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Screening of plant extracts for a microcosm in vivo test of inhibition of the toxic bloom-forming cyanobacteria *Chrysosporum ovalisporum*

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ABSTRACT

Freshwater eutrophication is an increasing ecological and public health concern. 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 investigate a nature based solution to this environmental hazard. Our approach consisted in the evaluation of the potential of aquatic plants to control the growth and viability of *Chrysosporum ovalisporum,* a cyanobacteria species associated with HABs and that produces toxic metabolites such as cylindrospermopsin. First, we carried out a screening with 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 this cyanobacteria. For this purpose, methanol/water (70:30, v:v) mixture extracts were prepared and tested, at various concentrations, in *C. ovalisporum* cultures. Exposure to *L. punctata* extracts at the highest concentration tested (2 mg L^{-1}) showed significant cellular abundance inhibitory effects on *C. ovalisporum*. Secondly, a microcosm system was assembled to evaluate the effects of co-growth of *C. ovalisporum* and *L. punctata*. In this experiment, we tested the effect of the presence of this plant on *C. ovalisporum* cell abundance, under a nutritional surplus and equivalent cyanobacterial light exposure in between test and control groups. The results confirmed the allelopathic effect of *L. punctata* on *C. ovalisporum*. This work demonstrates that the allelopathic properties of aquatic plants could be exploited to control the growth of harmful algae, and could be used to develop novel, eco-friendly and practical solutions, for treatment of water contaminated with *C. ovalisporum*.

1. Introduction

Algal blooms are characterized by an increase in the growth of microalgae reaching high abundances [1]. Algal blooms can further be defined as harmful due to the impairments caused in the environment, public health and economy [2]. Many HAB forming species produce toxins that can cause severe poison effects in other organisms [3]. Anoxia can affect invertebrates and vertebrates, overshading affects the phytoplankton community, macroalgae and submersed marcophytes. All these effects can lead to changes in the food-web, heavily impacting the local biota [4]. Furthermore, HABs can produce noxious odours and irritating particles, affecting recreational use of water bodies with economic impacts on the tourism industry [5,6].

The increased frequency and intensity of HABs worldwide is a major consequence of the eutrophication of aquatic systems. The natural eutrophication of a water body is representative of its aging process [7]. Additionally, meteorological conditions, with emphasis to wind and temperature, also contribute to natural episodic and seasonal eutrophication events, through resuspension of nutrients by water column vertical mixing [8,9]. On the other hand, cultural eutrophication can be defined as the acceleration of the nutrient enrichment of water through human influence. Aquaculture intentional water enrichment to increase productivity [10,11] industrial, sewage and other urban effluents or agricultural runoffs aggravated by excessive fertilization and manure production [12] are some examples of anthropogenic sources of nutrients.

While environmental measures, such as wastewater treatment and wastewater pollutant control, can and should be taken to prevent severe

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eutrophication of water bodies, other strategies are needed to prevent the rise of HAB outbreaks in water bodies [13]. These can be categorized in physical, chemical, and biological strategies [14].

Physical control of HABs involves methods such as pumping and mixing water to break water column stability [15], sonication [16] or the release of materials in the water, e.g. clay, to flocculate HAB cells, facilitating its removal by sedimentation [17]. Physical strategies can be limited to applications in small volumes of water [14]. Furthermore, some can be harmful to other aquatic organisms [18]. Chemical control consists in the use of chemicals that affect the viability of HAB species. Pesticides, like diquat and terbutryn are common chemical treatments used in the control of cyanobacteria [19]. These two non-selective compounds, affect any type of organism while other algicides, can have a more selective effect, such as atrazine and diuron, which inhibit the electron flow in photosystem II, therefore impairing mainly photosynthetic organisms [20,21]. Even though these chemicals have a more cyanobacteria-specific action, other organisms could be also affected, and in the case of diuron, it has a high persistence in the sediments and its degradation generate genotoxic substances which results in even more organisms being affected in the long term [21]. 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 [22–24]. 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 [25]. Lastly, biological control consist in the use of living organisms to suppress HABs or diminish its impacts. This approach has led to the study and identification of several HAB control candidate species that offer a potential cost effective and green alternative to other more ecologically adverse methods. For instance, algal grazing by certain species of fish, bivalve molluscs and zooplankton [26,27], contributes to control and balance phytoplankton in aquatic environments. Furthermore algal antagonistic properties, have been identified in microorganisms such as bacteria, protozoa, and fungi [28–30]. These microorganisms can act indirectly on algae, through nonspecific secretion of metabolites that inhibit algal growth or through the release of cyanobacteriolytic substances that cause cell death [31,32]. Viruses also play a role in the control of blooms. While still a growing research field, cyanophages may provide another potential HAB control method, with the advantage of being highly specificity or selective [33,34]. Finally, many aquatic plants have shown allelopathic effects on cyanobacteria, with hundreds of allelochemicals having been extracted and identified [35]. Seaweeds in particular, are an important source of bioactive compounds with known anti-cyanobacterial activity [36]. Telluric plants can also produce allelochemicals effective against algae, such as artemisin, isolated from *Artemisia annua*, which has shown strong inhibitory effects on *Microcystis aeruginosa* [37]. The major plant components with algicidal properties are phenolic acids, fatty acids/esters, alkaloids, terpenoids and flavonoids. These compounds however degrade quickly by photodegradation and oxidation reactions [35,38]. This disadvantage must be considered, notably in applications of the isolated allelochemicals. In-vivo allelopathic activity has also been reported, for example L. *trissulca* impaired growth of *Dolichospermum flos-aquae*, *Raphidopsis raciborskii* and *M. aeruginosa* while, simultaneously reducing cyanotoxin concentrations [39]. Macrophytes 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 including microalgae and cyanobacteria [40]. These characteristics, as well as the ability of certain species to uptake, accumulate and/or metabolize toxins and heavy metals, can be applied in environmental-friendly applications like constructed wetlands (CWs) and ecological floating beds (EFBs) as a means to purify aquatic ecosystems [41,42]. 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 the cyanobacteria [43].

