos previamente a capacidade competitiva de cada espécie para o recurso limitante empregado nesses ensaios (fosfato). Trabalhos anteriores mostraram que, se houver um *trade-off* entre a atividade alelopática e a afinidade pelo recurso limitante, o vencedor da competição será determinado pela abundância relativa inicial das espécies concorrentes. *Phormidium* sp. resultou ser melhor competidor por fosfato do que uma das estirpes empregadas, e pior do que as outras. Portanto, nesse caso posterior, pudemos demonstrar que a exclusão ecológica das cianobactérias tóxicas foi de facto causada por alelopatia. Um modelo de competição interespecífica incluindo apenas competição por fosfato e uma interação alelopática foi capaz de descrever com precisão os padrões de dinâmica populacional observados nos nossos ensaios, apoiando as nossas conclusões relativas á competição por fosfato e efeito alelopático.

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## Abbreviations list

| | |
|---|---|
| ATP | Adenosine Triphosphate |
| BBE | Blue Biotechnology and Ecotoxicology |
| $CaCl_2 \cdot 2H_2O$ | Calcium chloride dihydrate |
| CE | Competition experiment |
| $CoCl_2 \cdot 6H_2O$ | Cobalt chloride hexahydrate |
| $CuSO_4 \cdot 5H_2O$ | Cooper (II) sulfate pentahydrate |
| DNA | Deoxyribonucleic acid |
| Fe-EDTA | Ferric ethylenediaminetetraacetic acid |
| $H_3BO_3$ | Boric acid |
| $H_{PO4}$ | Half-saturation constant for uptake |
| $K_2HPO_4$ | Dipotassium hydrogen phosphate |
| KBr | Potassium bromide |
| $KNO_3$ | Potassium nitrate |
| $K_{PO4}$ | Half-saturation constant for growth |
| LEGE-CC | Blue Biotechnology and Ecotoxicology Culture Collection |
| LPS | Lipopolysaccharides |
| mA | Allelopathic effect |
| $MgSO_4 \cdot 7H_2O$ | Magnesium sulfate heptahydrate |
| $MnCl_2 \cdot 4H_2O$ | Manganese (II) chloride tetrahydrate |
| $Na_2MoO_4 \cdot 2H_20$ | Sodium molybdate dihydrate |
| $NaHCO_3$ | Sodium bicarbonate |
| $V_2O_5$ | Vanadium pentoxide |
| $V_{max}$ | Maximum uptake rate |
| $ZnSO_4 \cdot 7H_2O$ | Zinc sulfate heptahydrate |
| $\mu_{max}$ | Maximum growth rate |

# 1. Introduction

## 1.1. Biology of Cyanobacteria

Cyanobacteria are a group of gram-negative aerobic prokaryotes, that were the first traces of life that were found on our planet due to the appearance of stromatolites and microfossils that indicate that these microorganisms existed about 3.500 million years ago (Summons et al., 1999) and were probably the first oxygen producers as photoautotrophs. Its vital processes require only water, carbon dioxide, inorganic substances and light, with photosynthesis being its main source of energy (Chorus & Bartram, 1999). Many of them are also nitrogen fixers. They possess diverse metabolic and physiological adaptations, allowing them to colonize most of the earth ecosystems, being especially relevant in certain aquatic environments. These organisms have a variety of morphologies, e.g. unicellular, colonial or filamentous (Dworkin et al., 2006; Whitton & Potts, 2007) and exhibit a variety of colors, including various shades of blue, green, yellow, red and violet (Mur et al., 1999; Six et al., 2007; Stomp et al., 2007). In the aquatic environment, they inhabit planktonic and benthic habitats, being an important component of biofilms and some filamentous species form large mats.

These microorganisms were initially termed as blue-green algae and they were divided into five major orders: Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales and Stigonematales. This topic has been much debated and new orders have emerged (Hoffmann et al., 2005; Komárek et al., 2014): Gloeobacterales is characteristic for being the only one that does not have thylakoids, making its photosynthesis directly in the cytoplasm. The order Synechococcales is a complicated order in terms of morphology that integrates species with different types of cells from coccoids to trichomes. With only one-member family, the order Spirulinales has parietal tilacoides and is composed of wavy filaments, without sheath. The orders Chroococcales are distinguished by the fact that the cells of the various constituent species are unicellular and divide by asymmetric binary fission. Species of the Oscillatoriales and Nostocales orders are filamentous species that may or may not have a sheath. Unlike species of the order Oscillatoriales, which are not differentiated, species of the order Nostocales capable of forming heterocysts and acinetes. The orders Pleurocapsales and Chroococcidiopsidales present an irregular cell division and/or in multiple planes, containing cells, colonies and polarized pseudo-filaments forming baeocytes.

Cyanobacteria are ubiquitous. An important feature of some cyanobacterial groups is their ability to produce chemical compounds that are toxic for vertebrates and humans. They

can be found in all kinds of aquatic habitats, but also dry surfaces like rocks and soil, with low exposure to light, extreme environments (ice, hot springs, deserts). However, the predominant habitats of cyanobacteria are in the aquatic environment. They play an important role in open ocean waters, mainly in the picoplankton fraction (< 2µm, e.g. genus *Synechococcus* and *Prochlorococcus*) but the filamentous genus *Trichodesmium* is also very important in certain areas of the ocean, it is a nitrogen fixer responsible for one half of the total nitrogen fixation in the ocean (Fu et al., 2005). Cyanobacterial mats are relevant in certain shallow coastal areas, they are conformed by multi-layered structure composed of several cyanobacterial species and other microorganisms, being the filamentous cyanobacteria those who create the macroscopic structure (Stal et al., 1985). In freshwater habitats, cyanobacteria are relevant in all habitats: planktonic (e.g. genus *Microcystis, Anabaena, Aphanizomenon, Nodularia, Gloeocapsa*) or benthic biofilms (e.g. *Phormidium*, *Oscillatoria*) or mats. Its abundance changes seasonally, like all phytoplankton groups, and it is affected by several factors such as light intensity, water temperature, nutrient availability, water column stability (turbulence) and abundance of zooplankton grazers.

The microorganisms used for this work include species of the genera *Phormidium, Anabaena, Chrysosporum, Cylindrospermopsis* and *Nodularia* isolated by the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) group*. Phormidium* sp. (strain LEGE 05292) is a non-toxic cyanobacteria, unlike *Anabaena* sp. (strain LEGE X-002), *Chrysosporum ovalisporum* (strain LEGE X-001), *Cylindrospermopsis raciborskii* (strain LEGE 97047) and *Nodularia* sp. (strain LEGE 06071) which are toxin-producing strains. *Phormidium sp*., belonging to the order *Oscillatoriales*, are benthic mat-forming cyanobacteria with a simple, undifferentiated and filamentous morphology, where the cells are practically isodiametric in curved trichomes and surrounded by a colorless sheath (McAllister et al., 2016; Ramos et al., 2018). *Anabaena* sp., *Chrysosporum ovalisporum, Cylindrospermopsis raciborskii* and *Nodularia* sp. they are species of the order *Nostocales* and their main ecophysiological processes are nitrogen fixation and cyanotoxin production.

Nitrogen fixation is an energetically expensive process, carried out on the nitrogenase enzyme complex, and which can be irreversibly inactivated in the presence of oxygen (Stal, 2017). In this way, some genera form specialized cells that separate nitrogen fixation from photosynthetic oxygen production, the heterocysts. These are cells with thicker walls that allow a sufficient influx of nitrogen for their fixation, do not produce oxygen during photosynthesis and maintain high respiration rates, and consequent oxygen consumption (Stal, 2009) and suppress the diffusion of oxygen in the cell (Walsby, 1985). Nitrogen fixing cyanobacteria have a competitive advantage over non-diazotrophic cyanobacteria in

nitrogen-limited waters, where they can develop dense blooms if there is an abundance of other nutrients, especially iron and phosphorus. Akinetes or gaseous vesicles (visible in Figure 1) are hollow protein structures filled with gas that provide buoyancy for various species of bloom-forming cyanobacteria, so that they can emerge in the water column. In stagnant waters with little wind mix, the accumulation of many cyanobacteria on the water surface can lead to the development of intense flowering of species that form large colonies or aggregates (Huisman et al., 2018; Whitton, 1992).

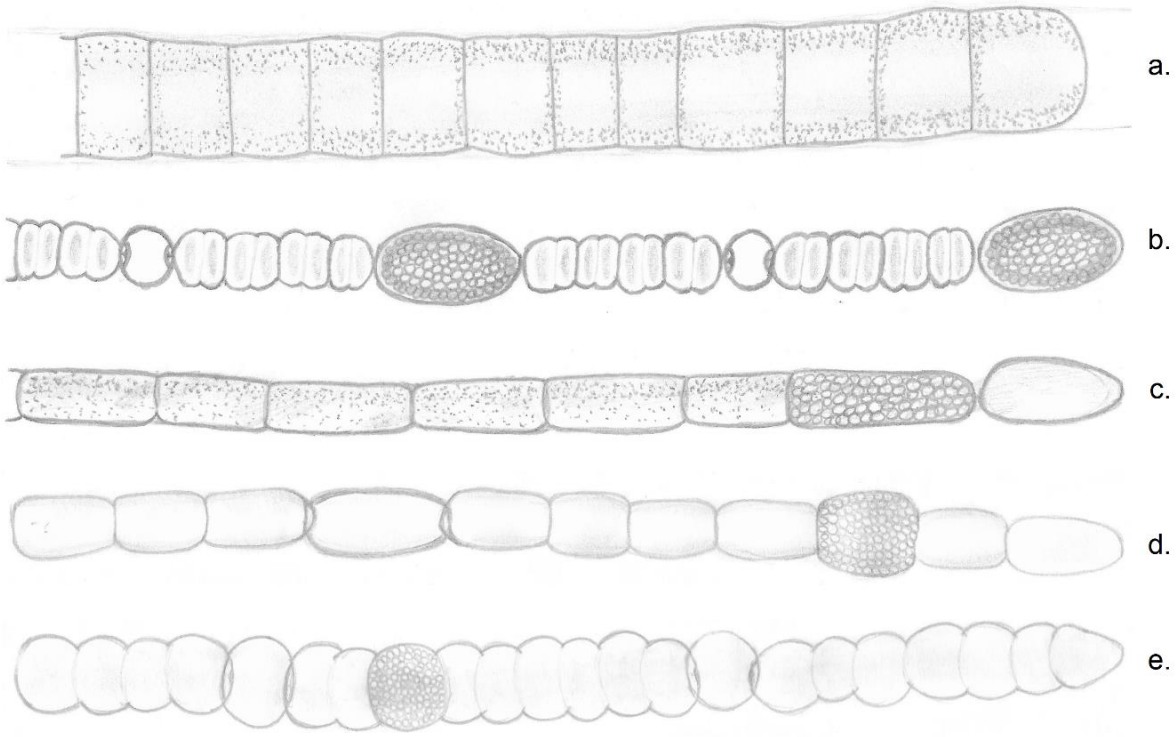



Figure 1. a. *Phormidium* sp.; b. *Chrysosporum ovalisporum*; c. *Cylindrospermospis raciborskii*; d. *Anabaena* sp.; e. *Nodularia* sp..

## 1.2. Cyanobacterial Blooms

Proliferations of phytoplankton populations to uncommon high abundances are usually termed as “blooms”. These blooms often produce a marked discoloration of water, sometimes with the appearance of foams, particularly when they are caused by cyanobacteria (Huisman et al., 2018; Mur et al., 1999). Blooms could be promoted by natural conditions (optimal levels of solar radiation, high temperature, elevated nutrient loads, low turbidity) or favored by anthropogenic disturbances (eutrophication, climate change). These blooms, when formed by toxin producing species, are a big problem of environmental health, affecting human health and economic activities (water consumption, aquaculture, fisheries, tourism, recreational uses of water). This is very often the case with

cyanobacteria (Kurmayer et al., 2004). Hypoxia or anoxia are secondary problems that can also be caused by blooms and could be responsible for increased mortality of fish and plants (Chorus & Bartram, 1999).

Genera associated with common blooms include *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Dolichospermum*, *Microcystis*, *Nodularia*, *Planktothrix* and *Trichodesmium*, of which many species produce toxins (Buratti et al., 2017; Hudnell, 2008; Huisman et al., 2018; Vasconcelos, 1999). Changes in the composition of bloom-forming strains can produce a variety of more concentrated toxic metabolites, harmful to plants, invertebrates and vertebrates, including humans, commonly known as cyanotoxins, and can cause liver, digestive and neurological diseases when ingested by organisms of upper trophic chain levels (Carmichael et al., 2001; Huisman et al., 2018; Jochimsen et al., 1998).

Under the natural seasonal cycles, in regions located at latitudes higher than the tropics, cyanobacteria usually dominate the summer phytoplankton in eutrophic and hypertrophic freshwaters, because of the high temperature and solar radiation as well as low water turbulence. As winter approaches, in most freshwater bodies the wind causes more turbulence, temperatures decrease, as does solar incidence. All this results in a decrease in the abundance of cyanobacteria. In the tropics, seasonal differences in environmental factors are generally not large enough to alter the abundance of cyanobacteria.

However, recently, the appearance of cyanobacterial blooms has increased world-wide due to two main factors of anthropic origin: eutrophication and global change. Eutrophication is a process by which water bodies receive excess nutrients, usually phosphorus and nitrogen, which naturally stimulate the proliferation of algae and cyanobacteria. Nutrients can originate from several natural and/or anthropogenic sources, such as domestic, rural and industrial effluents, agricultural fertilizers and pesticides and soil erosion (fires and consequent leaching). This process of eutrophication is one of the major drivers of blooms of cyanobacteria or algae. Phosphorus is the main nutrient that controls the occurrence of cyanobacterial blooms, although nitrogen compounds are sometimes relevant in determining the abundance of cyanobacteria. However, because some cyanobacteria can fix atmospheric nitrogen, they can avoid being limited by this element, favoring the domination of these species (Chorus & Bartram, 1999; Mur et al., 1999). Global change, mainly the rise in global average temperatures, and increased solar radiation, has also favored the development of cyanobacterial blooms (Paerl & Huisman, 2008).

