Exploring the allelopathic properties of plants to control harmful algal blooms

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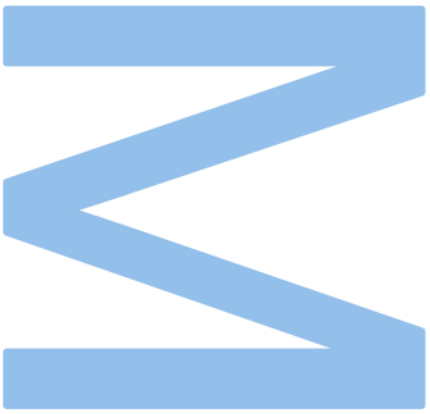

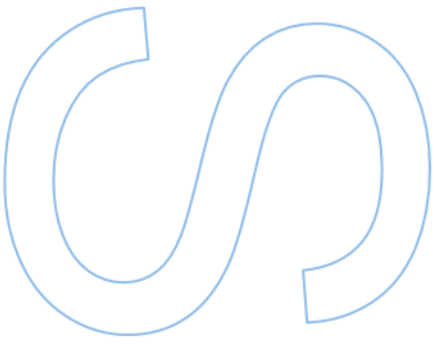

Dedicated to my family for their unconditional support

Sworn Statement

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By submitting this dissertation, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

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Pedro Nascimento

30th of September of 2022

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Resumo

A eutrofização de água doce é uma preocupação ecológica e de saúde pública cada vez maior. Foi demonstrado que fontes antrópicas, como efluentes industriais e domésticos, aquacultura e escoamentos agrícolas, aceleram, drasticamente, o enriquecimento nutritivo da água. Uma das principais consequências da eutrofização é a formação de fluorescências algais nocivas (em inglês, harmful algal blooms - HABs), e o rápido aumento e acumulação populacional de microrganismos, nomeadamente algas e cianobactérias. Neste projeto, pretendemos investigar uma solução baseada na natureza para este problema. A estratégia proposta consiste em avaliar os efeitos diretos de plantas aquáticas no crescimento e viabilidade de duas espécies de cianobactérias associadas a HABs: *Microcystis aeruginosa* e *Chrysosporum ovalisporum*. Ambas as espécies produzem metabolitos tóxicos, representando um risco para os ecossistemas, saúde pública e atividades agropecuárias. Na fase inicial deste projeto, rastreámos diferentes espécies de plantas – *Iris pseudacoros*, *Typha latifolia*, *Sparganium erectum*, *Alisma plantago-aquatica*, *Alisma lanceolatum*, *Nasturtium officinale*, *Landoltia punctata*, *Lemna minor* e *Wolffia arrhiza* - para o efeito alelopático dos seus extratos nestes microrganismos. Para este fim, extratos preparados com uma mistura de metanol e água, num rácio de 70:30 (v:v), foram testados, a diferentes concentrações, em culturas das duas espécies de cianobactérias. A exposição a extratos de *Landoltia punctata*, *Typha latifolia* e *Iris pseudacoros*, mostrou efeitos significativos de inibição na abundância celular de *C. ovalisporum*. Nenhum extrato revelou este efeito nas culturas de *M. aeruginosa*. Na segunda fase do nosso estudo, um sistema de microcosmos foi concebido para avaliar os efeitos do co-crescimento da *C. ovalisporum* com a planta que mostrou melhor potencial alelopático, *L. punctata*. Nesta experiência, testou-se o efeito da presença desta planta na abundância celular da cianobactéria, sem limitações de nutrientes. Os resultados deste estudo confirmaram o efeito alelopático por parte da planta. Concluindo, este trabalho vem contribuir para o conhecimento da alelopatia entre plantas e cianobactérias, e ainda para o desenvolvimento de soluções ecológicas e práticas para o tratamento de corpos de água doce eutróficos e contaminados por *C. ovalisporum*.

Palavras-chave: Alelopatia de plantas; Macrofitas aquáticas, *Microcystis aeruginosa*; *Chrysosporum ovalisporum*; Biorremediação; *Landoltia punctata*; Florescências algais nocivas.

Abstract

Freshwater eutrophication is an increasing ecological and public health concern. Anthropogenic inputs, such as sewage and industrial wastewater effluents, aquaculture and agriculture runoffs, have been shown to greatly accelerate the nutrient enrichment in water bodies. One of the major consequences of eutrophication, is the formation of harmful algal blooms (HABs), a rapid population increase and accumulation of microorganisms, namely algae and cyanobacteria. In this project, we aim to find a nature based solution to this problem. Our approach is to evaluate the direct and indirect effects of aquatic plants on the growth and viability of two cyanobacteria species associated with HABs: *Microcystis aeruginosa* and *Chrysosporum ovalisporum*. Both cyanobacteria produce toxic metabolites, representing a risk to the ecosystem, to public health and to agricultural and livestock activities. In the initial phase of this project, we screened different plant species – *Iris pseudacoros*, *Typha latifolia*, *Sparganium erectum*, *Alisma plantago-aquatica*, *Alisma lanceolatum*, *Nasturtium officinale*, *Landoltia punctata*, *Lemna minor* and *Wolffia arrhiza* - for the allelopathic effect of their extracts on these microorganisms. For this purpose, methanol/water 70:30 (v:v) mixture extracts were prepared and tested, at various concentrations, in the culture growing media. Exposure to *Landoltia punctata*, *Typha latifolia* and *Iris pseudacoros*, extracts showed significant cellular abundance inhibitory effects on *C. ovalisporum*. This effect was not observed for any extract in *M. aeruginosa* cultures. In the second phase of our study, a microcosm system was assembled to evaluate the effects of co-growth of *C. ovalisporum* and the plant that previously showed more allelopathic potential, *L. punctata*. In this experiment, we tested the effect of the presence of this plant on cyanobacterial cell abundance, under a nutritional surplus. The results confirmed the plant's allelopathic effect on *C. ovalisporum*. The results of this work constitute an important contribution to the knowledge regarding allelopathic effects between plants and cyanobacteria and provide novel, ecofriendly and practical solutions, for treatment of water contaminated with *C. ovalisporum*.

Keywords: Plant allelopathy; Aquatic macrophytes; *Microcystis aeruginosa*; *Chrysosporum ovalisporum*; Bioremediation; *Landoltia punctata*; Harmful algal blooms;

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List of Abbreviations

BI	BIGGER INOCULUM
CYN	CYLINDROSPERMOPSIN
HAB	HARMFUL ALGAL BLOOM
MC	MICROCYSTINS
N	NITROGEN
P	PHOSPHOROUS
SI	SMALLER INOCULUM

1. Introduction

1.1. Eutrophication

The natural eutrophication of a water body is representative of the aging process of the aquatic ecosystem (**Burkholder,** 2003). Such an example is the gradual sedimentation on the bottom of a deep, nutrient deficient lake that leads to shallower depths and higher nutritional contents, becoming more productive in plant and animal life. Additionally, meteorological conditions, namely wind, also contribute to natural episodic and seasonal eutrophication events, through resuspension of nutrients by water column vertical mixing (**Stainsby et al.**, 2011; **Joensuu et al.**, 2020). On the other hand, cultural eutrophication can be defined as the acceleration of the nutrient enrichment of water through human influence. There are numerous anthropogenic sources, such as aquaculture intentional water enrichment to increase productivity (**Boyd and Tucker,** 1998; **Mazumder and Edmundson,** 2002) industrial, sewage and other urban effluents or agricultural runoffs aggravated by excessive fertilization and manure production (**Carpenter et al.**, 1998). Most consequential are the inputs of growth limiting nutrients, namely, phosphorous (P) and nitrogen (N), the first having a bigger impact in the growth of photosynthetic organisms in freshwater systems than the latter, which plays a bigger role in the eutrophication of marine waters (**Anderson et al.**, 2002). **Schindler** (1974), reported rapid eutrophication in lakes supplemented with P and N, and an almost immediate recovery in similarly tested lakes where only the phosphorous supplement was discontinued.

The cultural eutrophication phenomena was recognized by the beginning of the 20th century and while certain measures, like the ban on phosphates in detergents, showed improvements, a new wave of global nutrient pollution has been observed with some regions facing recurrent episodes of eutrophication and new regions now suffering from the same problem (**Le Moal et al.**, 2019). The constant increase in global population and associated urban and agro-industrial developments, makes this an almost ubiquitous environmental problem, justifying the need for systematic research on its causes, consequences and respective mitigation strategies.

1.2. Harmful Algal Blooms

The major consequence of eutrophication of aquatic systems, is the escalated formation of algal blooms. These are defined by a rapid increase and/or accumulation of a population of algae, cyanobacteria or other unicellular microorganisms (**Lincoln et al.**, 1998). While this definition can be applied to any bloom, for the context of this thesis, we're referring to those that are particularly promoted by anthropogenic nutrient inputs, and more specifically harmful algal blooms (HABs).

Algal Blooms can be defined as harmful based on the impairments caused in the environment, public health and economy (**Smayda**, 1997). HAB forming species can be categorized by two major harmful characteristics: toxin producing species and high biomass output species (**Masó and Garcés**, 2006). The first group is characterized by the production of toxic compounds and subsequent release, whether by secretion or cell death. These compounds can act directly on local fauna or flora, or indirectly through vector organisms that may accumulate and cause adverse effects in the food web (**Smayda**, 1997). Additionally, while not strictly toxic, some released compounds such as reactive oxygen species (ROS), mucilage or polyunsaturated fatty acids, can be damaging and even lethal to other organisms (**Anderson et al.**, 2012; **Diaz and Plummer**, 2018). The non-toxic group of HAB forming species acts in a more indirect way, through high biomass production which can lead to damage to the environment and to fishery resources, through oxygen depletion, over shading, particle irritation, starvation or migration changes, among others (**Anderson**, 2009; **Berdalet et al.**, 2016). Furthermore, besides the high algae biomass, these HABs are often associated with scums and noxious odours, which can affect recreational facilities and have economic impacts on the tourism industry (**Anderson et al.**, 2012; **Smith et al.**, 2019).

1.2.1. *Microcystis aeruginosa*

One of the most common and well known species responsible for the formation of toxic algal blooms is *Microcystis aeruginosa*, a unicellular, colony-forming cyanobacterium, mostly found in freshwater ecosystems (**Harke et al.**, 2016). *M. aeruginosa* is responsible for the production of microcystins (MC), initially known as Fast Death Factor (**Bishop et al.**, 1959). These hepatotoxins form irreversible covalent bonds with protein phosphatases, leading to excessive phosphorylation, which results in a cascade of molecular events, such as cytoskeletal changes and subsequent cell death which, ultimately, translates to liver disease (**Campos and Vasconcelos**, 2010). Furthermore, MC can cause alterations in antioxidant systems and induce oxidative stress in cells and mitochondria (**Pavagadhi and Balasubramanian**, 2013). Wildlife, livestock and aquatic species are the most associated with fatal or severe poisonings by microcystins, but cases of human illness attributed to consumption of contaminated water and food or skin exposure in recreational water activities are well documented **(Campos and Vasconcelos,** 2010**; Harke et al.**, 2016).

Geographically, *M. aeruginosa* has been reported in every continent but Antarctica (**Zurawell et al.**, 2005). In a review of available records, **Harke et al.** (2016) found reports of blooms in 108 countries, with a smaller number of blooms reported in less developed countries, which can be representative of lack of monitoring programs, making it very possible that the extent of *M. aeruginosa* pervasiveness around the world is far greater than reported. Given the severe injuries caused by this toxic cyanobacteria species on animals and humans health it is of utmost importance to find counter-measures for the presence of this species in the environment.

1.2.2. *Chrysosporum ovalisporum*

Previously known as *Aphanizomenon ovalisporum* (**Zapomělová et al.**, 2012), *Chrysosporum ovalisporum* is a cyanobacteria belonging to the Nostocales order, of filamentous and heterocystic nature and capable of toxin production (**Fadel et al.**, 2014). Among other cyanobacteria, like *Cylindrospermopsis raciborskii,* from which the toxin acquired its name, it is one of the main producers of cylindrospermopsin (CYN) (**Messineo et al.**, 2010) a highly water-soluble tricyclic alkaloid (**Ohtani et al.**, 1992; **Chiswell et al.**, 1999).

First identified in 1979, in context of 148 human cases of severe hepatoenteritis on Palm Island, Queensland, Australia (**Griffiths and Saker**, 2003), CYN is traditionally classified as a hepatotoxin but it can also affect other organs such as the eye, lungs, heart, spleen and kidneys (**Guzmán-Guillén et al.**, 2017). Besides cytotoxicity, it has been associated with genotoxicity and carcinogenic effects, with its known toxicity mechanisms including protein synthesis inhibition and increased oxidative stress (**Pichardo et al.**, 2017). While the effects of CYN uptake in plants are still relatively unknown, it has been shown to inhibit protein synthesis at similar intensity as in mammal cells (**Terao et al.**, 1994; **Runnegar et al.**, 2002). Interestingly, **Pinheiro et al.** (2016) observed increased CYN toxicity by interaction with MC in *Chlorella vulgaris* when, individually, MC showed no toxic effects, even when present in high concentrations. Therefore, risk assessment of CYN blooms must consider the co-presence of MC, and other possible interactions with different cyanotoxins should be studied. Finally, while not as widely spread as *M. aeruginosa*, *Chrysosporum spp* toxic blooms have been reported in many countries, e.g. Turkey, Israel, Greece, Spain, Italy, Australia and Portugal (**Akcaalan et al.**, 2014; **Fadel et al.**, 2014; **Moreira et al.**, 2021).

1.3. Control methods of HABs

While environmental measures can and should be taken to prevent severe eutrophication of water bodies, reducing anthropogenic sources of nutrients, other approaches must be achieved to prevent the formation and to control HAB outbreaks in water bodies. These can be categorized in distinct types of strategies: physical, chemical, and biological (**Anderson**, 2009). Physical control involves methods such as pumping and mixing water to break water column stability (**Gallardo-Rodríguez et al.**, 2019), sonication (**Park et al.**, 2017) or the dispersion of substances, e.g. clay, to flocculate HAB cells, facilitating its removal by sedimentation (**Sengco and Anderson**, 2004). Physical strategies can be limited to applications in small volumes of water (**Anderson**, 2009). Furthermore , can be harmful or lethal to other aquatic organisms (**Rensel and Whyte**, 2004).

Chemical control consists in the use of compounds with a direct effect on the HAB species. The major drawback of this approach is the difficulty in finding an adequate compound with minimal effects on the surrounding ecosystems.

Pesticides, like diquat and terbutryn are common chemical treatments used in the control of cyanobacteria (**Cobo**, 2015). These two non-selective compounds, affect any type of organism while other algicides, can have a more restricted effect, such as atrazine and diuron, which inhibit the electron flow in photosystem II, therefore impairing photosynthetic organisms (**Kolmakov**, 2006; **Matthijs et al.**, 2016). Even though these chemicals have a more cyanobacteria-specific action, other flora in the environment are still harmed, and in the case of diuron, it has a high persistence in the sediments and its degradation leads to the formation of genotoxic substances which results in even more organisms being affected in the long term (**Matthijs et al.**, 2016). Other compounds, such as hydrogen peroxide and copper sulphate are also used in bloom mitigation, though cyanobacteria have been observed to acquire resistance to the latter (**Shavyrina et al.**, 2001; **Huang and Zimba**, 2020; **Zamyadi et al.**, 2020). Sensitivity to hydrogen peroxide is also dependent on environmental conditions, with nutrient limitation and low light intensities negatively affecting its effect as a control agent (**Sandrini et al.**, 2020).

Lastly, biological control is the use of living organisms to suppress HABs or diminish its impacts. We will introduce these methods in the next section.

1.3.1. Biotic Control

The use of conventional water treatment stations to treat aquatic systems such as ponds, lakes and reservoirs implicates high costs in energy, infrastructures, specialized labour and equipment which can make it economically unfeasible. Furthermore, environmental concerns can be raised by the usage of certain physical and chemical methods that entail damage to local fauna and flora. This poses a challenge to find effective, low cost and eco-friendly strategies, to prevent and control HABs. A contender that has shown great potential is biotic control, the use of other organisms as agents against HAB forming species.

This approach has spawned a myriad of green technologies from different biology fields mainly involving the use of algicidal microorganisms (**Wang and Coyne**, 2020), stocking of algal grazers and employment of aquatic plants (**Sun et al.**, 2018; **Pal et al.**, 2020).

Plants have an important role in the remediation of pollutants. Green technology applications like constructed wetlands (CWs) and ecological floating beds (EFBs) take advantage of the presence of plants and their ability to uptake, accumulate and/or metabolize toxins and heavy metals, as a means to purify aquatic ecosystems

(**Pflugmacher et al.**, 2015; **Samal** et al., 2019). CWs are complex water treatment, composed of substrates, plants and microorganisms (Figure 1), which mimic the physical, biochemical and biological processes that occur on natural wetlands (**Bavithra et al.,** 2020).

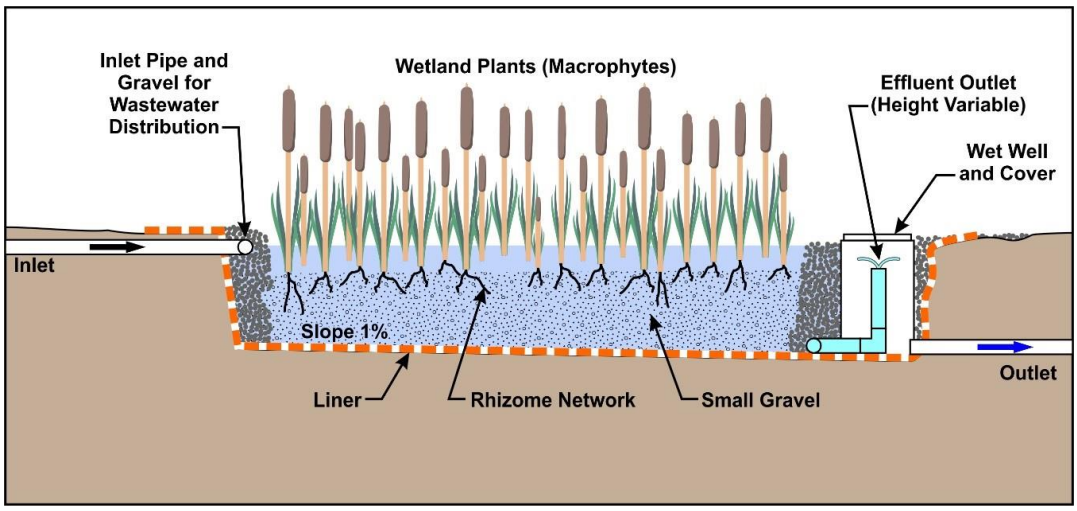

Figure 1 - Schematic layout of a constructed wetland water treatment system (Adapted from **Tilley et al.** (2014)).

On the other hand, EFBs rely on the use of macrophytes growing in a floating mat, supported by a floating device (Figure 2), with their root system emerged in the water facilitating the uptake of nutrients and pollutants as well as the release of bioactive compounds (**Samal et al.**, 2019).

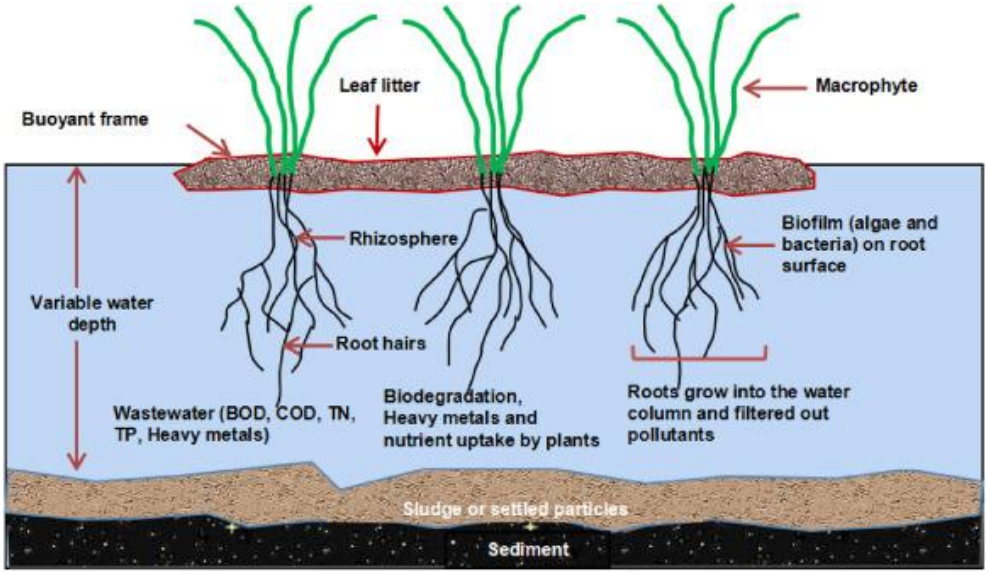

Figure 2 - General schematic layout of an ecological floating bed used for water treatment (Adapted from **Samal et al.** (2019)).

Both applications can also rely on the formation of a network of roots, rhizomes and attached biofilms to entrap, filter and biochemically degrade debris, pollutants and other organisms (**Bi et al.**, 2019). Due to their physiological characteristics and typical zonation patterns, macrophytes have a great adaptability to these ecological applications (Figure 3).