Bloom-forming cyanobacteria produce a diverse variety of secondary metabolites, several of which are toxic to plants, invertebrates and vertebrates, including humans, in natural concentrations. *Anabaena* sp., *Chrysosporum ovalisporum*, *Cylindrospermopsis raciborskii* and *Nodularia* sp. used for this study are toxin producers, called cyanotoxins. Some of the toxins produced by these species are described in Table 1.

Table 1. Toxins produced by our toxic species (Chorus & Bartram, 1999; Codd et al., 1999; Huisman et al., 2018).

| Toxins produced | Toxic species | | | |
|---|---|---|---|---|
| | *Anabaena* sp. | *C. ovalisporum* | *C. raciborskii* | *Nodularia* sp. |
| Hepatotoxic: | | | | |
| Cylindrospermopsin | | X | X | |
| Microcystin | X | | | |
| Nodularin | | | | X |
| Neurotoxic: | | | | |
| Anatoxin-a | X | | | |
| Anatoxin-a(s) | X | | | |
| Saxitoxin | X | | X | |
| Irritant: | | | | |
| Lipopolysaccharides (LPS) | X | X | X | X |

Cyanotoxins, in addition to being a cause of death for some aquatic species, can be potentially dangerous to human health in drinking water reservoirs. For freshwater and/or saltwater cyanobacteria, there are at least 20 common bloom-forming genera that are known to produce cyanotoxins (Paerl & Otten, 2013). Microcystins and nodularins are cyclic peptides that inhibit phosphatases, which can cause severe damage to the liver and other tissues of birds, fish and mammals, including humans, and originate tumors. Cylinderspermopsins are guanidine alkaloids that inhibit protein synthesis and affect multiple organs and tissues in animals and plants, also causing damage to DNA and cell death. Other alkaloid cyanotoxins include anatoxin-a and anatoxin-a(s), which are agonists of nicotinic acetylcholine receptors at neuromuscular junctions, causing acute neurotoxicity in mammals and birds, the saxitoxins that have the mechanism of action to block the sodium channels preventing the transmission of action potentials and causing rapid paralysis and the lipopolysaccharides that are integral components of cells and that can cause skin irritations in human and animal tissues (Buratti et al., 2017; Carmichael, 2001; Falconer & Humpage, 1996; Funari & Testai, 2008; Merel et al., 2013).

## 1.3. Cyanobacterial bloom remediation

Since human and environmental health is an inherent concern of these environmental events, there are several methods and strategies tested to combat these cyanobacterial blooms, such as control of nutrients by dredging, sealing of sediments, use of flocculants or hypolimnetic reaction and methods of mechanical (e.g. surface water filtration and ultrasonication), chemical (e.g. nutrient scavengers and reactive oxygen forms) and biological (e.g. viral and bacterial agents and plant material and extracts) remediation.

Visser et al. (1996) used a mechanical method of compressed air bubbles to vertically mix water and prevent the growth of *Microcystis* and other bloom-forming cyanobacteria. Also in the Netherlands, Matthijs et al. (2012) sought the selective suppression of cyanobacteria with the addition of hydrogen peroxide without strongly affecting the other biota in a lake with dense flowering of the cyanobacterium *Planktothrix agardhii*, producer of microcystins. Recently, Yang et al. (2017) investigated the algicidal mechanism of prodigiosin, against *Microcystis aeruginosa*.

In addition to the limited effectiveness, the high application costs and the changes that can be caused in the ecosystems are some of the disadvantages of most of the strategies tested against these blooms (Cobo, 2015). In this way, an effective, economical, sustainable, and environmental-friendly solution for freshwater and marine ecosystems to control these environmentally damaging blooms seems increasingly important.

## 1.4. Allelochemical compounds

Microalgae and cyanobacteria produce a huge variety of chemical compounds with different useful bioactivities anticancer, antimicrobial, anti-inflammatory, protease inhibit (Berry et al., 2004; Demay et al., 2019; Kaya et al., 2002; Nagarajan et al., 2013; Sousa et al., 2020). Some cyanobacteria produce allelopathic compounds to compete with other cyanobacteria, algae and bacteria.

Small peptides such as nostoclamide (Jüttner et al., 2001) and spiroidesin (Kaya et al., 2002) showed an allelopathic effect on toxic strains belonging to the genera *Microcystis* and *Anabaena*, however some of these compounds are produced by toxic strains which may also cause toxic blooms. Leão et al. (2010) identified and characterized four small cyclic peptides (portoamides A-D) as secondary metabolites of the freshwater cyanobacteria *Phormidium* sp. strain LEGE 05292 (previous *Oscillatoria* sp.) with a selective effect on some green algae and cyanobacteria and cytotoxicity to lung cancer cells H460 when tested in a mixture of portoamides A and B. Thus, some studies have been developed on the

potential of using portoamides in industrial and biomedical applications (J. Antunes et al., 2019).

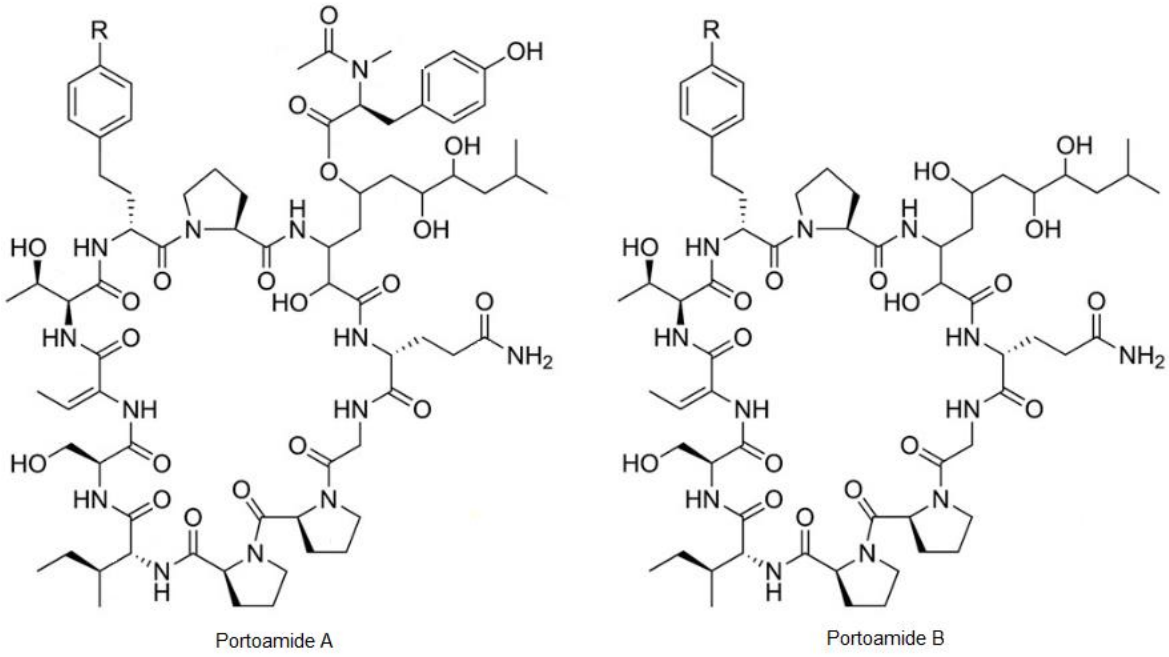



Figure 2. Molecular structure of portoamides A e B (adapted from Leão et al., 2010).

Portoamides A and B, generated in a 3:1 ratio, showed different toxicity effects on human carcinogenic and non-carcinogenic cell lines without causing oxidative stress in the cells. In addition, the mitochondrial reducing activity of cells treated with portoamide decreased, as did ATP levels, and affected the morphology of mitochondria (Ribeiro et al., 2017), with portoamides being recognized as mitochondrial toxins (Sousa et al., 2020).

Allelopathy is defined as the production of secondary metabolites by plants and microorganisms that affect positively or negatively the development of neighbor plants or microorganisms (Rice, 1984). Previous work has shown the allelopathic effect of portoamides on microbial planktonic organisms, such as microalgae and cyanobacteria (Barreiro & Vasconcelos, 2014; Barreiro Felpeto et al., 2017; Dias et al., 2017; Leão et al., 2012). In this work, we tested the potential of portoamides, as an environmentally friendly method to combat blooms of toxic cyanobacteria by its allelopathic effect.

## 1.5. Aims

The aims of this work are I) test with bioassays the sensitivity to the allelochemical effect of *Phormidium sp.* of four toxic cyanobacterial species; II) determine the competitive ability of all the species employed for inorganic phosphorus, and III) test in long-term competition

experiments with larger population abundances, using phosphorus as limiting resource, whether the allelopathic effect of *Phormidium sp.* is effective to outcompete the toxic species.

# 2. Materials and Methods

## 2.1. Cyanobacterial cultures

The species used in this work were: *Phormidium* sp. (strain LEGE 05292), formerly classified as *Oscillatoria* sp. (Barreiro & Vasconcelos, 2014; Barreiro Felpeto et al., 2017; Leão et al., 2009, 2010) *Chrysosporum ovalisporum* (strain LEGE X-001), *Anabaena* sp. (strain LEGE X-002), *Nodularia* sp. (strain LEGE 06071) and *Cylindrospermopsis raciborskii* (strain LEGE 97047), all from BBE laboratory culture collection. Cultures of these strains were maintained in flasks of 250 mL with a growth medium which composition is detailed in Table 2 (Barreiro & Vasconcelos, 2014). Medium was always prepared with ultrapure water (0.056 µS.cm at 23ºC) with a Lab Tower EDI 15 (Thermo Fisher Scientific®, Niederelbert, Germany) pro water purification system and sterilized in a Uniclave 88 (AJC Lda. Cacém, Portugal) autoclave system. Vitamins (filtered through 0.22 µm Millipore ®Express PES membrane filters) were added to the autoclaved medium after cooling, since some B vitamins are degraded at high temperatures, including thiamine-HCl and vitamin B12 (Bajaj & Singhal, 2020; Voelker et al., 2018). All tasks that required aseptic conditions were performed in a Bio II Advance (Telstar®, Terrassa, Spain) flow chamber.

Table 2. Detailed composition of the culture medium.

| Chemical compound | Final µM | Final mg $L^{-1}$ |
|---|---|---|
| Macronutrients | | |
| $KNO_3$ | 3200.000 | 32.352 |
| $K_2HPO_4$ | 200.000 | 3.480 |
| Micronutrients, iron, trace metals and others | | |
| $CaCl_2 \cdot 2H_2O$ | 250.000 | 36.760 |
| $MgSO_4 \cdot 7H_2O$ | 166.000 | 36.970 |
| Fe-EDTA | | 5.6 ($FeCl_3 \cdot 6H_2O$) + 7.41 (Na-EDTA) |
| $MnCl_2 \cdot 4H_2O$ | 2.100 | 0.5400 |
| $ZnSO_4 \cdot 7H_2O$ | 0.073 | 0.0287 |
| $CoCl_2 \cdot 6H_2O$ | 0.091 | 0.0300 |
| $Na_2MoO_4 \cdot 2H_20$ | 0.074 | 0.0230 |

| | | |
|---|---|---|
| $CuSO_4 \cdot 5H_2O$ | 0.038 | 0.0125 |
| KBr | 0.100 | 0.0120 |
| $V_2O_5$ | 0.007 | 0.0009 |
| $H_3BO_3$ | 74.397 | 4.560 |
| $NaHCO_3$ | 150.000 | 12.600 |
| Vitamins | | |
| Thiamine-HCl | 0.2965 | 0.10000 |
| d-Biotin | 0.0020 | 0.00050 |
| Cyanocobalamin ($B_{12}$) | 0.0004 | 0.00055 |

*Phormidium* sp. was employed as a source of the allelochemical compounds to be tested against the cyanobacterial strains. This strain of *Phormidium* sp. is known from producing allelochemicals called portoamides (Leão et al., 2010). The targeted strains of cyanobacteria were known to produce a series of toxins, as shown in Table 1.

A continuous flow chemostat consists in a closed circuit of culture medium in order to obtain stable physiological status of cultures. There is a constant input of nutrients through the renewal of culture medium, allowing the cells to reach an equilibrium, called “steady state”, between their growth rate and the rate of culture renewal (Huisman et al., 2002; Monod, 1978). For this purpose, following the chemostats principles and some previous work with chemostats (Barreiro Felpeto et al., 2017), 1000 mL Erlenmeyer flasks were used to set up the chemostat system, connecting it to a Minipuls 3 (Gilson®, Villiers Le Bel, France) peristaltic pump, and with constant air bubbling into the culture flask, 24 h of light, using cool-white fluorescent tubes (intensity of 60 µmol E $m^{-1}s^{-1}$), a dilution rate of 0.4 $d^{-1}$ and room temperature of 24ºC.

## 2.2. Allelopathy bioassays

The bioassays carried out to test the allelopathy of portoamides produced by *Phormidium* sp. were performed by testing the free-cell filtrate effect of *Phormidium* sp. on the four toxic strains.

In order to obtain cell-free filtrate for the assays, *Phormidium* sp. was grown in a chemostat maintained with a dilution rate that allowed permanent exponential growth. Portoamides are produced at higher rates during exponential growth (Barreiro et al. unpublished). *Phormidium* sp. is a biofilm-forming cyanobacteria and thus can form large aggregates of cell filaments. For this reason, in order to inoculate this strain in the

chemostat, it was necessary a previous homogenization of the inoculum, breaking filament aggregates with gentle sonication during almost 1 minute using a Vibra-cell™ (Sonics®, Sonics&Materials, Danbury, United States of America) sonicator.

The experimental set-up of the bioassays consisted in a treatment of different concentrations of the cell-free filtrate, in 8 mL glass vials containing 5 mL of cell suspensions of the target toxic strains. The concentrations ranged from 0 (control) to 60% of proportion of cell-free filtrate (Table 3). Five replicates were used for each concentration. The growth medium in the vials was the same as the on in Table 2, but with N:P concentrations of 640:40 µM). The initial preparation of the treatments was done in one pool per cell-free filtrate concentrations. So, each replicate was pipetted from the same pool, minimizing the initial variation among replicates.

Table 3. Cell abundances of the toxic species in the bioassays

| Toxic species | Initial abundance (cells $mL^{-1}$) | Tested concentrations (%) |
|---|---|---|
| *Anabaena* sp. | 63611,11 ± 1944,44 | 0 - 25 - 50 - 60 |
| *C. ovalisporum* | 256203,70 ± 8759,18 | 0 - 20 - 40 - 60 |
| *C. raciborskii* | 262847,22 ± 30436,87 | 0 - 20 - 40 - 60 |
| *Nodularia* sp. | 244351,85 ± 13056,54 | 0 - 25 - 50 - 60 |

Initial abundances of the target strains are shown in Table 3. When ready, the vials were transferred to a growth chamber with the conditions detailed above. Cell growth was estimated for all species after 48h, except for *C. raciborskii* which needed 120h due to its lower growth rate. Cell counts were performed on a Leica DM LB (Leica Microsystems, Wetzlar, Germany) microscope at 10x or 40x magnification, using a Neubauer chamber.

The growth rate was calculated as: $LN(n_2/n_1)/t_2\text{-}t_1$ being $n_i$ the abundances of the species in cells $mL^{-1}$ at t = i, and $t_i$ the time step in days. The allelopathic effect for each species was estimated by linear regressions between the dependent variable "growth rate" and the independent variable "% of cell-free culture filtrate" of *Phormidium* sp.. A significant negative slope of this regression indicates the existence of allelopathic effect. Pairwise Mann-Whitney tests were also performed. The *lm* and the *wilcox.test* functions available in the *stats* package of the R program (R Core Team, 2020) were employed to perform, respectively, the linear regression and the Mann-Whitney tests.

## 2.3. Growth assays

In order to estimate the growth parameters of these five species, they were performed experiments in batch cultures with phosphate as limiting nutrient. Growth conditions and medium composition were the same as above and in Table 1, but with a N:P ratio of 3200:20 µM (160:1). Cell abundances and phosphate concentration were monitored every 24 h. For the phosphate analysis, two replicates of 7 mL from the batches were filtered through 0.22 µm membrane filters (Millipore® Express PES) and were kept in the freezer at -20ºC until being analyzed in a nutrient autoanalyzer Skalar Sanplus using the method Skalar M461-318 (EPA 353.2). For the cell counts, all five strains of cyanobacteria were counted in the same way as described previously. The growth rate was calculated as indicated in the previous point. Monitoring was ended at 14 days, a few days after each species reached the stationary phase. It was assumed that population growth rate follows the Monod model, where $\mu_{max}$ is the maximum growth rate and $K_{PO_4}$ the half saturation constant.

$$\mu = \frac{\mu_{max}\, PO_4}{K_{PO_4} + PO_4} \quad (1)$$

The maximum growth rate of each species was estimated as the slope of a linear regression performed with cell densities (log-transformed) against time, during the period when the cultures showed faster growth and the estimates of the half saturation constant was obtained by fitting this non-linear Monod model to our real data using a non-linear least squares algorithm implemented in the *nls* function, from the stats package in R software (R Core Team, 2020).

## 2.4. Phosphate uptake assays

The kinetics of phosphate uptake were estimated for the 5 cyanobacteria of this study. First, a 50 mL flask of batch culture from each species was precultured during 6 days with the medium composition shown in Table 2, but with low phosphate concentration (1 µM). Then, the whole 50 mL cultures were transferred to a 250 mL culture flask, adding up to 250 mL of the same medium without phosphate. These cultures were kept under these conditions during 24 h. After this time, a pulse of approximately 5 µM of phosphate was added to these cultures and phosphate concentrations were monitored during 18h, in the time steps detailed in Table 4. Cell abundances were estimated only at time 0, assuming a neglectable growth during 18 h. During this 18h, light was permanently on. 3 replicates for phosphates at each time step. Samples were taken and analyzed following the same procedure as detailed above.

Table 4. Time steps for monitorization of phosphate uptake.

| Sample | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| Time (h) | 0 | 0.25 | 0.75 | 1.75 | 3.75 | 6.75 | 11 | 18 |

The uptake of phosphate as assumed to follow a Monod model, where $V_{max}$ is the maximum uptake rate and $H_{PO_4}$ the half-saturation constant:

$$V = \frac{V_{max}\, PO_4}{H_{PO_4} + PO_4} \quad (2)$$

For the estimate of the maximum uptake rate of each species, it was calculated the slope of a linear regression performed between external phosphate concentration and time, considering only the first hours of the experiment, when the uptake is faster. Half-saturation constant estimates were obtained by fitting our experimental data to the above equation using a nonlinear least-squares regression method, with the *nls* function coded in the *stats* package from R (R Core Team, 2020).

## 2.5. Long-term competition experiments in chemostats

In order to determine the effectiveness of *Phormidium* allelopathy as a mechanism for inter-specific competition, long-term experiments were set up in chemostats. In these experiments, *Phormidium* was competing against each of the toxic cyanobacteria strains. Here the population abundances of the toxic cyanobacteria reached higher abundances than in the bioassays. This contributes a stronger test of the potential of allelochemical compounds released by *Phormidium* for bloom remediation. In these experiments, allelopathy, in interplay with competition for the limiting resource will determine the winner of interspecific competition. In this case, the resource set to limit cyanobacterial growth was phosphate. In this kind of long-term experiments, it is common to limit growth by the main sources of macronutrients (nitrate or phosphate) or light. Estimating the competitive ability for light is logistically more complex, and among the macronutrients, we selected phosphate because all our strains have the ability to fix nitrogen.

A scheme of the chemostat system employed is shown in Figure 3. It was constructed with 1 L Erlenmeyer flasks, one of them containing the culture, other the fresh medium and other is the waste, were the excess of culture is leaking. The peristaltic pump pushes the fresh medium through the system, from the flask with fresh medium to the culture flask. Before reaching the culture flask, a 60 mL syringe is used as a “bacterial trap”, were the fresh medium is dropped and pushed by an air inlet (filtered with a 0.22 µm membrane filter) to the culture flask. This “bacterial trap” avoids, mainly, growth of non-photosynthetic

microbes (but also photosynthetic) that, starting from the tubes in the culture flask would go backwards to the fresh medium flask by growing attached to the tubes. The syringe, then, constitutes a break in this circuit. The fresh medium enters the culture flask pushed by the air, which also helps to homogenize the medium and increase the surface of exchange of gases (especially important to avoid $CO_2$ limitation) The culture flask also contains a small magnet used to prevent the growth of species on the flask wall, improving culture homogenization, being the cultures scraped at least three times a week. The third flask is the one that received the excess of culture, pushed by through an outlet in the culture flask "on top" of the culture volume.

The species are inoculated in the culture flask under aseptic conditions. The *Phormidium* strain was always sonicated (as detailed above) prior to the inoculation. For those toxic cyanobacteria that resulted to be better competitors for phosphate than *Phormidium*, two kind of experiments were performed: A) Higher initial relative abundance of *Phormidium* relative to the toxic strain and B) Higher initial relative abundance of the toxic strain relative to *Phormidium*. The rationale behind these experiments is that if the toxic strain is a better competitor for the limiting resource, in agreement with Ecological theory of competition for resources (Tilman, 1977) the best competitor will win competition irrespective of the relative initial abundances of both species. However, according to models and experiments involving allelopathy in interplay with resource competition (Barreiro Felpeto et al., 2017) the relative initial abundances would actually determine if the winner of competition is the best competitor for the resource, or the allelopathic species. For the experiments A, the allelopathic (*Phormidium* sp.) is expected to win competition and in the experiments B, the toxic strain, better competitor for phosphate, is expected to win competition.

Growth conditions were the same as detailed above, and culture medium composition was the same as in Table 2, but with a nitrate concentration of 3200 µM and phosphate of 20 µM, so a N:P ratio of 160:1, one order of magnitude above the theoretical optimal Redfield ratio (16:1). This ensures that only phosphate is limiting growth during the whole period of our long-term experiments. Cell abundances were estimated every day, and counts were made in Neubauer and Sedgewick-Rafter chambers if the population abundances were high or low, respectively.

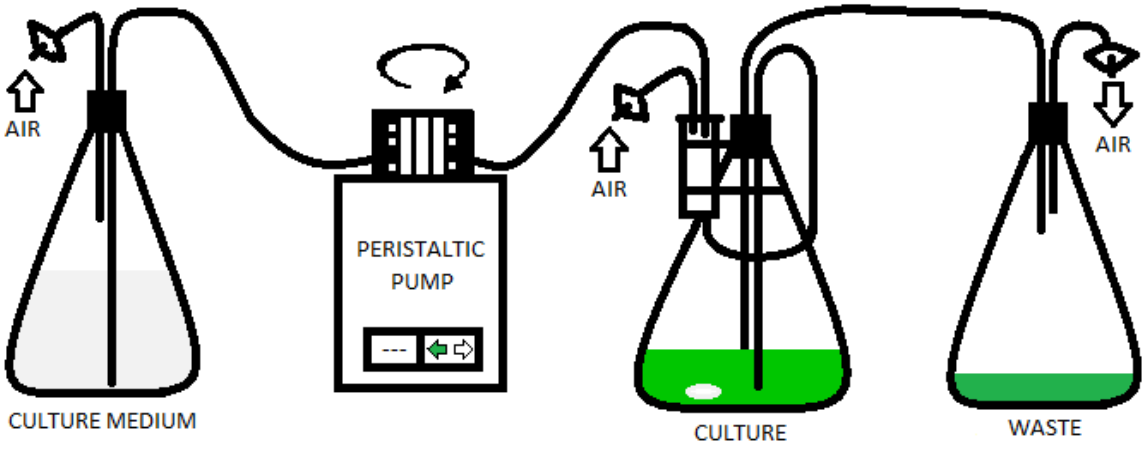



Figure 3. Continuous flow chemostat

## 2.6. Competition model

### *2.6.1. Model formulation*

In order to represent, predict and analyze the results of the long-term competition experiments describe above, we formulated, optimized and simulated a model constituted by coupled ordinary differential equations. This model represents the dynamics of the populations of the two competing species based in two mechanisms: competition for the limiting resource (phosphate) and the allelopathic effect of *Phormidium* sp. against the other species. The model formulation is as follows:

$$\frac{dP}{dt} = D(P_0 - P) - F_1(P)\frac{1}{\eta_1} - F_2(P)\frac{1}{\eta_2} \quad \text{where,} \quad F_i(P)(i = 1,2) = \frac{\mu_i S_i P}{K_i + P} \tag{3}$$

$$\frac{dS_1}{dt} = F_1(P) - \gamma S_2 S_1 - DS_1 \tag{4}$$

$$\frac{dS_2}{dt} = F_2(P) - DS_2 \tag{5}$$

where, equation 3: $P$ is the actual phosphate concentration in the culture, $D$ is the dilution rate of the chemostat system, $P_0$ is the inflowing phosphate concentration, $F_i(P)$ are the growth function for species, where i = 1 is one of the toxic cyanobacteria and 2 is a *Phormidium* sp., $\mu_i$ is the maximum growth rate of species $i$, and $K_i$ the half saturation constants for phosphate of species $i$. Parameters $\eta_i$ are the yield coefficients of species $i$, as a ratio of mass of cells to mass of nutrient consumed.

Equations 4 and 5 express the change in species abundance for toxic cyanobacteria and *Phormidium* sp., respectively. $S_i$ are the population abundances of toxic cyanobacteria (i =

1) and *Phormidium* sp. (*i* = 2). *γ* represents the allelopathic effect, that is, the rate of reduction in population growth of $S_1$ caused per cell of $S_2$.

*2.6.2. Model parameterization and optimization*

The initial parameter estimates for $\mu_i$, and $K_i$ come from the growth experiments. Other parameters were set experimentally ($D$ = 0.4 day$^{-1}$, $P_0$ = 20 µM). The rest of the parameters were unknown, and their initial values were set tentatively for estimation.

Parameter optimization was performed in two steps, first by applying a global optimization method, the simulated annealing, and then, with the estimates obtained from it, a local optimization method based in the Nelder-Mead algorithm (Nelder and Mead 1965). The simulated annealing was implemented with the *GenSA* function from *GenSA* R package. The Nelder-Mead algorithm was implemented with the *optim* function from the stats R package. In both global and local optimization, the objective functions were set according to a version of the Levenberg-Mardquart minimization criterion (Levenberg, 1944):

$$E = \sum_{t=1}^{t=n} [(\frac{|P_{1\,pred} - P_{1\,obs}|}{P_{1\,obs}} \times \frac{1}{CV_{P1}}) + (\frac{|P_{2\,pred} - P_{2\,obs}|}{P_{2\,obs}} \times \frac{1}{CV_{P2}})] \quad (6)$$

The parameter set providing the minimum value of $E$ is considered the best estimate. In this formula, $P_{ipred}$ (being $i$ = 1 each toxic cyanobacteria, 2 for *Phormidium* sp.) correspond to the abundances of each species predicted by the model for times $t = 1$ to $t = n$, and $P_{iobs}$, the same abundances from the real data. $CV_{Pi}$ is the coefficient of variation of the observed population abundances. For the values of the observed population abundances were employed smoothed with a non-parametric regression method (Ellner et al., 2002).