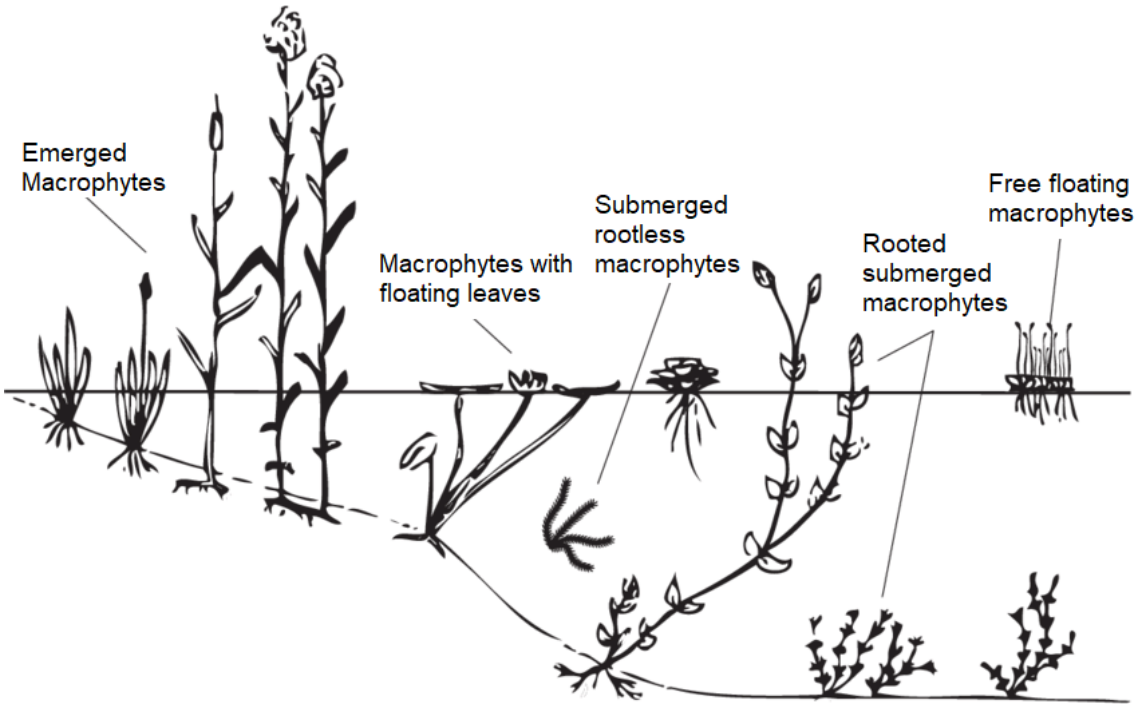

Figure 3 - Classic zonation pattern of aquatic macrophytes (Adapted from **Esteves** (1998)).

Additionally, many aquatic plants have shown allelopathic effects on cyanobacteria, with hundreds of allelochemicals having been extracted and identified (**Zhu** et al., 2021). Seaweeds in particular, are an important source of bioactive compounds with known anti-cyanobacterial activity (**El Amrani Zerrifi et al.**, 2020). In-vivo allelopathic activity has also been reported, for example *Lemna trissulca* impaired growth of *Dolichospermum flos-aquae*, *Raphidopsis raciborskii* and *M. aeruginosa* while, simultaneously reducing cyanotoxin concentrations (**Kucała et al.**, 2021). Furthermore, macrophytes can act as nutrient scavengers, making them ideal choices for bioremediation of eutrophic, cyanobacteria contaminated waters by acting both directly and indirectly on cyanobacteria (**Liu et al.**, 2018).

2. Aims

With climate change and the constantly expanding global population, the pressures on freshwater resources is increasing exponentially. It is of utmost importance that science and technology keeps up with these needs, discovering and developing ways to maintain and even increase, water availability and quality. To that end, this project aimed to tackle the problem of eutrophication and cyanobacteria contamination of freshwater ecosystems, establishing the following as the main objectives:

- Investigate the allelopathic activity of different aquatic plants on the growth of two toxic cyanobacteria: *Microcystis aeruginosa* and *Chrysosporum ovalisporum*;
- Validate the allelopathic activity of a selected macrophyte, in a co-growth experiment (microcosm).

Furthermore, the results of this investigation should give us insight on potential macrophyte species that can be applied in various nature based technologies, namely EFBs and CWs, pursuing biological control of cyanobacterial blooms.

3. Materials and Methods

3.1. Experimental Procedures

3.1.1. Plant collection and sample preparation

The selected plants were obtained through collection in local gardens and other natural environments. Some were also commercially obtained. The majority of plant species were granted by the Porto Botanical Garden, including *Iris pseudacoros*, *Alisma lanceolatum*, *Alisma plantago-aquatica* and duckweeds *Wolffia arrhiza* and *Landoltia punctata*. The third duckweed, *Lemna minor* was collected from two small ponds in Parque da Cidade, Porto. *Typha latifolia* specimens were collected from the margins of two lakes also located in this park. *Sparganium erectum* was obtained from the riverbank of a shallow water area in Ribeira da Granja, Porto. *Nasturtium officinale* was purchased from a local market. For the extract assays, plants were obtained in late autumn of 2021, while the *L. punctata* used in the microcosm assay was collected in the summer of 2022.

In order to maximize sample representativeness, both young and adult specimens of *T. latifolia* and *S. erectum* were collected. *L. minor* was also collected randomly in the ponds from Parque da Cidade. In contrast, due to reduced availability during this period, only single adult specimens were obtained for *I. pseudacoros*, *A. lanceolatum* and *A. plantago-aquatica*. Furthermore, due to the difficulty in separating different duckweeds present in the various tanks of the Botanical Garden, both duckweeds were each collected from a single water basin, in which they could be found relatively isolated.

Each plant sample was thoroughly washed with running water to remove dirt and other debris. With the exception of duckweeds, leaves and root systems were separated from each plant and cut in smaller pieces. When more than one sample of a species was available, tissues from each sample were mixed, in equal portions, to provide for a more generic metabolic profile of their respective species. The plant material was stored at -20 ºC until further processing. The material was transferred to -80 ºC for at least 2 hours, before being put into a freeze dryer for no less than 3 days. Finally, the lyophilized plant material was macerated by mortar and pestle and stored at room temperature.

3.1.2. Plant extracts preparation

The plant extracts were prepared using a mixture of methanol and water, in a 7:3 (v:v) ratio. This method was chosen to enhance the extraction of a wider range of polar compounds, increasing the representativeness of the plant's estimated activity, through the release of water soluble compounds, on the cyanobacteria (**Liu**, 2008). A volume of 50 mL of solvent mixture was used for every 200mg of grounded dry plant tissue. To further increase solvent penetration, the biomass was soaked for 72h with constant agitation and at room temperature. The resulting extract was then vacuum filtered through grade 1 Whatman filter paper. Finally, the solvents were removed by rotary evaporator and the resulting product was stored at -20 ºC until required for the bioassay.

3.1.3. Cyanobacterial growth and maintenance

The two species of cyanobacteria were provided by the LEGE-CC culture collection, owned by the Blue Biotechnology and Ecotoxicology research group from CIIMAR. The toxin producer strains of *Microcystis aeruginosa* LEGE 91094 and *Chrysosporum ovalisporum* LEGE X-001, were transferred into a flask containing 40 mL of Z8 culture media, prepared as in **Kotai** (1972), and grown for 1 month . Afterwards, roughly half of these initial cultures volume was transferred into 400 mL culture flasks. From the 400 mL flasks, gradual upscaling was performed up to 20 L. Due to the increasingly larger biomass and its aerobic requirements, aeration was added to increase water oxygenation and improve nutrient access through the resulting medium flow.

The cultures were grown at 25 ºC, with a light intensity of 22 µmol m^{-2} s^{-1}, under a light/dark cycle of 14/10 h. To ensure the integrity, viability and growth of the cyanobacteria used in the assays, regular medium replenishment and sample microscopic observation was performed. In order to replenish nutrients and oxygen levels, roughly 20% of the cultures volume was discarded and the same volume of fresh Z8 medium was added. Every culture manipulation occurred under sterile conditions to avoid contamination.

3.1.4. Cellular concentration estimation

In order to quickly assess cyanobacterial cellular concentration, optical density calibration curves were determined for both cyanobacteria (Figure 4). These curves were calculated by measuring the absorbance at 420 nm of 7 different known cellular concentrations in Z8 growth medium. The various solutions were diluted from a stock sample of culture that had its concentration determined through cell counting using a light microscope and a Neubauer chamber. Due to the filamentous nature of *C. ovalisporum,* the samples had to be homogenised by sonication, using a Bandelin Ultrasonic Homogeneiser GM 2070.2 with a MS72 probe at 20 KHz frequency and 10% amplitude for 45 seconds. Microscopy observation confirmed this process did not affect the cells integrity.

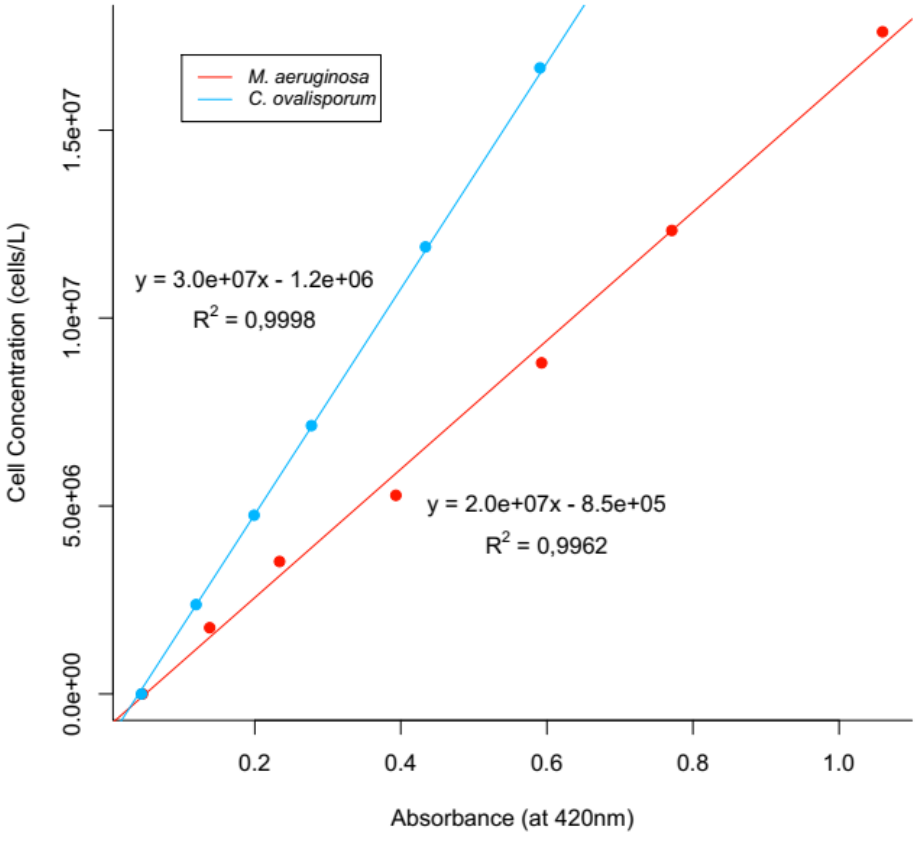

Figure 4 - Calibration curves determined for cyanobacterial cellular concentration through absorbance measurements.