*2.6.3. Model simulation*

When a set of optimal parameter estimates was obtained for the experimental data of each pair toxic cyanobacteria-*Phormidium*, the model was extrapolated to test the outcome of inter-specific competition under a wider range of initial conditions (relative proportions of the competing species). The aim of this extrapolation is to check whether the predictions hold for a wider range of conditions, and it will be particularly interesting only if there is a trade-off between competition for the limiting resource and allelopathy, since, in this case, the winner of competition will depend on the initial relative proportions of the species, as explained above.

The model simulations were performed on a grid with all possible combinations of initial abundances of the two competing species from 1.000 to 3.000.000 cells $mL^{-1}$, in steps of 1000. This makes a total of 9.000.000 simulations. The simulations were initially run for 100-time steps (days), to eliminate initial transient phases, and, taking the values at day 100 as new initial values, they were run for additional 200 days. The density of each species at day 200 was then assumed to be the steady-state equilibrium abundance for the winner of competition when the abundance of the species excluded is 0.

# 3. Results and Discussion

## 3.1. Allelopathic effects on toxic strains by cell-free filtrate from *Phormidium* sp.

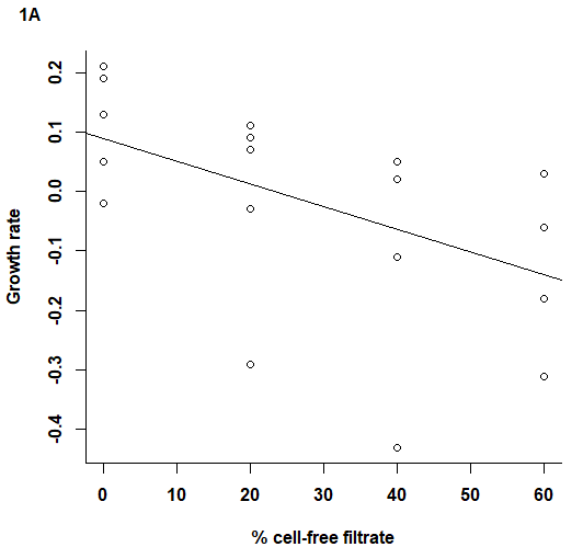
Cell-free filtrate from a culture of *Phormidium* sp. showed a significant allelopathic effect on each of our tested species in bioassays, which are summarized in Figure 4. We consider an allelopathic effect when there is a significant reduction in the growth of the target species. This negative effect is supported by the statistical significance of the slope of a linear regression between the concentration of the filtrate and the growth rate, shown in the legend of Figure 4. The slope of the regressions for the different species (Figure 4) showed similar values. The conditions of these assays were highly standardized between target species because: I) target species abundances employed in these assays yielded similar biomass levels (a bit lower only for *Anabaena*, Table 3); II) target species cultures were maintained in semi-continuous growth conditions, and III) the cell-free filtrate employed came from a *Phormidium* culture in constant exponential phase. Considering this, it is possible to compare the values of the slopes, and hence say that the strength of the negative allelopathic effect found was of the same magnitude across the target species.

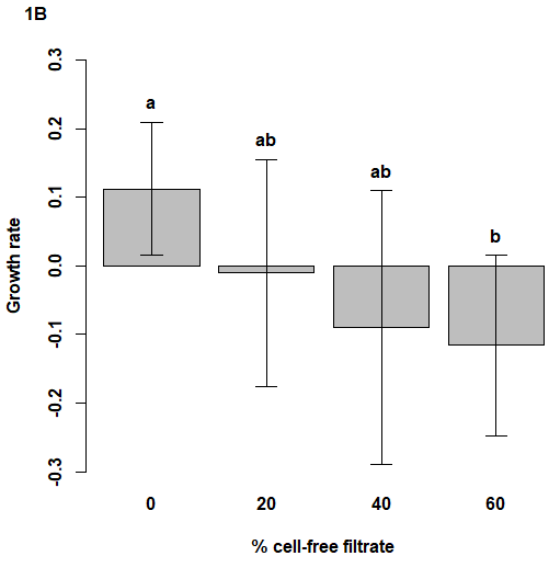
In this kind of experiments, however, the strength of the allelopathic effect detected is usually lower than the potential, because the source of allelochemical compounds is not renewed during the experiment, and the duration of the experiment was 48 h except for *C. raciborskii* (see methods).

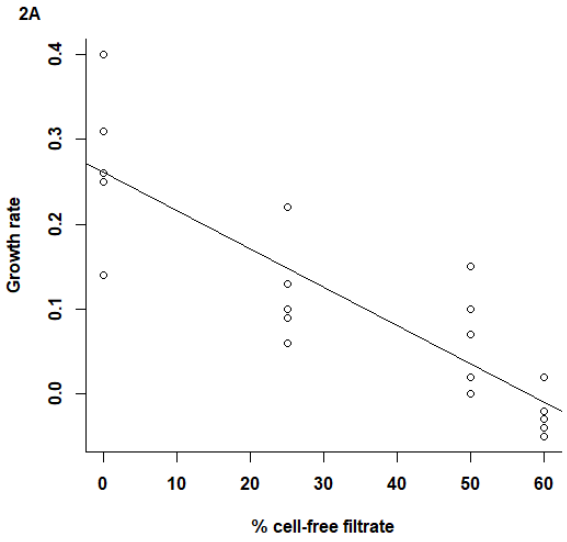
Besides the linear regression analysis, the non-parametric Mann-Whitney tests performed to compare all pairs of filtrate concentrations (Figure 4, column B) always showed significant differences between the control and the highest concentration of filtrate. These differences were the main cause of the significant slope of the regressions and hence the significant allelopathic effect. However, not always were found these differences between all other pairs of concentrations (Figure 4, column B).

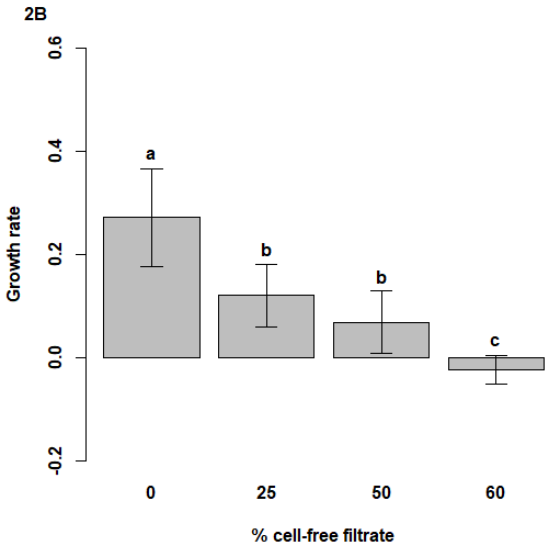
Specifically, differences were found in the following cases: between the control and 25%, in the case of *Anabaena* sp. and *Nodularia* sp. ($p$ = 0.016 and $p$ = 0.028) and between the

control and 40% in the case of *C. raciborskii* ($p$ = 0.011). However, for *C. ovalisporum*, this effect, despite being expressive, only shows significance with 60% of filtrate volume, since the standard deviation of growth rates is large compared to that of the other species.



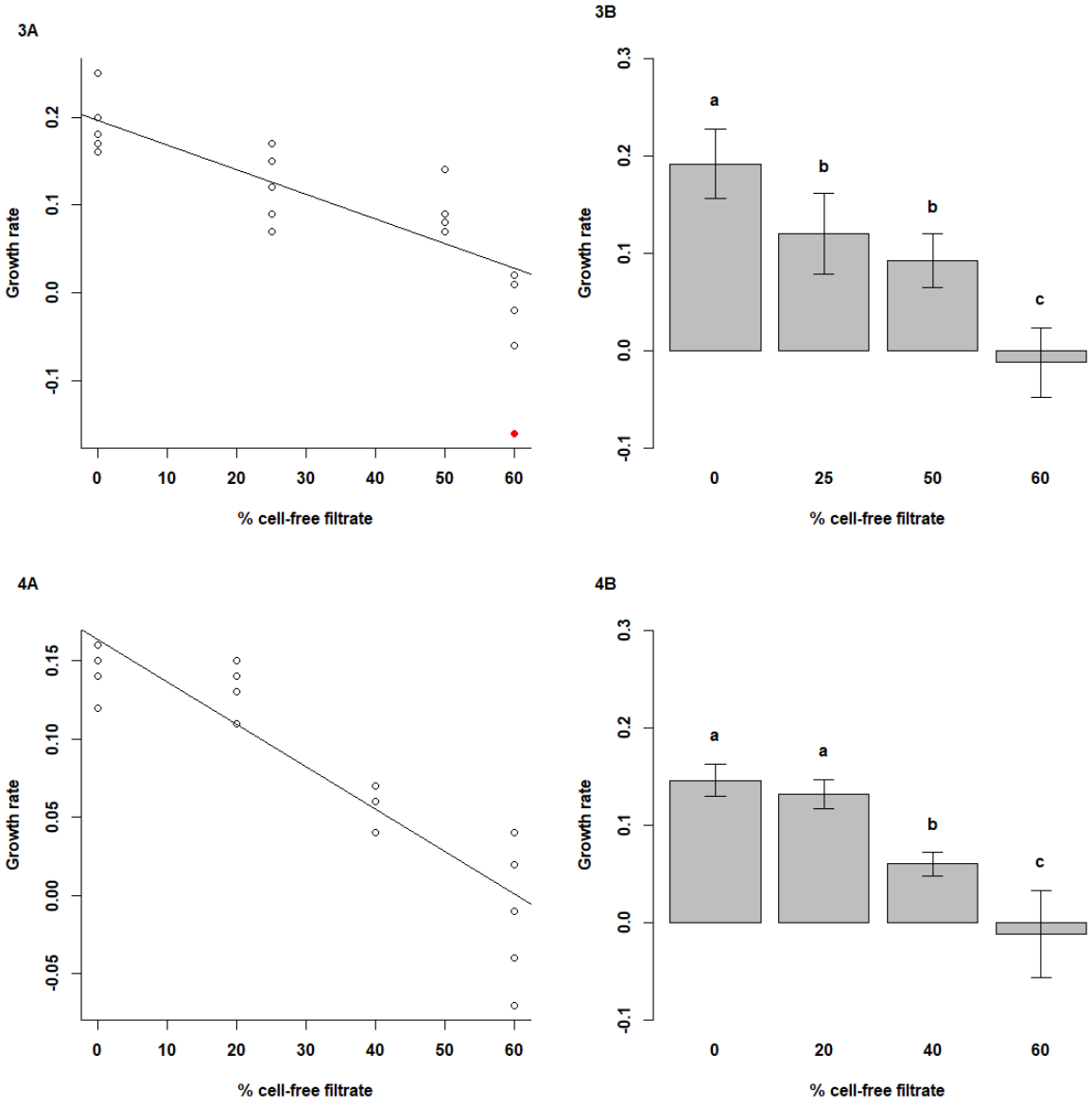



Figure 4. Allelopathic effect by cell-free filtrate on each of the target species. column A shows the linear regressions including all independent experimental replicates and column B the barplots of mean ± SD. *C. ovalisporum* (1) slope = -0.004, t-student = -2.603, $p = 0.018$); *Anabaena* sp. (2) slope = -0.004, t-student = -7.053, $p < 0.001$; *Nodularia* sp. (3) slope = -0.003, t-student = -5.819, $p < 0.001$) and *C. raciborskii* (4) slope = -0.003, t-student = -9.295, $p < 0.001$). The letters on top of each bar in the B column indicate significant differences according to Mann-Whitney tests.

The negative allelopathic effect of this strain of *Phormidium* was well know against specific microalgae, like *Chlorella* and *Ankistrodesmus* (Barreiro & Vasconcelos, 2014; Barreiro Felpeto et al., 2018; Leão et al., 2010) but not against cyanobacteria. It was already reported as having an effect in the general structure of phytoplanktonic communities, but without clarifying the target species and whether if the effects have positive or negative sign (Leão et al., 2012). On the other hand, there were species of microalgae reported to be

resistant, such as *Chlamydomonas reinhardtii* and *Selenastrum capricornutum* (Barreiro & Vasconcelos, 2014).

After exhaustive searches, portoamides A, B, C and D seem to be the only allelopathic compounds produced by this strain of *Phormidium* (Leão et al., 2010). The pure mixture of these molecules showed allelopathic effects in microplanktonic communities (Dias et al., 2017) and anti-bacterial properties (J. Antunes et al., 2019). We hence have strong reasons to assume that portoamides are the agents causing the negative allelopathic effect that we detected against our four toxic cyanobacterial species.

Among phytoplanktonic organisms, cyanobacteria are the group for which more examples of allelopathy are known. This could be due to the fact that cyanobacteria are more frequently screened for allelopathy, but also because it is a larger and more diverse group than all others. Examples of relevant genera of cyanobacteria in which allelopathy was detected are *Anabaena*, *Aphanizomenon*, *Microcystis*, *Cylindrospermopsis*, *Oscillatoria*, *Planktothrix*, *Lyngbya*, *Nostoc*, *Synechococcus* (Antunes et al., 2012; Becher et al., 2005; Brilisauer et al., 2019; Ma et al., 2015; Puschner, 2018; Suikkanen et al., 2004). The allelopathic compounds are not always known, but, besides the portoamides, there are some other known allelopathic and/or cytotoxic compounds first described in some of the previous relevant genera of cyanobacteria: 7-deoxysedoheptulose (*Synechococcus*, Brilisauer et al., 2019), lyngbyatoxin A (*Lyngbya*, Berman et al., 1999), nostocyclamide (*Nostoc*, Todorova et al., 1995), homoanatoxin-a (*Oscillatoria*, Puschner, 2018), microviridin (*Microcystis*, Puschner, 2018).

Our finding with these allelopathy bioassays is remarkably interesting because all the four species employed can cause toxic blooms in the natural environment, and hence the potential of portoamides in bioremediation of toxic blooms deserves to be further studied.

### 3.2. Uptake and growth of each species

The results of external phosphate concentration versus uptake during our experiments are shown in Figure 5. According to the estimated parameters (Table 5), the species with the most affinity for phosphate appears to be clearly *C. ovalisporum* and the one with the least affinity seems *C. raciborskii*. Among *Anabaena*, *Nodularia* and *Phormidium*, the differences are less clear. Affinity, according to this equation, not only depends on a single parameter, but on the proportion between maximum uptake rate ($V_{max}$) and the half-saturation constant ($H_{PO4}$) sp. The higher the maximum uptake rate, the more the affinity, and the same for the lower the half-saturation constant. Despite the relatively low number of data, two of the five estimated half-saturation constants were statistically significant

(*Phormidium* and *C. ovalisporum*) and all others showed values very close to statistical significance (Table 5).

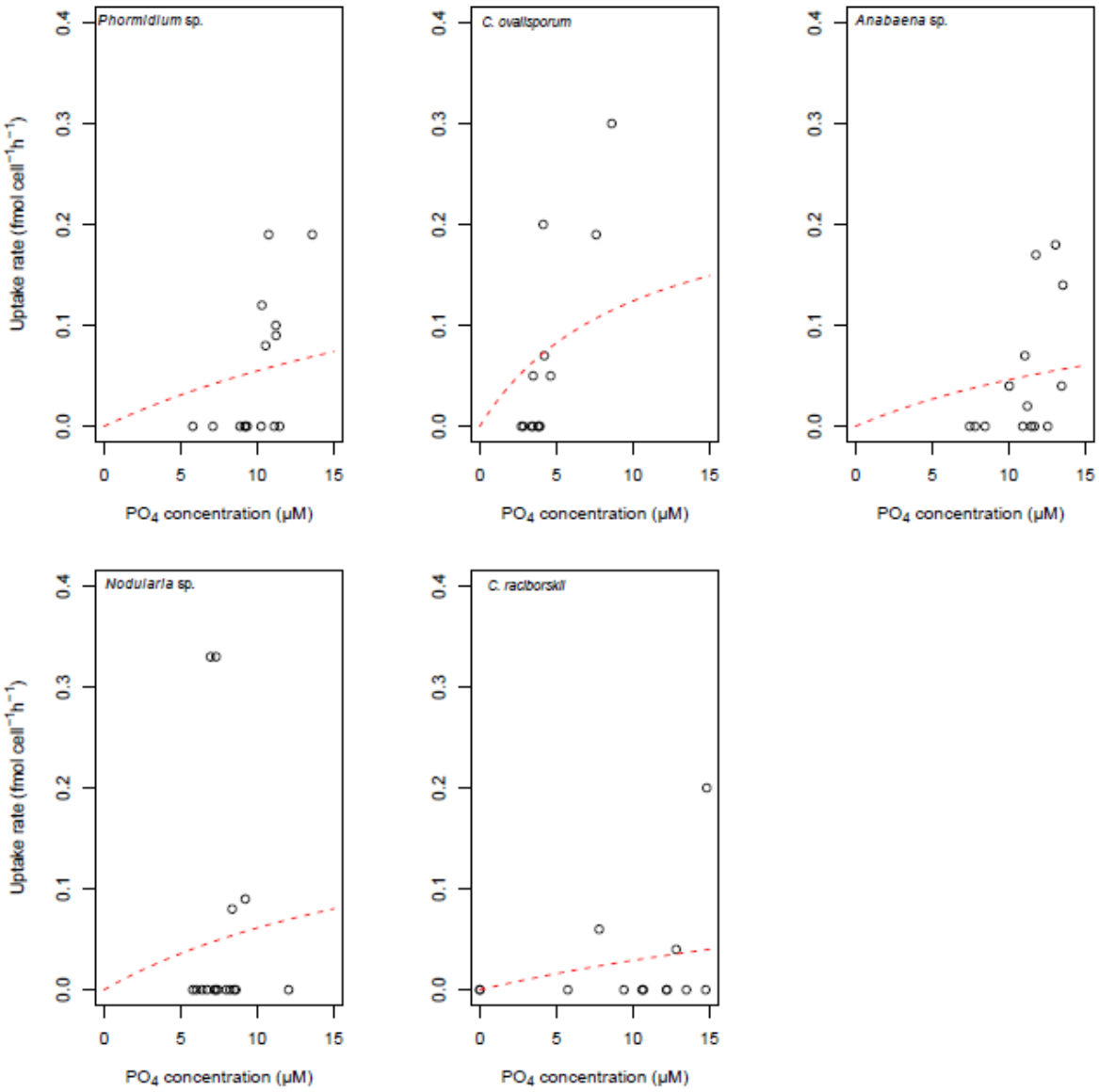



Figure 5. Phosphate concentrations in the external medium during the 18 hours consumption experiments versus the calculated uptake rates. The red hyphened lines represent the Monod model fits with the parameters from Table 5.

There are very few literature reports on parameters of phosphate uptake and growth for cyanobacteria and phytoplankton in general. The scarcity of these data leaded to the development of phylogenetic methods for the estimates of these parameters when needed (Bruggeman et al., 2009). Phosphate limitation is the macronutrient that more often limits primary production in freshwater environments (Elser et al., 2007). Considering this, and since our five species are ecologically very relevant, the data that we present here are a

relevant contribution for the literature in this aspect, that could be further employed by other authors in community studies (Wentzky et al., 2020).

Table 5. Best parameter estimates for the uptake Monod equation obtained from the uptake experiment, indicating the statistical significance for the estimated half-saturation constants (students-t value and associated *p* value).

| Species | $V_{max}$ (fmol cell$^{-1}$h$^{-1}$) | $H_{PO4}$ (µM) | t | *p* |
|---|---|---|---|---|
| *Anabaena* sp. | 0.16 | 24.67±12.44 | 1.98 | 0.07 |
| *C. ovalisporum* | 0.25 | 10.13±2.67 | 2.16 | 0.05 |
| *C. raciborskii* | 0.16 | 44.95±26.17 | 1.72 | 0.11 |
| *Nodularia* sp. | 0.21 | 24.16±17.80 | 1.36 | 0.19 |
| *Phormidium* sp. | 0.24 | 33.64±13.82 | 2.44 | 0.03 |

The results of the growth experiments in continuous cultures, using phosphate as a limiting resource, are shown in Figure 6, and the fitted parameters of the growth model (Eq. 1) in Table 6. The ratio of the maximum growth rate to the half-saturation constant could be sued as an approximate indicator of better competitors. According to this ratio, it is clear that *Nodularia* is the best competitor and Anabaena the worst. This situation can be clearly observed in Figure 6, with the highly steep growth curve fitted for *Nodularia*, reaching also saturation at relatively high level, and the opposite for Anabaena. Among the rest of the species, the values are very close and, in this situation, this ratio is not a reliable indicator.

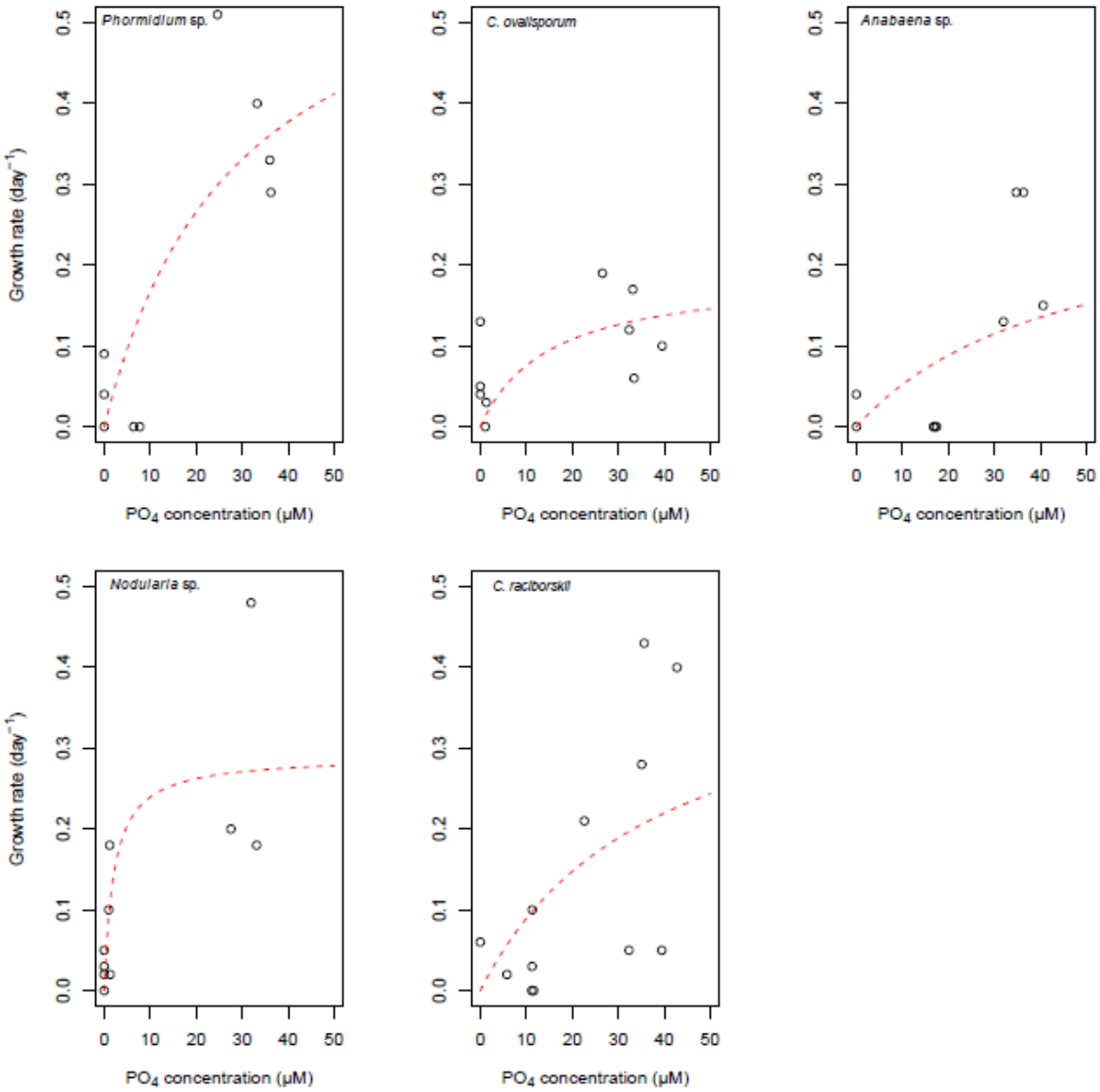



Figure 6. Phosphate concentration in the external medium versus daily growth rate from the growth experiments. The hyphened red lines are the respective Monod model fits employing parameter values from Table 6.

Same as for the uptake experiments, two out of the five half-saturation constants were found statistically significant (*Phormidium* and *C. raciborskii*). The rest of the values are also not far from being significant. In this kind of equations, estimating a significant value of half-saturation constant is difficult, because it is a very sensitive parameter to small errors in the nutrient concentration, since it is the parameter that defines the rise in the Y axis of the curve. It becomes particularly difficult when its value is low, since the test applied (students' *t*) what is determining specifically is its significant difference from 0. This is likely the reason why the p value is the highest for the lowest K value (*Nodularia*). In addition, estimating growth parameters with phosphate as limiting resource is particularly difficult, since this

macronutrient, when in excess, tends to be accumulated inside the cells as polyphosphate chains, and so its apparent uptake and external concentration are not always in equilibrium with the cell growth rates. To avoid this problem was developed time ago the quota growth model (Droop, 1968; Ducobu et al., 1998) that considers the internal concentration of the nutrient (quota). However, this model is mathematically more complex and not necessary in our context, in which we only need to predict a simple outcome of competition (winner or loser) in continuous cultures, where nutrient concentrations inside the cell are in equilibrium during steady-state, so during most of our experiments.

Table 6. Best parameter estimates for the growth Monod equation obtained from the growth experiment, indicating the statistical significance for the estimated half-saturation constants (students-t value and associated p value). The ratio $\mu_{max}/K$ is an approximate indicator of the better competitor (the higher the value, the better competitor).

| Species | $\mu_{max}$ ($day^{-1}$) | $KPO_4$ (µM) | t | p | $\mu_{max}/K_{PO4}$ |
|---|---|---|---|---|---|
| *Anabaena* sp. | 0.29 | 45.67±25.45 | 1.79 | 0.11 | 0.02 |
| *C. ovalisporum* | 0.19 | 15.13±9.97 | 1.52 | 0.16 | 0.01 |
| *C. raciborskii* | 0.43 | 38.30±15.46 | 2.48 | 0.03 | 0.006 |
| *Nodularia* sp. | 0.29 | 2.11±1.62 | 1.30 | 0.23 | 0.14 |
| *Phormidium* sp. | 0.65 | 28.93±8.96 | 3.23 | 0.01 | 0.01 |

As already mentioned for the uptake parameters, reports of growth parameters with phosphate in the scientific literature is scarce, although they are very important as ecological traits for studies of dynamics of plankton communities (Litchman & Klausmeier, 2008). Then, our data are also a relevant source of information for further studies in this field.

### 3.3. Long-term competition between *Phormidium* sp. and toxic species

The results of these experiments, as well as the corresponding competition model fits, are represented in Figure 7. The best parameter estimates are detailed in Table 7. The aim of these experiments was first, to show the effectiveness of the allelopathic effect detected in the previous bioassays in a higher culturing scale, both in terms of volume, time and abundance of the toxic target species. The other aim was to show, if possible, that high abundances of the target toxic species can virtually be excluded from the culture due to allelopathy and not competition for the limiting nutrient. This would be only possible if there is a trade-off between the allelopathic effect and competition for nutrients.