In the microcosm assays, due to possible unaccountable contaminations and other factors that would render optical density measuring unreliable in terms of its relation to cyanobacterial concentration, its abundance was estimated through manual cell count.

As a consequence of lower cellular abundances, cellular concentration was estimated through cell count in Utermöhl chambers, using an inverted microscope and 1 mL culture samples.

3.1.5. Allelopathy screening - extract bioassay

To screen the allelopathic activity of the different plants against both cyanobacteria, assays using the previously prepared extracts, were performed. The methodology was adapted from **Tazart et al.** (2018). The experiments were prepared in 24 well plates. The wells were inoculated with the cyanobacteria culture volume necessary to obtain 7 x 10^6 cells/mL as final concentration, in a volume of 2,5 mL. Different extract concentrations (0,125 mg/mL, 0,25 mg/mL, 0,5 mg/mL, 1 mg/mL and 2 mg/mL) were added. The treatment solutions were obtained by diluting the previously prepared extracts in Z8 medium and subsequently centrifuging them to remove any insoluble particles that might accumulate in the bottom of the wells and therefore affect the absorbance measurements. These treatments and a negative control with no extract added, were replicated 3 times (n = 3). Finally, the last row of wells in the test plates contained each extract concentration, without cyanobacteria, including one well with only Z8 medium. Besides determining the medium's blank value of absorbance, this allowed us to estimate the absorbance from the extract alone, both initially and at the end of the experiment, since some of the extracts experienced physical or chemical changes under the experimental conditions, which altered their absorbance. These assays were set under the same growth conditions as previously described in the culture methodology, and had a total duration of 72 hours.

3.1.6. Allelopathy bioassay in microcosm environment

In the screening phase, cyanobacterial abundance inhibition was observed in three extracts. On account of limited resources, it would not be possible to proceed with the microcosm experiments with the three potentially allelopathic species. Hence, the duckweed *L. punctata*, whose extract had shown activity against *C. ovalisporum*, was chosen for the next part of the study. The duckweed has advantages over the other

potentially allelopathic species, it is easy to handle and maintain in the laboratory and grows fast (**Ziegler et al.**, 2015).

The methodology employed consisted in the co-culture of the cyanobacteria with the aquatic plants in 250 mL beakers in a greenhouse (Figure 5). The plants were tested against 2 different concentrations of *C. ovalisporum*: 1 x 10^3 cells/mL (small inoculum, SI) and 1 x 10^6 cells/mL (big inoculum, BI). To ensure that any decrease in cyanobacterial growth was not due to nutrient depletion, the Z8 medium was adapted with the double macronutrient and iron concentrations (**Kotai**, 1972). For the experiments 1g (fresh weight) of duckweed was utilized. Controls (both for SI and BI) without plants were covered with aluminium foil on the top in order to mimic the light shading effect of *L. punctata*. Small holes were made in the foil to allow some of the evaporation that occurred in the treatment groups. Each experimental condition was replicated 5 times (n = 5).

Figure 5 - *L. punctata* and *C. ovalisporum* co-culture assays, as initially set up.

Prior to the actual experiment, *L. punctata*, obtained from the original source, were washed and transferred to beakers with the adapted Z8 medium and grown for 1 week

under the same conditions as in the assays. This period allowed the plants to adapt to the experimental conditions.

The experiment had a duration of 35 days. 1,5 mL samples were taken every 3 days to count cyanobacteria cells. Lugol iodine solution was added for preservation. Sampling for nutrient quantification was performed initially, on the 10th day and at the end of the experiment. For this purpose, two 6 mL samples were taken from each replica, filtrated through a 0,22 µm pore size syringe filter and frozen until analyse. To ensure that cyanobacterial growth was not affected by nutrient deficiencies, nutrient replenishment was performed on the 10th day, after sampling had been performed. Z8 macronutrient and iron components were added to each medium, making up the same concentrations as in the initial conditions. Additionally, sterile water was added regularly, to compensate the evaporation and minimizing water loss discrepancies between experimental groups.

3.1.7. Nutrient quantification

Nitrate ion (NO^{3-}) and Phosphate ion (PO_4^{3-}) levels were determined through colorimetry. These nutrients quantifications were performed with a Skalar Sanplus Segmented Flow Analyser, using respectively, the Skalar methods: M461-318 (EPA 353.2) and M503-555R (Standard Method 450-P I). The analytic procedures were validated by doping a few samples with known quantities of NO^{3-} and PO_4^{3-}.

3.2. Statistical Analysis

All statistical analysis were performed with R software (**R Core Team**, 2022). For the extract bioassay data, simple linear regressions were fit using the function *lm* from the *stats* package. The *plot* function, from *Graphics* package was used to test the general diagnostics of the general linear models, while the function *shapiro.test*, from *stats* package was used to test the assumption of model residual normality. Bonferroni outlier detection test was performed with the function *outlierTest*, from *car* package. In those cases of extracts with significant negative slope in the linear regression, further tests to detect differences between concentrations were carried out. Specifically, Kruskal-Wallis tests were performed in order to detect global effects, using extract concentration as a

grouping factor. When global effects were significant, a post-hoc Nemenyi test was performed for the pairwise comparisons between concentrations. These analyses were performed with the *kruskal.test* function, from the stats package and the *kwallPairsNemenyiTest* function, from the *PMCMRplus* package.

For the microcosms experiment data, several general and generalized linear models were fit employing the functions *lm* and *glm* (the latter for two generalized linear models, with Poisson error distribution and with gamma error distribution) from the *stats* package and *glm.nb* (generalized linear model with negative binomial error distribution) from *MASS* package. The function *dispersiontest*, from *AER* package was employed to test for over-dispersion in the generalized linear model with Poisson error distribution. The function *simulateResiduals* from *DHARMa* package and *plot*, from *Graphics* package were employed to test the general diagnostics from the generalized and the general linear models, respectively. The function *ks.test*, from *stats* package was employed to perform the one-sample Kolmogorov-Smirnov test in order to test residual normality in the general linear model. Transformation of the dependent variable to optimize normality for the general linear model was performed with the function *bestNormalize*, from the homonym package. The function *Anova* from c*ar* package was employed to perform an analysis of variance (in the general linear model) or an analysis of deviance (in the generalized linear models). The function *AIC*, from the package *stats* was employed to apply the Akaike Information Criterion for model selection. As before, the function *outlierTest*, from *car* package, was employed to perform Bonferroni outlier detection test.

4. Results

4.1. Extract Assays

Growth of *C. ovalisporum* and *M. aeruginosa*, in the plant extract experiments, was analysed using simple linear regression models in which the dependent variable was the abundance of cyanobacteria in cells mL^{-1} and the independent variable were the different concentrations of plant extract. A single outlier was detected and removed from the four following assays: extracts of *Iris pseudacoros'*s roots and *Wolffia arrhiza*, tested against *M. aeruginosa*; extracts of *Nasturtium officinale*'s leaves and *L. punctata*, tested against *C. ovalisporum*. Table 1 and 2 show the coefficients of each linear regression model fit for the data obtained from the plant extract assays.

Table 1 - Results of the linear regression fits for the slopes for the plant extract bioassays against *M. aeruginosa*. Negative slopes represent a decrease in cyanobacterial cell abundance, with positive ones representing an increase in cell abundance. These results were statistically significant for *p*-values <0,05.

***Microcystis aeruginosa* bioassays**					
Plant Extract	**Slope**	**Std Error**	**t-value**	**Df**	***p*-value**
Iris pseudacoros (Leaves)	0.1433	0.0215	6.659	16	< 0.001
Iris pseudacoros (Roots)	0.0754	0.0194	3.888	15	< 0.01
Lemna minor	0.1933	0.0987	1.959	16	0.0678
Nasturtium officinale (Leaves)	0.1438	0.0237	6.079	16	< 0.001
Nasturtium officinale (Roots)	-0.0334	0.0240	1.392	16	0.183
Sparganium erectum (Leaves)	-0.0134	0.0699	-0.191	16	0.851
Sparganium erectum (Roots)	0.0182	0.0103	1.767	16	0.0963
Landoltia punctata	0.0705	0.0367	1.919	16	0.07297
Typha latifolia (Leaves)	-0.0107	0.0146	-0.736	16	0.472
Typha latifolia (Roots)	0.0510	0.0175	2.915	16	< 0.05
Wolffia arrhiza	0.0370	0.0062	6.004	15	< 0.001

While exposure to 3 of the extracts, namely the ones made from *N. officinale* roots, *S. erectum* leaves and *T. latifolia* leaves, respectively, showed a negative effect on the growth of *M. aeruginosa* none of them was statistically significant for an α = 0,05 (Table 1). On the contrary, at the same confidence levels, the extracts made from *I. pseudacoros* leaves and roots, *N. officinale* leaves, *T. latifolia* roots and *Wolffia arrhizal*, respectively, showed a statistically significant positive effect in the growth of *M. aeruginosa*. This could be explained, by an increase of the presence of macronutrients (and metabolites including growth promoters) from the plant tissues, which would result in a growth stimulation.

Table 2 - Results of the linear regression fits for the slopes for the plant extract bioassays against *C. ovalisporum*. Negative slopes represent a decrease in cyanobacterial cell abundance, with positive ones representing an increase in cell abundance. These results were statistically significant for *p*-values <0,05.

***Chrysosporum ovalisporum* bioassays**					
Plant Extract	**Slope**	**Std Error**	**t-value**	**Df**	***p*-value**
Iris pseudacoros (Leaves)	0.22638	0.01611	14.053	16	< 0.001
Iris pseudacoros (Roots)	**-0.06255**	0.02790	-2.242	16	< 0.05
Lemna minor	0.42220	0.16970	2.488	16	< 0.05
Nasturtium officinale (Leaves)	0.12224	0.04295	2.846	15	< 0.05
Nasturtium officinale (Roots)	0.07070	0.02086	3.389	16	< 0.01
Sparganium erectum (Leaves)	0.00133	0.04137	0.032	16	0.9748
Sparganium erectum (Roots)	0.13739	0.02754	4.99	16	< 0.01
Landoltia punctata	**-2.53230**	0.27130	-9.333	15	< 0.001
Typha latifolia (Leaves)	**-0.23832**	0.03252	-7.329	16	< 0.001
Typha latifolia (Roots)	0.15937	0.02923	5.452	16	< 0.001
Wolffia arrhiza	0.15560	0.02292	6.789	16	< 0.001

Regarding the *C.ovalisporum* assays, 3 extracts showed significant inhibitory effects on growth of this cyanobacteria: *I. pseudacoros* root extract, *L. punctata* extract and *T. latifolia* leaves extract (Table 2, Figures 6, 7 and 8). Otherwise, with the exception of the non-statistically significant effect of *Sparganium erectum* roots extract, every other

extract had a positive effect on *C. ovalisporum* growth. Once again, this could be explained by the assimilation of nutrients present in the plant extracts.