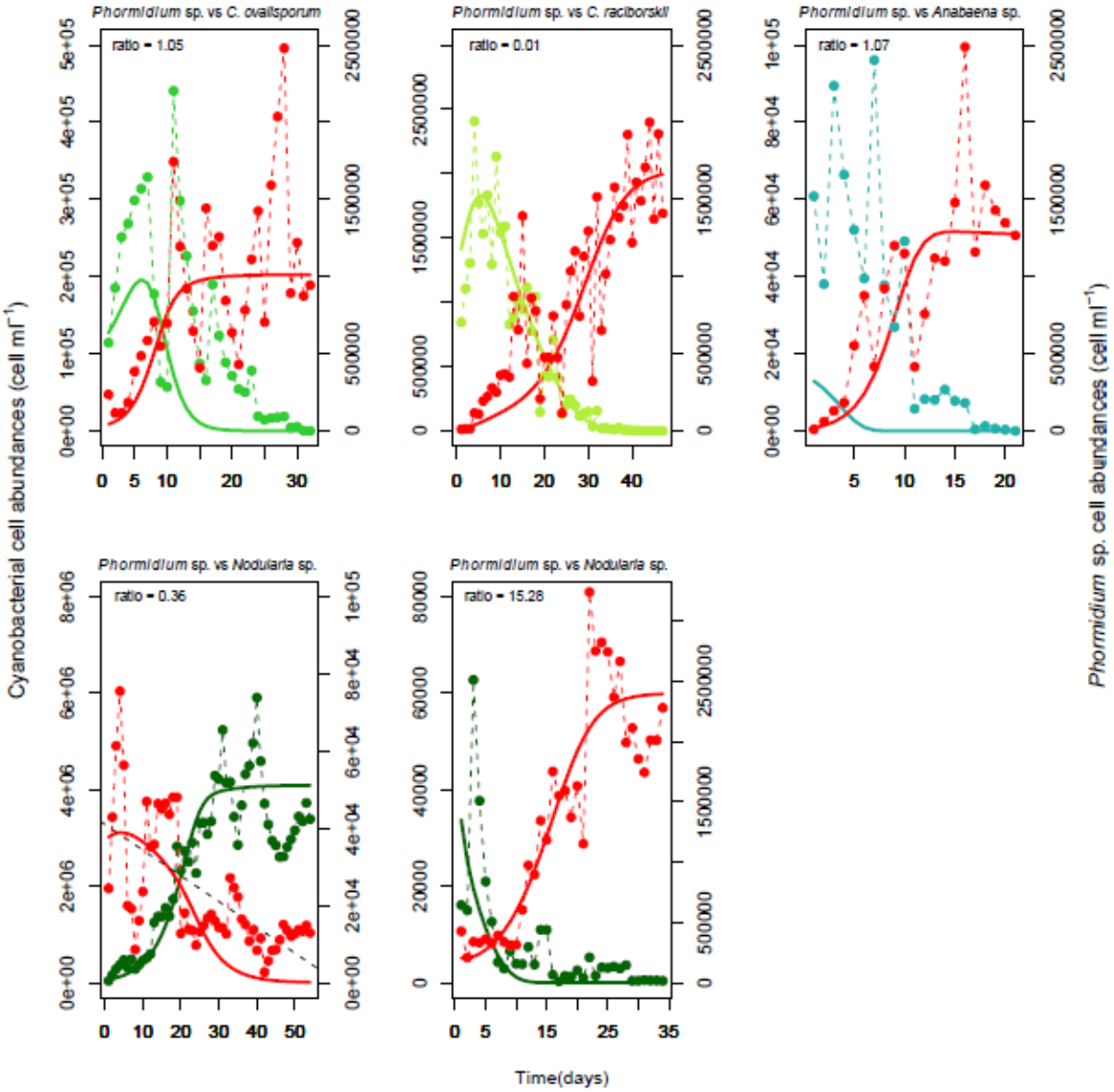



Figure 7. Outcomes of long-term competition experiments between *Phormidium* sp. (red) and toxic species (green). Dots with hyphened lines represent the real data from the experiments, as averages of four independent samples. Solid lines represent the model fits according to the parameter values shown in Table 7. Numbers in the top let corner indicate the ratio of initial *Phormidium*:toxic species as an average over the first three days. Panel 4 plots a linear regression fit for the abundance of *Phormidium* over time (hyphened line), which showed a significant negative slope (-667, $p > 0.001$).

According to the classical theory or resource competition in ecology (Tilman, 1977) the initial abundance of each competing species is a factor that has no influence in determining the best competitor for nutrients. The only important factors are the maximum growth rate ($\mu_{max}$) and the half-saturation constant for growth with the limiting resource (K). However, according to more recent works, this situation changes in the presence of an interaction like allelopathy in trade-off with competition for nutrients, in which the initial relative abundance

of the species determines whether the winner of competition is the allelopathic species or the best competitor (Barreiro Felpeto et al., 2018; Roy, 2009).

Regarding the results of each competition test in Figure 6, in CE4 there was exclusion of *Nodularia* from the chemostat by the action of the allelopathic effect (parameter *mA*) generated by *Phormidium*, since *Nodularia* was better competitor than *Phormidium*, but the initial relative abundances favors *Phormidium* by far. The same does not happen in CE1, CE2 and CE3 where Phormidium wins the competition, and, because *Anabaena* is clearly a worse competitor for phosphate than *Phormidium* (Table 6), it cannot be demonstrated that exclusion would happen only with the allelopathic effect. However, the exclusion caused only for lack of the limiting nutrient is often much slower than the one caused by allelopathy (50-70 days in comparable experiments, Barreiro Felpeto et al., 2018). In the cases of CE1-CE3, the exclusion is quite fast, suggesting that allelopathic effect was important. This seems particularly true for *C. ovalisporum and C. raciborskii*, because the growth parameter estimates showed a situation in which they were similar as competitors for phosphate than *Phormidium*. So, if exclusion was only caused by competition for the limiting resource, in these two cases, it would have taken a lot of time. With these two later species, we performed some experimental trials (not shown) in which it was aimed to show exclusion of *Phormidium,* and it did not happen. This could be explained because the competitive abilities comparing these pairs of species are so close that, even if *Phormidium* was a worse competitor, its allelopathy was enough to allow this species to maintain its population. Finally, in CE5, *Nodularia* wins competition against *Phormidium* because it is a better competitor and the initial relative abundance of *Phormidium* was quite low. However, in this case, *Phormidium* did not disappear completely from the culture, but the fact that the exclusion will simply take longer is shown by the significant regression in CE4 of Figure 7. exclusion So, for the case of *Nodularia*, it was the second experimental demonstration that allelopathy can reverse the expected outcome of competition for a limiting nutrient. The first one was in Barreiro Felpeto et al. (2018).

Table 7 shows the model parameters estimated for these experiments. The relationship ranking who is the best competitor does not change much, except for the case of *C. ovalisporum*, but the values are always very close, and as said before, this $\mu_{max}$/K ratio for evaluating who is the better competitor is not accurate when values are so close. In general, the growth of the species in these long-term experiments was more efficient than in the short growth experiments. This is why all the fitted maximum growth rates were larger than those in Table 6, and most of the $K_{PO4}$ values lower.

Table 7. Best fit parameters of the competition model for each of the long-term competition experiments (Ph = *Phormidium* sp.; Chr = *Chrysosporum ovalisporum*; Ana = *Anabaena* sp.; Nod = *Nodularia* sp.; Cyl = *Cylindrospermopsis raciborskii*). The winner of competition on each trial is shown in bold. Last column shows for each species pair their rank as competitor for phosphate according to the $\mu_{max}/K_{PO4}$ ratio calculated with these parameters.

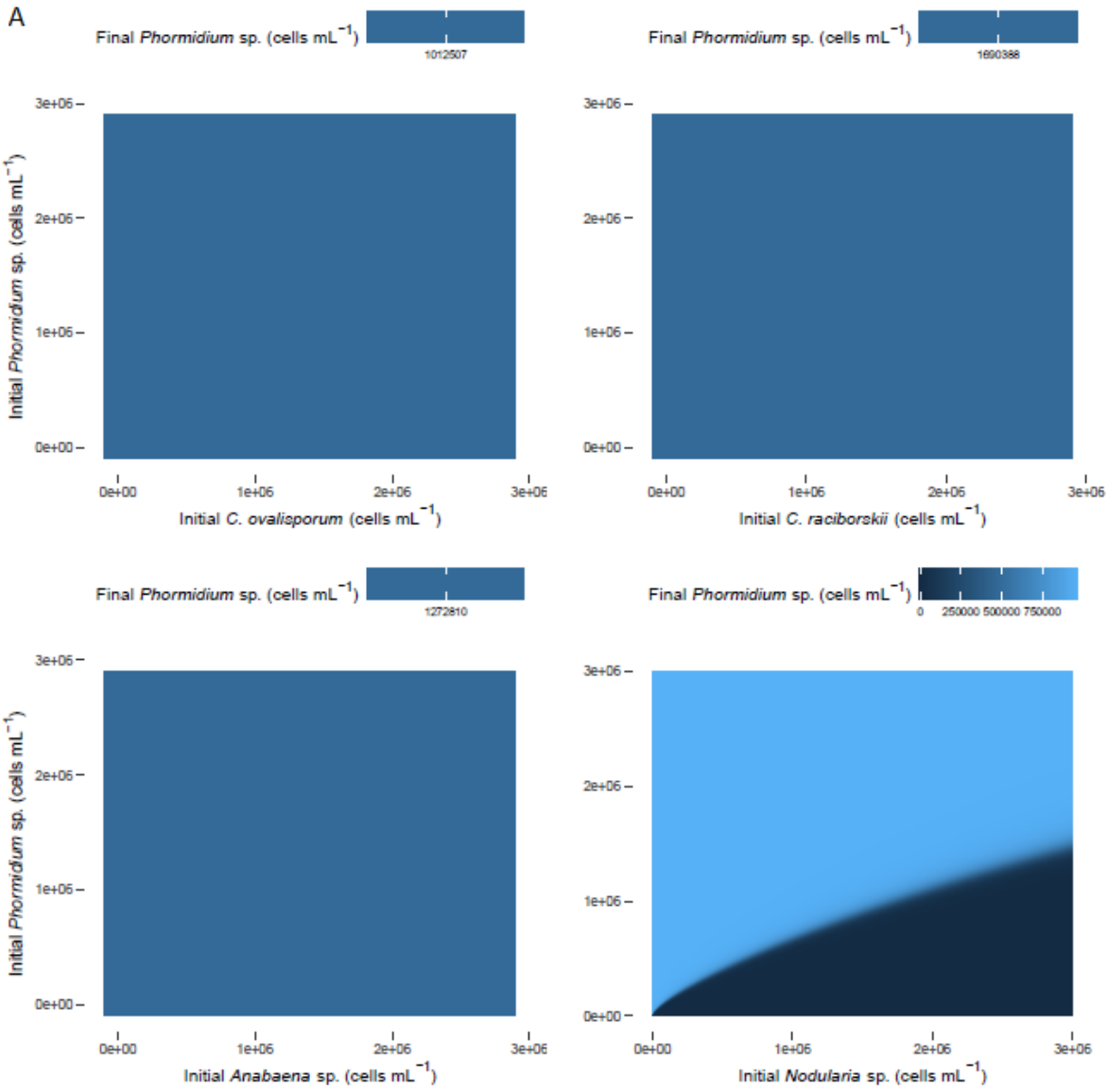
| **Competition experiment (CE)** | | | **Y** (cell µM$^{-1}$) | **$\mu_{max}$** (cell day$^{-1}$) | **$K_{PO4}$** (µM) | ***mA*** (cell day$^{-1}$) | **Rank as competitors** |
|---|---|---|---|---|---|---|---|
| **CE1** | ***Phormidium* sp.** vs *C. ovalisporum* | Ph | 63262 | 1.35 | 14.04 | 5.2e$^{-07}$ | 0.12 > 0.10 |
| | | Chr | 267319 | 0.62 | 5.02 | | Chr > Ph |
| **CE2** | ***Phormidium* sp.** vs *C. raciborskii* | Ph | 159824 | 1.09 | 24.92 | 2.4e$^{-07}$ | 0.04 > 0.03 |
| | | Cyl | 276102 | 0.56 | 18.44 | | Ph > Cyl |
| **CE3** | ***Phormidium* sp.** vs *Anabaena* sp. | Ph | 79731 | 1.14 | 11.27 | 5e$^{-06}$ | 0.10 > 0.02 |
| | | Ana | 336947 | 0.51 | 29.13 | | Ph > Ana |
| **CE4** | ***Phormidium* sp.** vs *Nodularia* sp. | Ph | 184438 | 0.78 | 11.24 | 1.2e$^{-06}$ | 0.08 > 0.07 |
| | | Nod | 135478 | 0.46 | 5.88 | | Nod > Ph |
| **CE5** | *Phormidium* sp. vs ***Nodularia* sp.** | Ph | 208132 | 0.39 | 7.73 | 4.9e$^{-07}$ | 0.16 > 0.05 |
| | | Nod | 251366 | 0.60 | 3.74 | | Nod > Ph |

### 3.4. Simulation of the competition model

In order to check if the results of our previous experimental trials are coherent in a wider scenario, in which we take a grid of combinations of initial relative abundances of the species, we performed 9 million simulations (see Methods) for each of the pairs *Phormidium*-toxic species. The parameters employed were the same as shown in Table 7, except for the simulation with *Nodularia*, in which a single set of parameters was needed to obtain the two outcomes. Then, we employed the parameter set fitted for CE5 with very few changes: reducing the $\mu_{max}$ of *Phormidium* to 0.53 and the allelopathic effect to 0.9 e$^{-7}$.

The results are shown in Figure 8 A and B. As we can see, when the best competitor is also the allelopathic species (*Phormidium*), the winner of competition is always *Phormidium*, and its equilibrium abundance is similar to those shown in Figure 6. But, when competing with *Nodularia*, there is a region of the grid of relative abundances when *Phormidium* wins and another one when *Nodularia* wins. These regions, however, are a bit displaced relative to the diagonal, being larger the region where *Phormidium* wins. This means that the effect of allelopathy by *Phormidium* is stronger than the competitive advantage conferred to

*Nodularia* as a better competitor for phosphate. Another explanation could be that the parameter set chosen for this simulation was mainly that of CE5, in which *Phormidium* wins. However, this explanation should be discarded since, as said above, we reduced the competitive advantage of *Phormidium* by reducing µmax and *mA* as much as one order of magnitude.



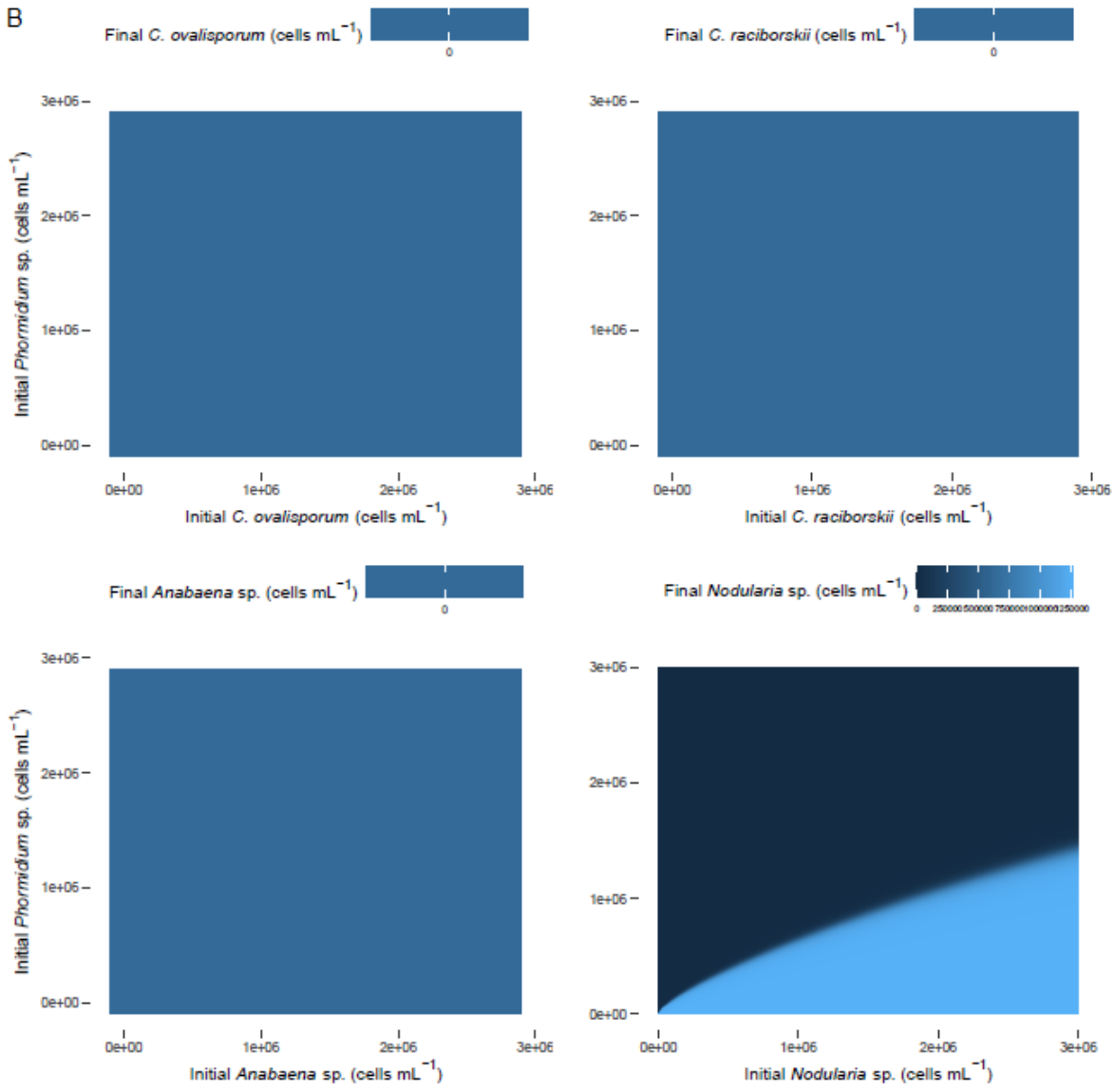



Figure 8. A - Results of the simulations of the competition model showing the equilibrium abundances of *Phormidium* sp. B - Results of the simulations of the competition model showing the equilibrium abundances of the four toxic cyanobacteria species.

### 3.5. Future perspectives

Considering the results obtained, it is necessary to continue with the study of the viability of *Phormidium* sp. and portoamides as a method of bioremediation of toxic cyanobacteria blooms, using both the purified molecules (portoamides) and the producing agent (*Phormidium*). Likewise, the microcosm experiment scale, which was developed in this work, should be increased to a mesocosm scale, which can be: I) laboratory containers of hundreds or thousands of liters that simulate real freshwater communities with blooms of toxic cyanobacteria; or II) plastic bags or containers located in situ (ponds, lakes, ponds, dams) isolating a part of the real community.

## 4. Conclusions

I) *Phormidium* allelopathy is effective against the four toxic species of cyanobacteria tested, although at high population abundances in long-term experiments, it was suggested, but not demonstrated for all the species.

II) The use of *Phormidium* sp. allelochemicals as a bioremediation method for toxic cyanobacterial blooms should be further studied at higher spatial and community scales.

III) The effectiveness of allelopathy as an ecological mechanism reversing the outcome of inter-specific competition for a limiting resource was demonstrated for the competition between *Nodularia* sp. and *Phormidium* sp.

# 5. References

Antunes, J., Pereira, S., Ribeiro, T., Plowman, J. E., Thomas, A., Clerens, S., Campos, A., Vasconcelos, V., & Almeida, J. R. (2019). A multi-bioassay integrated approach to assess the antifouling potential of the cyanobacterial metabolites portoamides. *Marine Drugs*, *17*(2), 1–19. https://doi.org/10.3390/md17020111

Antunes, J. T., Leão, P. N., & Vasconcelos, V. M. (2012). Influence of Biotic and Abiotic Factors on the Allelopathic Activity of the Cyanobacterium Cylindrospermopsis raciborskii Strain LEGE 99043. *Microbial Ecology*, *64*(3), 584–592. https://doi.org/10.1007/s00248-012-0061-7

Bajaj, S. R., & Singhal, R. S. (2020). Degradation kinetics of vitamin B12 in model systems of different pH and extrapolation to carrot and lime juices. *Journal of Food Engineering*, *272*, 109800. https://doi.org/10.1016/j.jfoodeng.2019.109800

Barreiro, A., & Vasconcelos, V. M. (2014). Interactions between allelopathic properties and growth kynetics in four freshwater phytoplankton species studied by model simulations. *Aquatic Ecology*, *48*(2), 191–205. https://doi.org/10.1007/s10452-014-9475-2

Barreiro Felpeto, A., Roy, S., & Vasconcelos, V. M. (2017). Allelopathy prevents competitive exclusion and promotes phytoplankton biodiversity. *Oikos*, *127*(1), 85–98. https://doi.org/10.1111/oik.04046

Barreiro Felpeto, A., Śliwińska-Wilczewska, S., Złoch, I., & Vasconcelos, V. (2018). Light-dependent cytolysis in the allelopathic interaction between picoplanktic and filamentous cyanobacteria. *Journal of Plankton Research*, *40*(2), 165–177. https://doi.org/10.1093/plankt/fby004

Becher, P. G., Beuchat, J., Gademann, K., & Jüttner, F. (2005). Nostocarboline: Isolation and synthesis of a new cholinesterase inhibitor from Nostoc 78-12A. *Journal of Natural Products*, *68*(12), 1793–1795. https://doi.org/10.1021/np050312l

Berman, F. W., Gerwick, W. H., & Murray, T. F. (1999). Antillatoxin and kalkitoxin, ichthyotoxins from the tropical cyanobacterium Lyngbya majuscula, induce distinct temporal patterns of NMDA receptor-mediated neurotoxicity. *Toxicon*, *37*(11), 1645–1648. https://doi.org/10.1016/S0041-0101(99)00108-7

Berry, J. P., Gantar, M., Gawley, R. E., Wang, M., & Rein, K. S. (2004). Pharmacology and

toxicology of pahayokolide A, a bioactive metabolite from a freshwater species of Lyngbya isolated from the Florida Everglades. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *139*(4), 231–238. https://doi.org/10.1016/j.cca.2004.11.005

Brilisauer, K., Rapp, J., Rath, P., Schöllhorn, A., Bleul, L., Weiß, E., Stahl, M., Grond, S., & Forchhammer, K. (2019). Cyanobacterial antimetabolite 7-deoxy-sedoheptulose blocks the shikimate pathway to inhibit the growth of prototrophic organisms. *Nature Communications*, *10*(1), 1–11. https://doi.org/10.1038/s41467-019-08476-8

Bruggeman, J., Heringa, J., & Brandt, B. W. (2009). PhyloPars: Estimation of missing parameter values using phylogeny. *Nucleic Acids Research*, *37*(SUPPL. 2), W179. https://doi.org/10.1093/nar/gkp370

Buratti, F. M., Manganelli, M., Vichi, S., Stefanelli, M., Scardala, S., Testai, E., & Funari, E. (2017). Cyanotoxins: producing organisms, occurrence, toxicity, mechanism of action and human health toxicological risk evaluation. *Archives of Toxicology*, *91*(3), 1049–1130. https://doi.org/10.1007/s00204-016-1913-6

Carmichael, W. W. (2001). Health effects of toxin-producing cyanobacteria: "The CyanoHABs." *Human and Ecological Risk Assessment (HERA)*, *7*(5), 1393–1407. https://doi.org/10.1080/20018091095087

Carmichael, W. W., Azevedo, S. M. F. O., An, J. S., Molica, R. J. R., Jochimsen, E. M., Lau, S., Rinehart, K. L., Shaw, G. R., & Eaglesham, G. K. (2001). Human fatalities form cyanobacteria: Chemical and biological evidence for cyanotoxins. *Environmental Health Perspectives*, *109*(7), 663–668. https://doi.org/10.1289/ehp.01109663

Chorus, I., & Bartram, J. (1999). *Toxic cyanobacteria in water : a guide to their public health consequences, monitoring and management / edited by Ingrid Chorus and Jamie Bertram* (p. 400). World Health Organization. https://apps.who.int/iris/handle/10665/42827

Cobo, F. (2015). Métodos de control de las floraciones de cianobacterias en agues continentales. *Limnetica*, *34*(1), 247–268.

Codd, G., Bell, S., Kaya, K., Ward, C., Beattie, K., & Metcalf, J. (1999). Cyanobacterial toxins, exposure routes and human health. *European Journal of Phycology*, *34*(4), 405–415. https://doi.org/10.1080/09670269910001736462

Demay, J., Bernard, C., Reinhardt, A., & Marie, B. (2019). Natural Products from

Cyanobacteria: Focus on Beneficial Activities. *Marine Drugs*, *17*(6), 320. https://doi.org/10.3390/md17060320

Dias, F., Antunes, J. T., Ribeiro, T., Azevedo, J., Vasconcelos, V., & Leão, P. N. (2017). Cyanobacterial allelochemicals but not cyanobacterial cells markedly reduce microbial community diversity. *Frontiers in Microbiology*, *8*(AUG), 1495. https://doi.org/10.3389/fmicb.2017.01495

Droop, M. R. (1968). Vitamin B12 and Marine Ecology. IV. The Kinetics of Uptake, Growth and Inhibition in Monochrysis Lutheri. *Journal of the Marine Biological Association of the United Kingdom*, *48*(3), 689–733. https://doi.org/10.1017/S0025315400019238

Ducobu, H., Huisman, J., Jonker, R. R., & Mur, L. R. (1998). Competition between a prochlorophyte and a cyanobacterium under various phosphorus regimes: Comparison with the droop model. *Journal of Phycology*, *34*(3), 467–476. https://doi.org/10.1046/j.1529-8817.1998.340467.x

Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., & Stackebrandt, E. (2006). The Prokaryotes. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, & E. Stackebrandt (Eds.), *A Handbook on the Biology of Bacteria, Volume 4* (3rd ed.). Springer Science & Business Media.