Despite the significantly negative effect predicted by our model, exposure to the *I. pseudacoros* root extract only showed a non-significant inhibition response on *C. ovalisporum* at the highest concentration of 2 mg/mL (Figure 6). In fact, at the lowest concentrations, there seems to an overall positive influence on the cyanobacteria growth rate. This drastic difference can be attributed to either a resistance to the allelopathic compound(s) at lowest concentration, coupled with the inherent addition of nutrients present in the extract , or possibly a hormetic effect, that is, the allelopathic compound being beneficial to *C. ovalisporum* at lower doses.

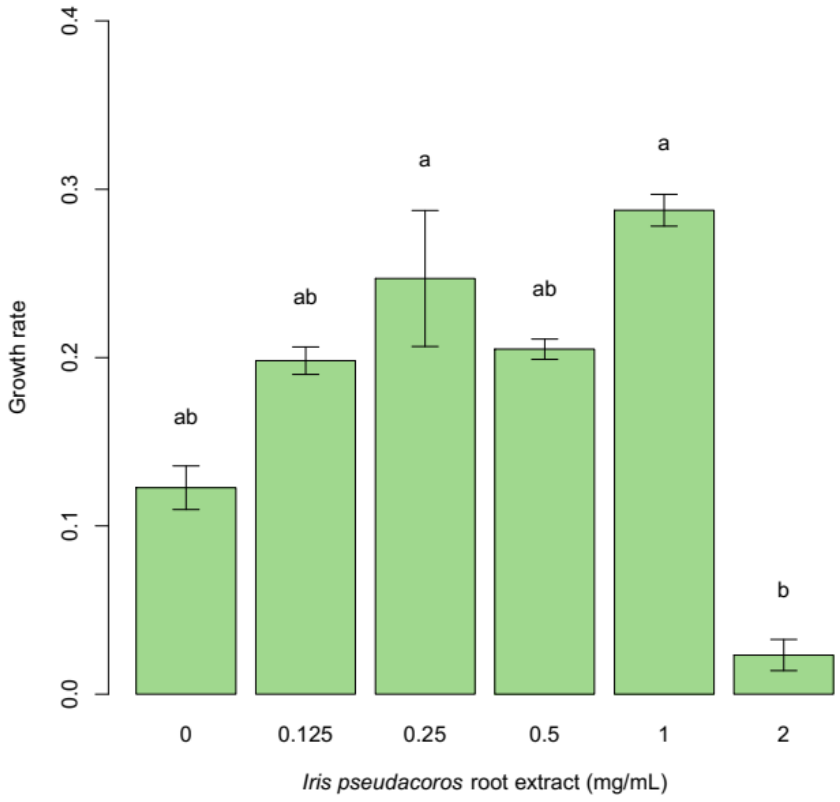

Figure 6 - Bar plot representing *C. ovalisporum* growth response to *I. pseudacoros* root extract.

The same low dose positive response was observed in *C. ovalisporum* treatment with *T. latifolia* leaves extract (Figure 7). In this case, however, only the two lowest concentrations (0,125 mg/mL and 0,25 mg/mL) caused positive growth. Additionally, at the highest concentration (2 mg/mL), cellular concentrations reached values lower than the initial, which could be considered an algicidal effect. Once again, despite the regression line's statistically significant negative slope, the most negative effect was still not significant when compared to the control group.

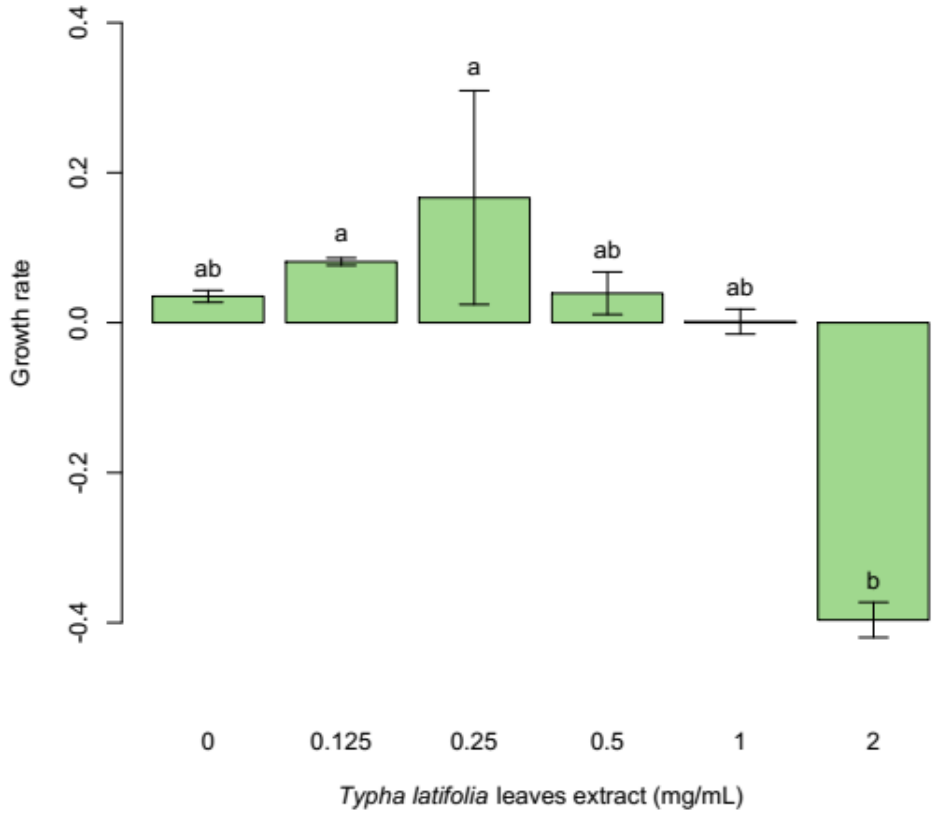

Figure 7 - Bar plot representing *C. ovalisporum* growth response to *T. latifolia* leaves extract.

Finally, exposure to *L. punctata* extract had a strong effect on *C. ovalisporum* growth (Figure 8). Cyanobacterial inhibition was observed at all tested extract concentrations, although only statistically significantly so, at the highest concentration of 2 mg/mL, in which the negative growth rate expresses a decline in the cyanobacteria population, compared to the initial cell abundance. These results led us to pursue further investigation on the allelopathic properties of *L. punctata*, selecting this macrophyte for the subsequent plant-cyanobacteria co-growth assays.

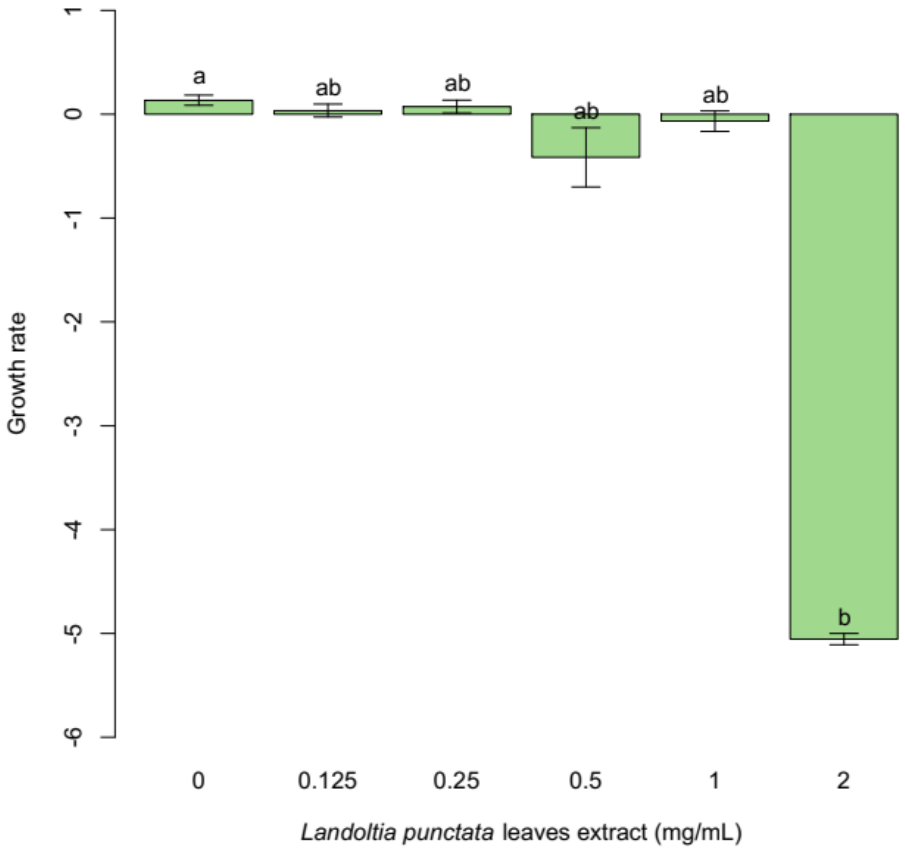

Figure 8 - Bar plot representing *C. ovalisporum* growth response to *L. punctata* extract.

4.2. Microcosm Assay

From the results of the plant extracts screening phase, the macrophyte which showed higher allelopathic potential was selected and tested in a microcosm experiment, to confirm the allopathic properties. Similar experiments could be replicated to validate the allelopathy of the other species, namely *T. latifolia* and *I. pseudacoros*.

Growth of *C. ovalisporum* in the microcosm experiment with *L. punctata* was analyzed using statistical models in which the dependent variable was the abundance of *C. ovalisporum* (cells mL^{-1}) over time, and the independent variables were time (day of experiment) as numerical covariate and presence/absence of plants and inoculum size (small/large) as categorical factors. An initial data exploration showed that the dependent variable followed a distribution similar to Poisson. For this reason, initially was fit a generalized linear model using Poisson distribution for the residual error. A dispersion test was applied to this model showing the existence of strong over-dispersion ($p < 0.001$). For this reason, the same model was fit using two alternative distributions: the gamma distribution (adding a constant to avoid 0) and negative binomial distribution, which can handle higher dispersion. Model formulations were optimized eliminating non-significant interactions. The most typical model diagnostics (quantil-quantil plots, normality test, residual dispersion tests and outlier tests) were not optimal for any of these two models, but particularly for the model with negative binomial distribution (Annex 1 and 2). In addition, a general linear model was fit after transforming the dependent variable to comply with normal distribution. This model showed better diagnostic than the previous two ones (Annex 3, and the residual normality assumption was met: Kolmogorov-Smirnov test of normality, $p = 0.71$). The Akaike Information Criterion (AIC) was employed to select the best among these three final models. This criterion considers the likelihood of each model, but penalizes complex models with a fixed penalty per parameter. For this reason, a complex model needs to be much better in terms of likelihood than a simpler one in order to counterbalance this penalty. The model with lower AIC will be the selected one. The application of this criterion resulted in a clear advantage towards the general linear model (Table 3).

Table 3 - Akaike Information Criterion values for each final model. Df are the degrees of freedom for the model, indicating the number of model parameters, and hence model complexity.

Models	AIC	df
GLM (Gamma)	3126.18	5
GLM (Negative Binomial)	944870.78	9
General linear	332.71	5

Table 4 shows the coefficients of the selected general linear model for the data obtained in the first 21 days of the experiment. After this period, the appearance and rapid propagation of contaminating species (*Chlorella spp*. and an unidentified filamentous cyanobacteria) may have had an effect on our target cyanobacteria. Because under these conditions, the effects on growth cannot be solely attributable to the allelopathic effect of L*. punctata*, the data from further days were eliminated from the statistical analysis. Despite this, both test groups showed a negative effect on *C. ovalisporum* abundance compared to the control groups, even with proliferation of the contaminating species (Figure 9).

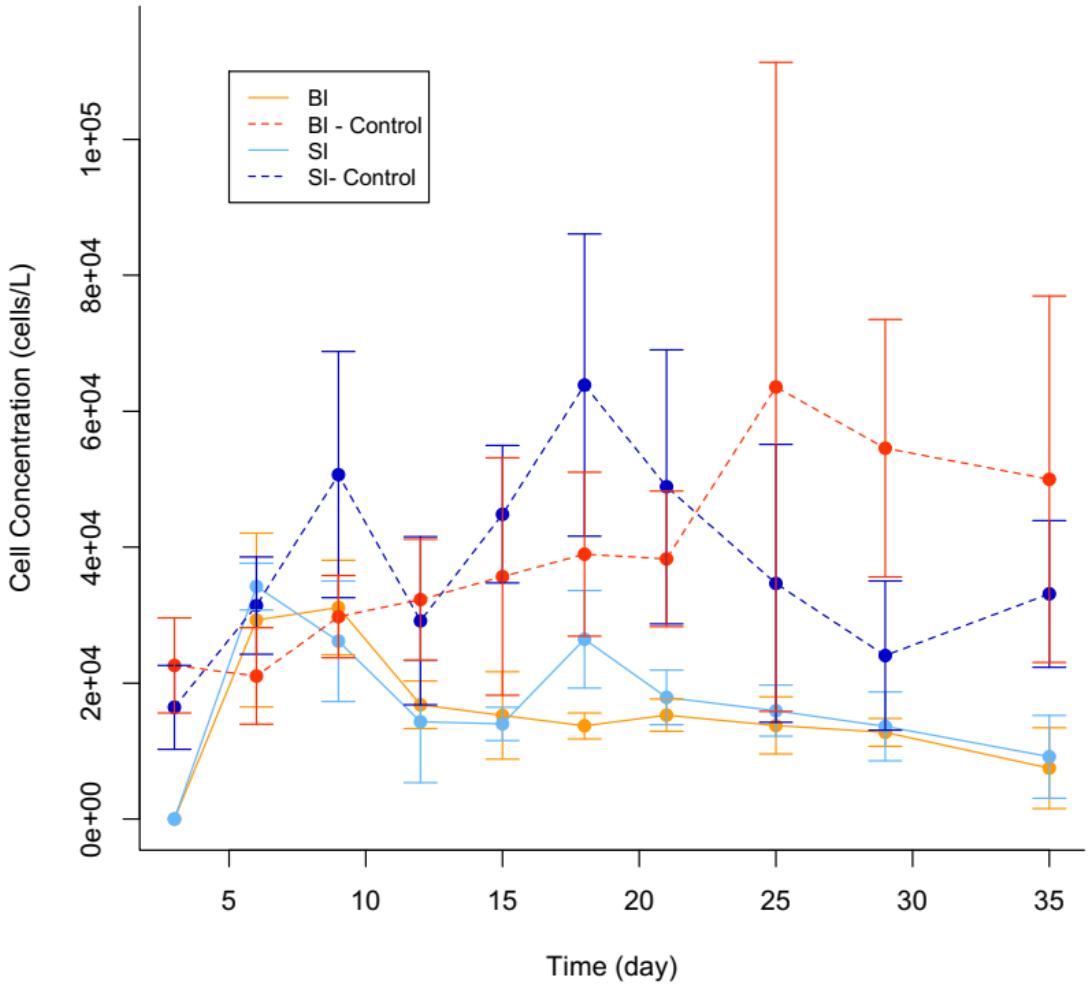

Figure 9 - *C. ovalisporum* cell abundance variation throughout the experiment duration. BI – Big Inoculum – denotes groups inoculated with 1 x 10^6 cells/mL. SI – Small Inoculum - denotes groups inoculated with 1 x 10^3 cells/mL. Data points represent the average values of 6 replicates.

The coefficients of the selected model (Table 4) showed a positive significant effect of time, and a lower mean growth of the cyanobacterium in the presence of *L. punctata*. Only the coefficient for the inoculum size was not statistically significant. This can be explained by the decreased effect of the initial cellular concentration on growth in the long-term. Under the same conditions, the growth rate would eventually equalize between inoculum sizes.

It was also expected time to have a statistically significant positive effect. This is reflected in the increase in the abundance of *C. ovalisporum* with time.

Finally, the presence of *L. punctata* revealed to have a significant negative effect on cyanobacteria growth in the 21 days of the assay. This result corroborates the inhibitory effect previously hypothesized in the extract assays.

Table 4 - Results of Analysis of variance applied to the selected general linear model. “Df” denotes degrees of freedom.

Source	Sum of Squares	Df	F value	*p*-value
Time	**16.913**	1	28.3091	**< 0.001**
Plant	**39.180**	1	65.5794	**< 0.001**
Inoculum Size	0.736	1	1.2322	0.26900
Time:Plant	0.474	1	0.7926	0.37493
Time:Inoculum Size	0.046	1	0.0774	0.78131
Plant:Inoculum Size	1.968	1	3.2944	0.07179
Time:Plant:Inoculum Size	0.822	1	1.3757	0.24295
Residuals	78.862	132		

The analysis of variance shows significant (< 0.05) *p*-values for factors Time and Plant. Since no interaction was significant, we can infer that these two factors influence the dependent variable, independently of each other. We can safely reject the null hypothesis – these factors do not influence growth rate of *C. ovalisporum* – and accept the alternative hypothesis that they, in fact, have an effect on cyanobacteria growth.

4.2.1. Nutrient quantification

Macronutrient analysis revealed no nutrient depletion throughout the experiment. While phosphate levels in the groups with plants reached a low point by the 10th day, the decrease in the controls was more sustained (Figure 10). This was due to the higher increase in cyanobacteria populations during the first 10 days. After nutrient replenishment (day 10), phosphate levels recovered in the groups with plants (Figure 10) but not in the controls. This was probably due to the inhibition effect of plants on cyanobacterial growth (Figure 9). During the whole course of the experiment, phosphate levels were considerably high, even more in the SI and BI groups than in the controls. In the groups with plants (SI and BI) it even increased from replenishment (day 10) to the end of the experiment (day 35). So, any *C. ovalisporum* inhibition occurring in-between the 10th and 35th day, could not be attributed to low phosphate levels.

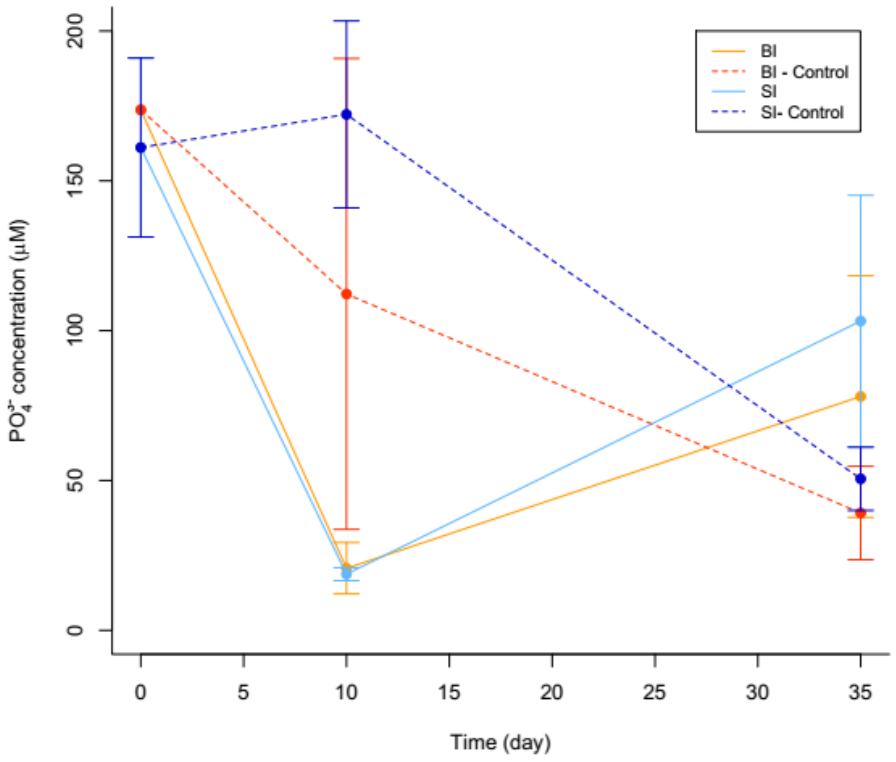

Figure 10 - Phosphate levels measured at 0, 10 and 35 days of the experiment. BI and SI are the same as in the previous plot. Data points represent the average values of 6 replicates in each group.

Regarding nitrate, its availability was not restricted at any point of the experiment (Figure 11). Analysis shows an increase in nitrate levels through the whole of the experiment's

duration. This can be attributed to unanticipated nitrogen inputs, specifically in the first 10 days of the experiment, when the differences were more accentuated and no supplementation had been performed. It should be noted that heterocyst formation was not observed at this point of the experiment. Regardless of the cause, this analysis proves there was no lack of NO_3^- that could impair *C. ovalisporum* growth and survivability.