Ellner, S. P., Seifu, Y., & Smith, R. H. (2002). Fitting Population Dynamic Models to Time-Series Data by Gradient Matching. *Ecology*, *83*(8), 2256–2270. https://doi.org/10.2307/3072057

Elser, J. J., Bracken, M. E. S., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., Ngai, J. T., Seabloom, E. W., Shurin, J. B., & Smith, J. E. (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecology Letters*, *10*(12), 1135–1142. https://doi.org/10.1111/j.1461-0248.2007.01113.x

Falconer, I. R., & Humpage, A. R. (1996). Tumour promotion by cyanobacterial toxins. *Phycologia*, *35*(SUPPL.), 74–79. https://doi.org/10.2216/i0031-8884-35-6s-74.1

Fu, F. X., Zhang, Y., Bell, P. R. F., & Hutchins, D. A. (2005). Phosphate uptake and growth kinetics of Trichodesmium (Cyanobacteria) isolates from the North Atlantic Ocean and the Great Barrier Reef, Australia. *Journal of Phycology*, *41*(1), 62–73. https://doi.org/10.1111/j.1529-8817.2005.04063.x

Funari, E., & Testai, E. (2008). Human Health Risk Assessment Related to Cyanotoxins

Exposure. *Critical Reviews in Toxicology*, *38*(2), 97–125. https://doi.org/10.1080/10408440701749454

Hoffmann, L., Komárek, J., & Kaštovský, J. (2005). System of cyanoprokaryotes (cyanobacteria) state in 2004. *Algological Studies/Archiv Für Hydrobiologie, Supplement Volumes*, *117*, 95–115. https://doi.org/10.1127/1864-1318/2005/0117-0095

Hudnell, H. K. (2008). Cyanobacterial harmful algal blooms: State of the science and research needs. In *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs*. https://doi.org/10.1007/978-0-387-75865-7

Huisman, J., Codd, G. A., Paerl, H. W., Ibelings, B. W., Verspagen, J. M. H., & Visser, P. M. (2018). Cyanobacterial blooms. *Nature Reviews Microbiology*, *16*(8), 471–483. https://doi.org/10.1038/s41579-018-0040-1

Huisman, J., Matthijs, H. C. P., Visser, P. M., Balke, H., Sigon, C. A. M., Passarge, J., Weissing, F. J., & Mur, L. R. (2002). Principles of the light-limited chemostat: theory and ecological applications. *Antonie van Leeuwenhoek*, *81*(1), 117–133. https://doi.org/10.1023/A:1020537928216

Jochimsen, E. M., Carmichael, W. W., An, J., Cardo, D. M., Cookson, S. T., Holmes, C. E. M., De Antunes, M. B. C., De Melo Filho, D. A., Lyra, T. M., Barreto, V. S. T., Azevedo, S. M. F. O., & Jarvis, W. R. (1998). Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. *New England Journal of Medicine*, *338*(13), 873–878. https://doi.org/10.1056/NEJM199803263381304

Jüttner, F., Todorova, A. K., Walch, N., & Von Philipsborn, W. (2001). Nostocyclamide M: A cyanobacterial cyclic peptide with allelopathic activity from Nostoc 31. *Phytochemistry*, *57*(4), 613–619. https://doi.org/10.1016/S0031-9422(00)00470-2

Kaya, K., Mahakhant, A., Keovara, L., Sano, T., Kubo, T., & Takagi, H. (2002). Spiroidesin, a novel lipopeptide from the cyanobacterium Anabaena spiroides that inhibits cell growth of the cyanobacterium Microcystis aeruginosa. *Journal of Natural Products*, *65*(6), 920–921. https://doi.org/10.1021/np010660x

Komárek, J., Kastovsky, J., Mares, J., & Johansen, J. R. (2014). Taxonomic classification of cyanoprokaryotes (cyanobacterial genera) 2014, using a polyphasic approach. *Preslia*, *86*, 295–335. https://doi.org/10.1353/ctt.0.0006

Kurmayer, R., Christiansen, G., Fastner, J., & Börner, T. (2004). Abundance of active and

inactive microcystin genotypes in populations of the toxic cyanobacterium Planktothrix spp. *Environmental Microbiology*, *6*(8), 831–841. https://doi.org/10.1111/j.1462-2920.2004.00626.x

Leão, P. N., Pereira, A. R., Liu, W. T., Ng, J., Pevzner, P. A., Dorrestein, P. C., König, G. M., Vasconcelos, V. M., & Gerwick, W. H. (2010). Synergistic allelochemicals from a freshwater cyanobacterium. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(25), 11183–11188. https://doi.org/10.1073/pnas.0914343107

Leão, P. N., Ramos, V., Vale, M., Machado, J. P., & Vasconcelos, V. M. (2012). Microbial Community Changes Elicited by Exposure to Cyanobacterial Allelochemicals. *Microbial Ecology*, *63*(1), 85–95. https://doi.org/10.1007/s00248-011-9939-z

Leão, P. N., Vasconcelos, M. T. S. D., & Vasconcelos, V. M. (2009). Allelopathy in freshwater cyanobacteria Allelopathy in freshwater cyanobacteria Pedro N. Leo et al. In *Critical Reviews in Microbiology* (Vol. 35, Issue 4, pp. 271–282). Taylor & Francis. https://doi.org/10.3109/10408410902823705

Levenberg, K. (1944). A method for the solution of certain non-linear problems in least squares. *Quarterly of Applied Mathematics*, *2*(2), 164–168. http://www.jstor.org/stable/43633451

Litchman, E., & Klausmeier, C. A. (2008). Trait-based community ecology of phytoplankton. In *Annual Review of Ecology, Evolution, and Systematics* (Vol. 39, Issue 1, pp. 615–639). Annual Reviews. https://doi.org/10.1146/annurev.ecolsys.39.110707.173549

Ma, Z., Fang, T., Thring, R. W., Li, Y., Yu, H., Zhou, Q., & Zhao, M. (2015). Toxic and non-toxic strains of Microcystis aeruginosa induce temperature dependent allelopathy toward growth and photosynthesis of Chlorella vulgaris. *Harmful Algae*, *48*, 21–29. https://doi.org/10.1016/j.hal.2015.07.002

Matthijs, H. C. P., Visser, P. M., Reeze, B., Meeuse, J., Slot, P. C., Wijn, G., Talens, R., & Huisman, J. (2012). Selective suppression of harmful cyanobacteria in an entire lake with hydrogen peroxide. *Water Research*, *46*(5), 1460–1472. https://doi.org/10.1016/j.watres.2011.11.016

McAllister, T. G., Wood, S. A., & Hawes, I. (2016). The rise of toxic benthic Phormidium proliferations: A review of their taxonomy, distribution, toxin content and factors regulating prevalence and increased severity. *Harmful Algae*, *55*, 282–294.

https://doi.org/10.1016/j.hal.2016.04.002

Merel, S., Walker, D., Chicana, R., Snyder, S., Baurès, E., & Thomas, O. (2013). State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. In *Environment International* (Vol. 59, pp. 303–327). Elsevier Ltd. https://doi.org/10.1016/j.envint.2013.06.013

Monod, J. (1978). La Technique de Culture Continue Théorie et Applications. In *Selected Papers in Molecular Biology by Jacques Monod* (pp. 184–204). Academic Press. https://doi.org/10.1016/b978-0-12-460482-7.50023-3

Mur, L. R., Skulberg, O. M., & Utkilen, H. (1999). Cyanobacteria in the Environment. *Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management*, 30.

Nagarajan, M., Maruthanayagam, V., & Sundararaman, M. (2013). SAR analysis and bioactive potentials of freshwater and terrestrial cyanobacterial compounds: A review. *Journal of Applied Toxicology*, *33*(5), 313–349. https://doi.org/10.1002/jat.2833

Paerl, H. W., & Huisman, J. (2008). Climate: Blooms like it hot. In *Science* (Vol. 320, Issue 5872, pp. 57–58). https://doi.org/10.1126/science.1155398

Paerl, H. W., & Otten, T. G. (2013). Harmful Cyanobacterial Blooms: Causes, Consequences, and Controls. *Microbial Ecology*, *65*(4), 995–1010. https://doi.org/10.1007/s00248-012-0159-y

Puschner, B. (2018). Cyanobacterial (Blue-Green Algae) Toxins. In *Veterinary Toxicology: Basic and Clinical Principles: Third Edition* (pp. 763–777). Elsevier. https://doi.org/10.1016/B978-0-12-811410-0.00057-X

R Core Team. (2020). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.r-project.org/

Ramos, V., Morais, J., Castelo-Branco, R., Pinheiro, Â., Martins, J., Regueiras, A., Pereira, A. L., Lopes, V. R., Frazão, B., Gomes, D., Moreira, C., Costa, M. S., Brûle, S., Faustino, S., Martins, R., Saker, M., Osswald, J., Leão, P. N., & Vasconcelos, V. M. (2018). Cyanobacterial diversity held in microbial biological resource centers as a biotechnological asset: the case study of the newly established LEGE culture collection. *Journal of Applied Phycology*, *30*(3), 1437–1451. https://doi.org/10.1007/s10811-017-1369-y

Ribeiro, T., Lemos, F., Preto, M., Azevedo, J., Sousa, M. L., Leao, P. N., Campos, A., Linder, S., Vitorino, R., Vasconcelos, V., & Urbatzka, R. (2017). Cytotoxicity of portoamides in human cancer cells and analysis of the molecular mechanisms of action. *PloS One*, *12*(12), e0188817. https://doi.org/10.1371/journal.pone.0188817

Rice, E. L. (1984). *Allelopathy*. Elsevier Science. https://books.google.pt/books?id=uqcH7AOXMCUC

Roy, S. (2009). The coevolution of two phytoplankton species on a single resource: Allelopathy as a pseudo-mixotrophy. *Theoretical Population Biology*, *75*(1), 68–75. https://pubmed.ncbi.nlm.nih.gov/19116158/

Six, C., Thomas, J. C., Garczarek, L., Ostrowski, M., Dufresne, A., Blot, N., Scanlan, D. J., & Partensky, F. (2007). Diversity and evolution of phycobilisomes in marine Synechococcus spp.: A comparative genomics study. *Genome Biology*, *8*(12). https://doi.org/10.1186/gb-2007-8-12-r259

Sousa, M. L., Ribeiro, T., Vasconcelos, V., Linder, S., & Urbatzka, R. (2020). Portoamides A and B are mitochondrial toxins and induce cytotoxicity on the proliferative cell layer of in vitro microtumours. *Toxicon*, *175*, 49–56. https://doi.org/10.1016/j.toxicon.2019.12.159

Stal, L. J. (2009). Is the distribution of nitrogen-fixing cyanobacteria in the oceans related to temperature?: Minireview. In *Environmental Microbiology* (Vol. 11, Issue 7, pp. 1632–1645). Environ Microbiol. https://doi.org/10.1111/j.1758-2229.2009.00016.x

Stal, L. J. (2017). The effect of oxygen concentration and temperature on nitrogenase activity in the heterocystous cyanobacterium Fischerella sp. *Scientific Reports*, *7*(1), 1–10. https://doi.org/10.1038/s41598-017-05715-0

Stal, L. J., Gemerden, H., & Krumbein, W. E. (1985). Structure and development of a benthic marine microbial mat. *FEMS Microbiology Letters*, *31*(2), 111–125. https://doi.org/10.1111/j.1574-6968.1985.tb01138.x

Stomp, M., Huisman, J., Voros, L., Pick, F. R., Laamanen, M., Haverkamp, T., & Stal, L. J. (2007). Colourful coexistence of red and green picocyanobacteria in lakes and seas. *Ecology Letters*, *10*(4), 290–298. https://doi.org/10.1111/j.1461-0248.2007.01026.x

Suikkanen, S., Fistarol, G. O., & Granéli, E. (2004). Allelopathic effects of the Baltic cyanobacteria Nodularia spumdigena, Aphanizomenon flos-aquae and Anabaena lemmermannii on algal monocultures. *Journal of Experimental Marine Biology and*

*Ecology*, *308*(1), 85–101. https://doi.org/10.1016/j.jembe.2004.02.012

Summons, R. E., Jahnke, L. L., Hope, J. M., & Logan, G. A. (1999). 2-Methylhopanoids as biomarkers for cyanobacterial oxygenic photosynthesis. *Nature*, *400*(6744), 554–557. https://doi.org/10.1038/23005

Tilman, D. (1977). Resource Competition between Plankton Algae: An Experimental and Theoretical Approach. *Ecology*, *58*(2), 338–348. https://doi.org/10.2307/1935608

Todorova, A. K., Jüttner, F., Linden, A., Plüiss, T., & von Philipsborn, W. (1995). Nostocyclamide: A New Macrocyclic, Thiazole-Containing Allelochemical from Nostoc sp. 31 (Cyanobacteria). *Journal of Organic Chemistry*, *60*(24), 7891–7895. https://doi.org/10.1021/jo00129a032

Vasconcelos, V. M. (1999). Cyanobacterial toxins in Portugal: Effects on aquatic animals and risk for human health. *Brazilian Journal of Medical and Biological Research*, *32*(3), 249–254. https://doi.org/10.1590/S0100-879X1999000300001

Visser, P. M., Ibelings, B. W., Van Der Veer, B., Koedood, J., & Mur, L. R. (1996). Artificial mixing prevents nuisance blooms of the cyanobacterium Microcystis in Lake Nieuwe Meer, the Netherlands. *Freshwater Biology*, *36*(2), 435–450. https://doi.org/10.1046/j.1365-2427.1996.00093.x

Voelker, A. L., Miller, J., Running, C. A., Taylor, L. S., & Mauer, L. J. (2018). Chemical stability and reaction kinetics of two thiamine salts (thiamine mononitrate and thiamine chloride hydrochloride) in solution. *Food Research International*, *112*, 443–456. https://doi.org/10.1016/J.FOODRES.2018.06.056

Walsby, A. E. (1985). The permeability of heterocysts to the gases nitrogen and oxygen. *Proceedings of the Royal Society of London - Biological Sciences*, *226*(1244), 345–366. https://doi.org/10.1098/rspb.1985.0099

Wentzky, V. C., Tittel, J., Jäger, C. G., Bruggeman, J., & Rinke, K. (2020). Seasonal succession of functional traits in phytoplankton communities and their interaction with trophic state. *Journal of Ecology*, *108*(4), 1649–1663. https://doi.org/10.1111/1365-2745.13395

Whitton, B. (1992). Diversity, Ecology, and Taxonomy of the Cyanobacteria. In *Photosynthetic Prokaryotes* (pp. 1–51). https://doi.org/10.1007/978-1-4757-1332-9_1

Whitton, B., & Potts, M. (2007). Introduction to the Cyanobacteria. In B. Whitton (Ed.),

*Ecology of Cyanobacteria II* (pp. 1–11). Springer Science & Business Media. https://doi.org/10.1007/0-306-46855-7_1

Yang, K., Chen, Q., Zhang, D., Zhang, H., Lei, X., Chen, Z., Li, Y., Hong, Y., Ma, X., Zheng, W., Tian, Y., Zheng, T., & Xu, H. (2017). The algicidal mechanism of prodigiosin from Hahella sp. KA22 against Microcystis aeruginosa. *Scientific Reports*, *7*(1), 1–15. https://doi.org/10.1038/s41598-017-08132-5