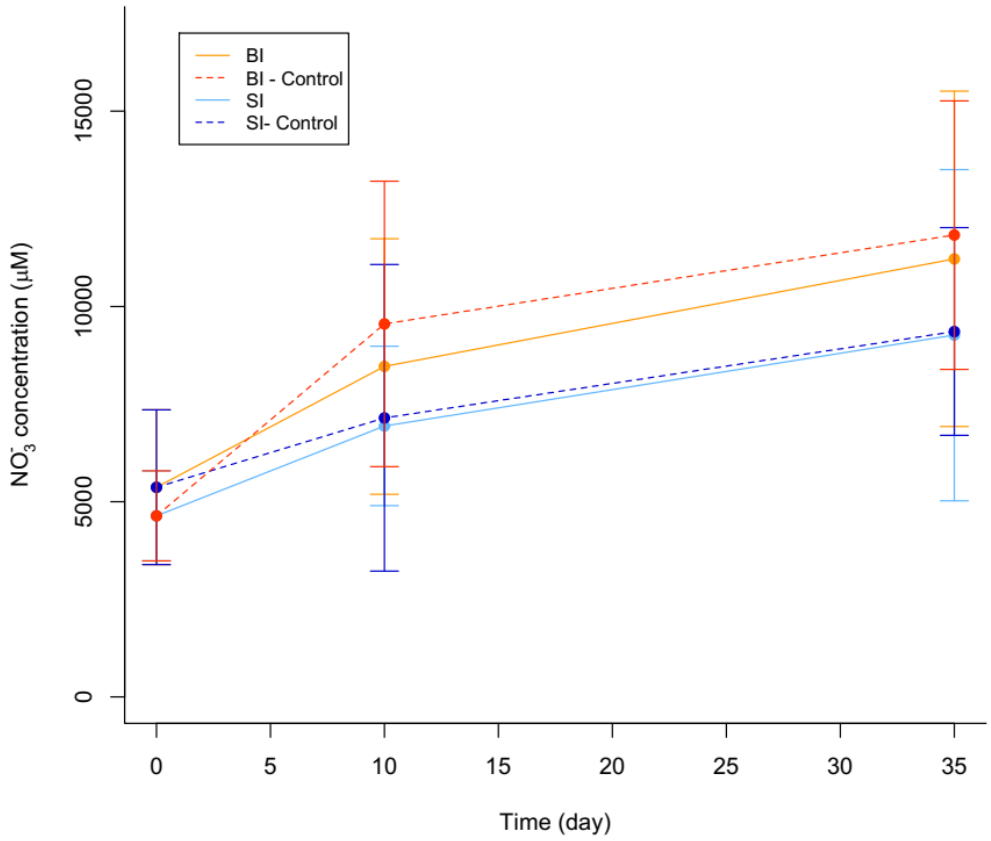

Figure 11 - Nitrate levels measured at 0, 10 and 35 days of the experiment. BI and SI are the same as in the previous plots. Data points represent the average values of 6 replicates in each group.

Overall, nutrient quantification further supports the thesis that *L. punctata* shows allelopathic activity towards *C. ovalisporum*.

5. Discussion

The main objective of this work was to find aquatic macrophytes with potential allelopathic effects that could be employed in the control of toxic cyanobacteria growth. With this in mind, a screening of aquatic macrophyte species, with putative allelopathic properties, was first carried out, through the use of plant extracts. Due to the difficulties in obtaining plant biomass, few individuals were collected from the majority of the plants species investigated. While care was taken to minimize this issue, the availability of specimens and/or different populations was low in most cases, which results in a lack of intra-species variability validation in this study. Furthermore, samples utilized to prepare the extracts were taken at a single time point, and thus reflect a particular physiological and metabolic condition of plants, which are strongly determined by the environment (and the season). Additionally, the use of extracts does not take into consideration the possibility of allelopathic mechanisms triggered or expressed exclusively in response to the presence of cyanobacteria in natural environment. In fact, *T. latifolia* was collected from ponds likely contaminated with cyanobacteria, which may have influenced the plant to respond by producing allelopathic compounds effective against *C. ovalisporum*. Notwithstanding all these considerations, the results of the extract bioassays showed significant inhibitory effects on the growth of *C. ovalisporum* when exposed to *I. pseudacoros* root extracts, *T. latifolia* leaves extracts and *L. punctata* extracts. The hormesis effect observed in the *I. pseudacoros* assay could possibly be caused by the presence of compound(s) which, in low concentrations, stimulate the growth of cyanobacteria, while being toxic at higher concentrations (Calabrese et al. 2007). It could also be explained by simple nutrient enrichment through macronutrients, such as sugars, present in the extract and the ineffectiveness of the active allelopathic compound(s) at lower concentrations. These explanations also apply to the less pronounced hormetic effect caused by exposure to *T. latifolia* leaves extract. Of the three macrophytes, *I. pseudacoros* shows the least allelopathic potential. Nevertheless, the presence of the allelopathic activity in extracts prepared from the tissues of its roots is of interest to EFB applications where the root system is in more contact with the cyanobacteria contaminated water. On the other hand, while *T. latifolia* active compound presence in aerial tissues does not invalidate the potential effectiveness on EFB/CW mediated bloom control, it does imply the necessity of an internal transport to the lower tissues, if not to rely on possible external vectors, such as the wind and the rain.

Overall, the best candidate from this screening phase, proved to be *L. punctata* having shown not only an inhibitory effect at the lowest concentrations, but a dramatic algicidal effect at the two highest concentrations. Moreover, the increased water contact and fast propagation of this small macrophyte is ideal for the purpose of bloom control through allelopathic compounds release. As such, investigation proceeded with this species.

In the microcosm assays, the difficulty in creating an environment more protected from biocontaminants was the major challenge. Ideally, *L. punctata* would have been micro propagated through multiple generations in sterilized medium, in order to minimize the presence of microorganisms in the surface of the plants. Even though a small, one week, period of acclimation to the growing medium allowed some removal of debris and other contaminants and the propagation of fresh individuals, microscopy observation of the various samples showed an increasing number and variety of microorganisms. While these did not have a significant impact in nutrient consumption and may even have been responsible for a slight increase in nitrogen levels, they may have directly affected cyanobacterial growth, through shading or even by their own allelopathic effects. Having this in consideration, data was only analysed for the first 21 days of the experiment, during which the growth of control plants could guarantee that conditions were still optimal, and inhibitory effects in the treatments only attributable to the release of compounds by L. *punctata*. The results obtained corroborate the existence of inhibitory effects already observed in the extracts assay phase for this species. Nutrient quantification validates the allelopathic inhibition hypothesis, having ensured no nutrient depletion occurred during the experiment.

While our treatment with *L. punctata* methanolic/aqueous extract did not show a significant inhibitory response on the growth of *M. aeruginosa,* **Dan et al.** (2022) observed lower biomass accumulation with petroleum ether, dichloromethane and ethyl acetate extract treatments, the latter of which also showed oxidative stress and photosynthetic systems interference, through lower chlorophyll a levels and phycobilin protein synthesis inhibition. It would be of interest to investigate the in-vivo activity of *L. punctata* against *M. aeruginosa*, as well as assess if effects similar to the ones caused by the plant's ethyl acetate extract on *M. aeruginosa* were responsible for *C. ovalisporum* growth inhibition in our co-culture assays.

Further research may involve testing the potential of this plant in an increased experimental scale in terms of space, cyanobacterial abundance and time of exposure. This could be performed employing methodologies such as EFBs and CWs, which could mitigate the effects of an active bloom and/or prevent their formation, while at the same

time, having limited detrimental effects to the local environment. Finally, the isolation, purification and characterization of the allelochemicals present in *L. punctata* should be conducted along with the investigation of their cyanobacteria inhibition mechanisms.

6. Conclusion

The main aim of this project was reached, by identifying three aquatic macrophyte species whose extracts show potential allelopathic effects against *C. ovalisporum,* although no plant extracts showed significant inhibitory effects against *M. aeruginosa*. Of these three species, one was confirmed to have in-vivo inhibitory effects on the cyanobacteria growth. This plant, *L. punctata,* showed potential as a biotic agent to control *C. ovalisporum* blooms.

Additionally, the two other potentially allelopathic plants, *T. latifolia* and *I. pseudacoros* are interesting subjects to a more in-depth investigation on their effect on *C. ovalisporum,* akin to the microcosm assays performed with *L. punctata*. All three macrophytes can also be tested against other common HAB forming cyanobacteria, not considered in this project. Furthermore, these studies should be extended to other relevant phytoplankton and zooplankton species to infer possible undesirable ecological effects with the use of these species in natural environments and to disclose the selective inhibition towards toxic cyanobacteria

Finally, while no significant negative result was found in the *M. aeruginosa* assays, the inexpensive and quick methodology developed in this work, allows for a continuation of the plant allelopathy investigation on the control of this and other cyanobacteria.

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Annexes

Annex 1 – Gamma distribution generalized linear model diagnostics

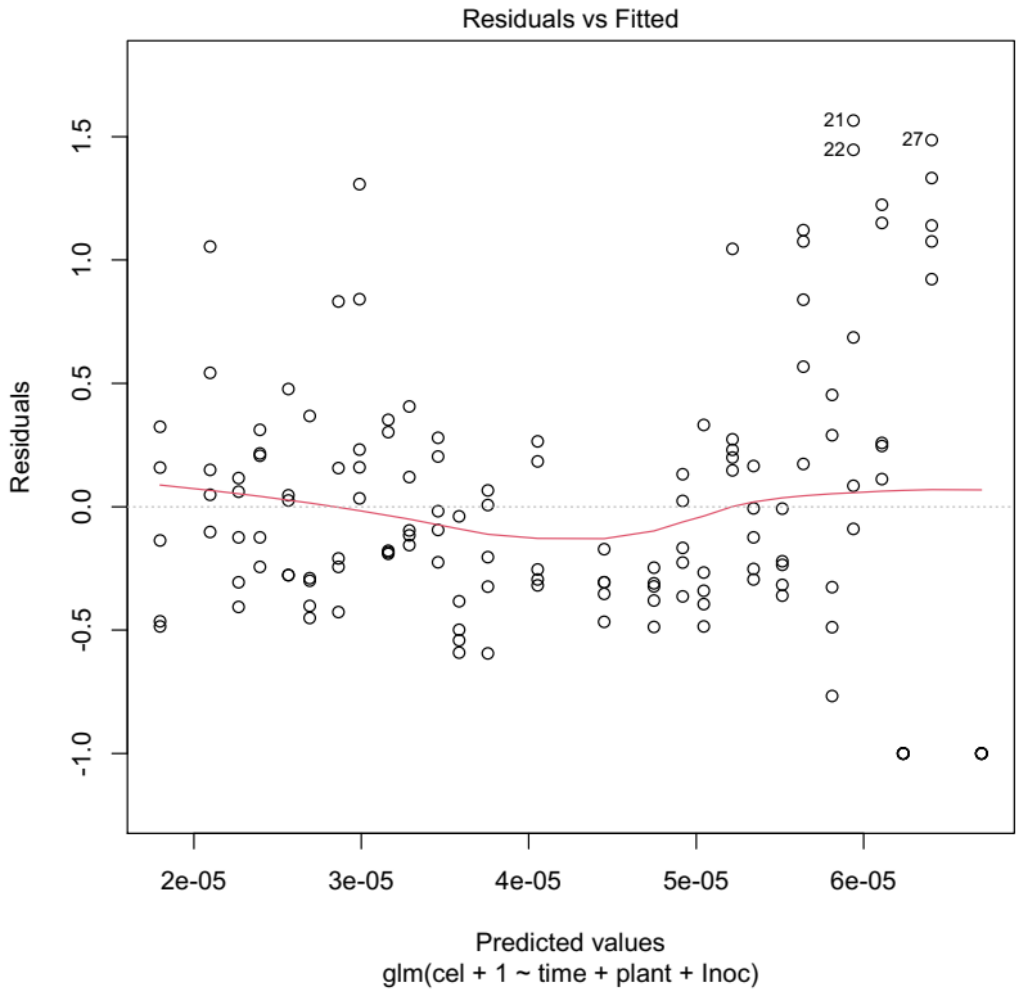

Figure 12 - Residuals vs Fitted plot for the Gamma distribution generalized linear model.

Annex 2 – Negative binomial distribution generalized linear model diagnostics

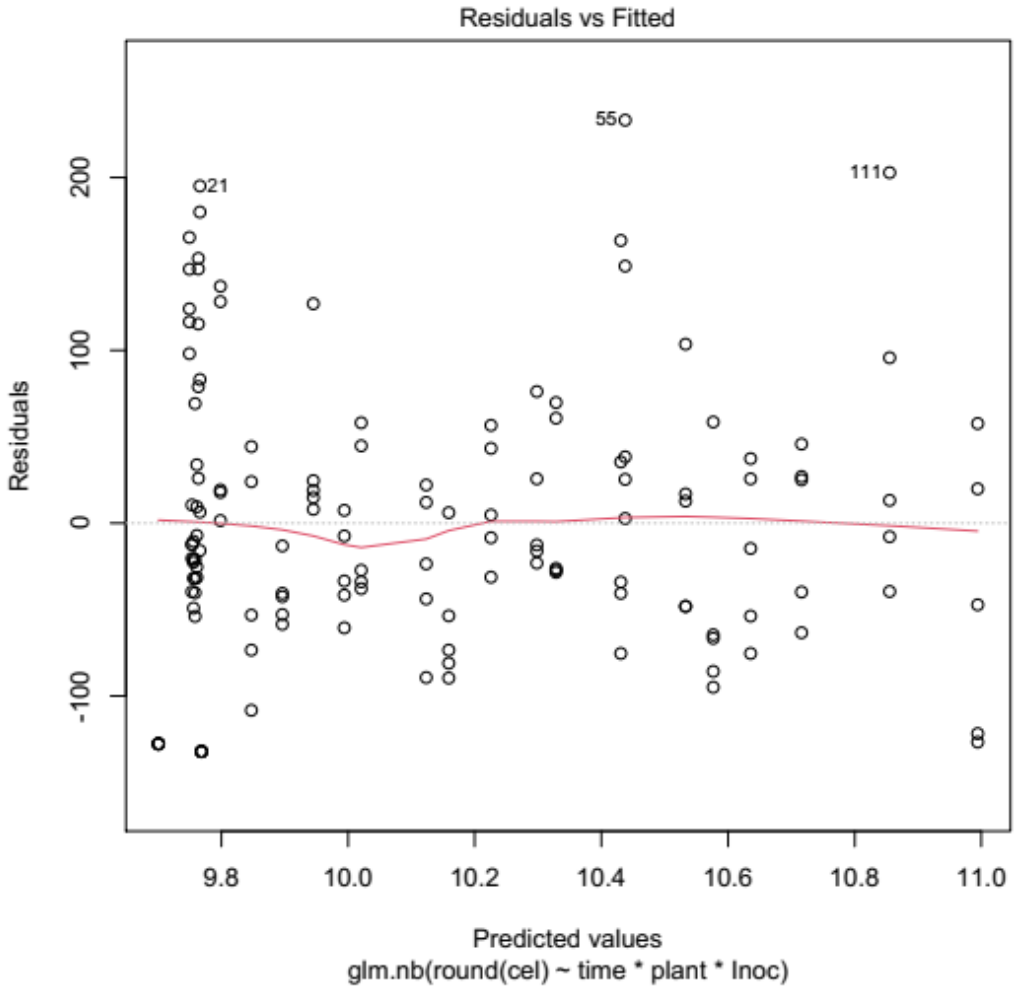

Figure 13 – Residuals vs Fitted plot for the negative binomial distribution generalized linear model.

Annex 3 – General linear model diagnostics

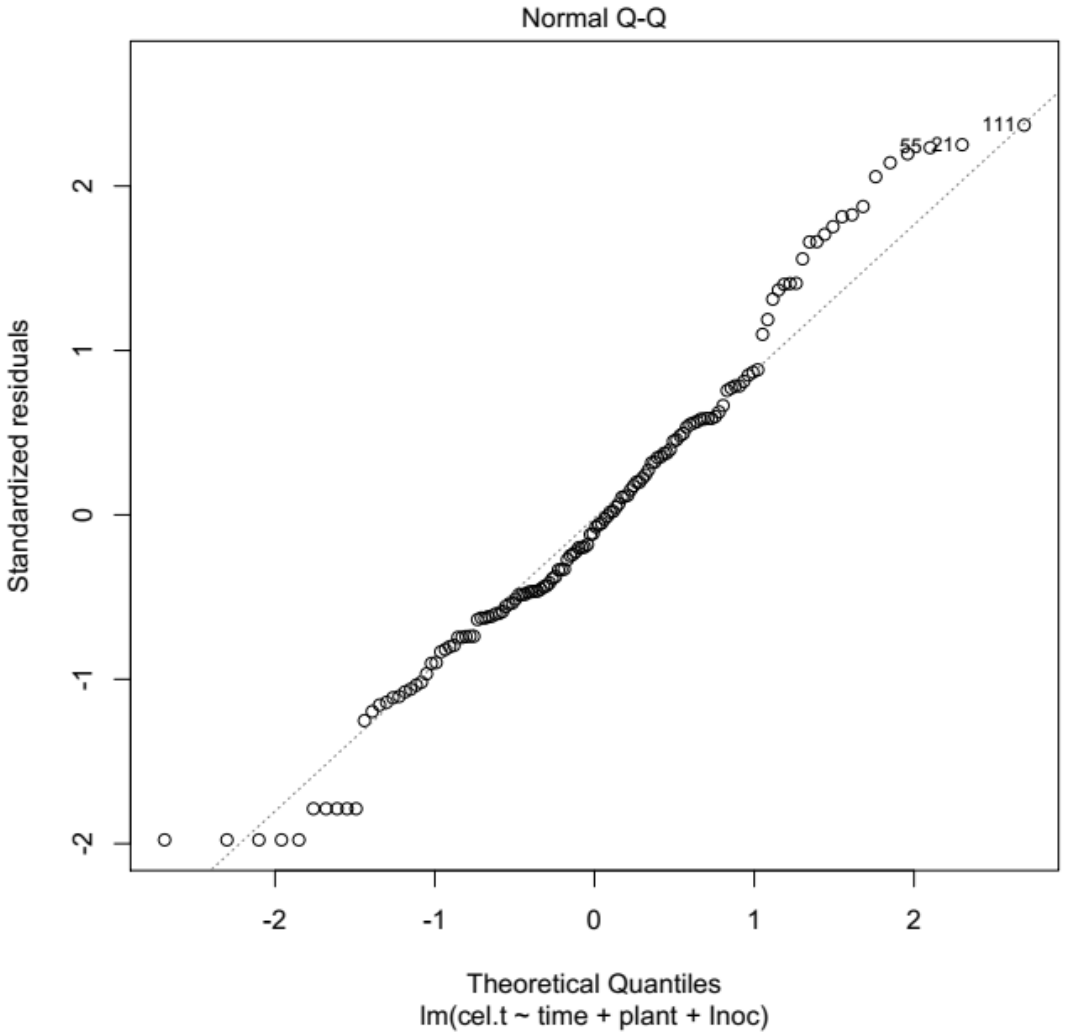

Figure 14 - Quantile-quantile plot for the selected general linear model.

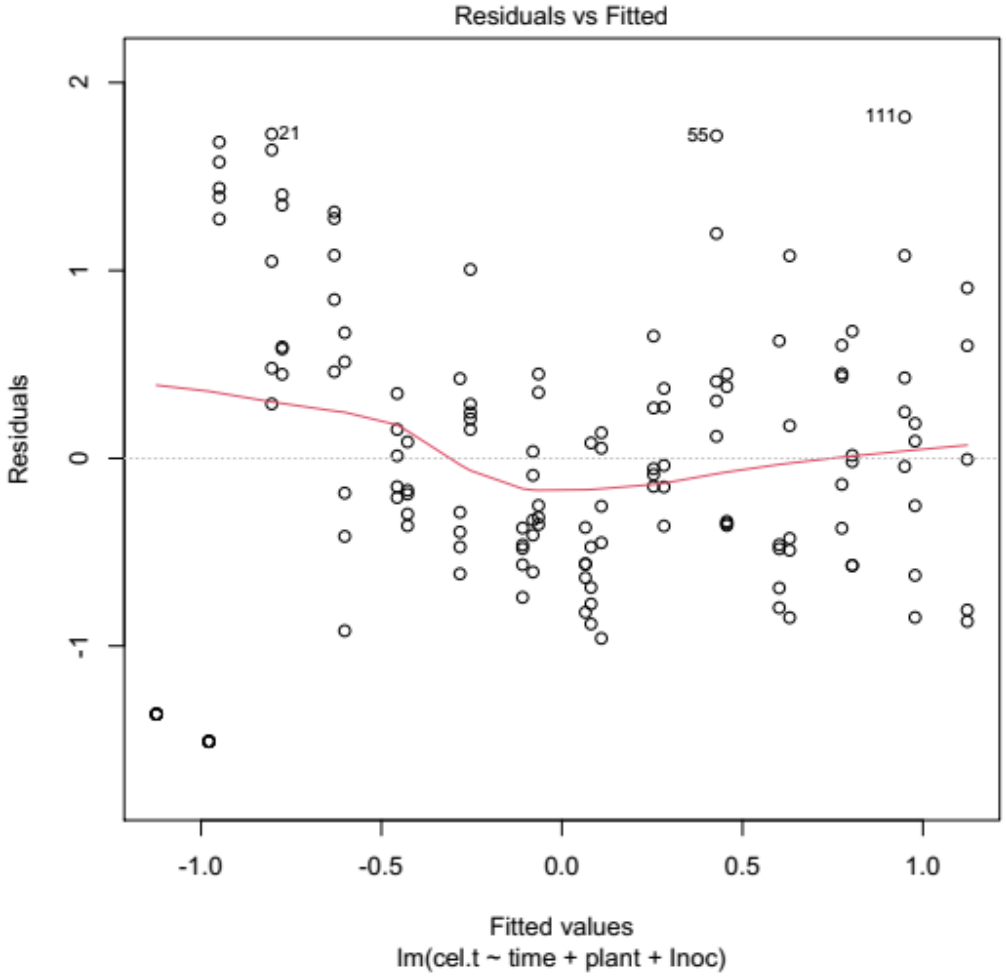

Figure 15 - Residuals vs Fitted plot for the selected general linear model.