Chrysosporum ovalisporum, previously known as *Aphanizomenon ovalisporum* [44] is a cyanobacteria belonging to the Nostocales order, of filamentous and heterocystic nature and capable of toxin production [45]. Among other cyanobacteria, like *Cylindrospermopsis raciborskii,* from which the toxin acquired its name, it is one of the main producers of cylindrospermopsin (CYN) [46] a highly water-soluble tricyclic alkaloid [47,48]. CYN is traditionally classified as a hepatotoxin but it can also affect other organs such as the eye, lungs, heart, spleen and kidneys [49]. Besides causing cytotoxic effects, it has been referred to be genotoxic and carcinogenic, with its known toxicity mechanisms including protein synthesis inhibition and increased oxidative stress [50–52]. *Chrysosporum* spp. toxic blooms have been reported in many countries, e.g. Turkey, Israel, Greece, Spain, Italy, Australia and Portugal [45,53,54].

With this in mind, we aimed to tackle the occurrence of HABs in the environment through the investigation of allelopathic activity of different aquatic plants on the growth of *C. ovalisporum.* After a screening for the best candidate macrophyte, *L. punctata,* whose extract showed highest allelopathic potential, was then used in a co-growth microcosm experiment. The results of this investigation should give us insight on macrophyte species with potential to develop nature based technologies, namely EFBs and CWs, pursuing biological control of cyanobacterial blooms.

2. Methods

2.1. Plant collection and sample preparation

Plant species were selected based on their natural environment, with only fresh water macrophytes being used, as these would stand to be the most probable to have evolved defence mechanisms towards the targeted cyanobacteria. The selection was further restricted by local availability and native classification. Specimens were harvested from different gardens in Porto city, Portugal. The majority of plant species were granted by Porto Botanical Garden, including *I. pseudacoros*, *A. lanceolatum*, *A. plantago-aquatica and* duckweeds *W. arrhiza* and *L. punctata*. The third duckweed, *L. minor* was collected from two small ponds in Parque da Cidade, Porto. *T. latifolia* specimens were collected from the margins of two lakes also located in this park. *S. erectum* was obtained from the riverbank of a shallow water area in Ribeira da Granja, Porto. *N. officinale* was purchased from a local market. For the cyanobacteria inhibition assays with plant extracts, plants were obtained in late autumn 2021, while the *L. punctata* used in the microcosm assay was collected in the summer 2022.

Only one adult specimen of *I. pseudacoros*, *A. lanceolatum* and *A. plantago-aquatica* was possible to acquire due to limited availability. For the other selected species, an increased number of specimens at different growth stages were collected to ensure a more representative sample. Individual specimen data was not registered, as the goal was not to compare intraspecific metabolic differences, but to estimate the species allelopathic potential, as a whole.

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. The plant material was stored at −20 °C for no >3 weeks, until further processing. The material was transferred to −80 °C for at least 2 h, before freeze drying for 3 days or more. Finally, the lyophilized plant material was macerated with mortar and pestle and stored at room temperature.

2.2. Plant extracts preparation

Plant extracts were prepared using a mixture of methanol and water, in a 7:3 (v:v) ratio. This solvent was chosen to enhance the extraction of a wider range of polar compounds, increasing the chemical diversity of the extracts and hypothetically their biological activity. A volume of 50

mL of solvent mixture was used for every 200 mg of plant tissue powder. To further increase solvent penetration, the biomass was soaked for 72 h 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, with a bath temperature set between 30 °C and 35 °C and under incrementally reduced pressure until full dryness. The resulting powder was weighed and stored at −20 °C until required for the bioassay.

2.3. Cyanobacteria growth and maintenance

Cyanobacteria was provided by LEGE-CC culture collection (https://lege.ciimar.up.pt/). *Chrysosporum ovalisporum* CYN producer strain (LEGE X-001), was transferred to 40 mL of Z8 culture media, prepared as in Kotai (1972), and grown for 1 month. Afterwards, cultures were upscaled initially to culture flasks with 400 mL and after to 20 L cultures. 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.

Cultures were grown at 25 °C, 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 cyanobacteria used in the assays, regular fresh medium replenishment and microscopic observations were performed. For nutrient and oxygen replenishment, roughly 20 % of old medium was discarded and the same volume of fresh Z8 medium was added. Every culture manipulation occurred under sterile conditions to avoid contamination.

2.4. Cell concentration estimation

An optical density calibration curve was determined to estimate cyanobacteria cell concentration (A 1). The calibration curve was determined by measuring the absorbance at 420 nm of 7 different known *C. ovalisporum* cellular concentrations in Z8 growth medium. The various concentrations were diluted from *C. ovalisporum* culture with cell 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 (Berlin, Germany) with a MS72 probe at 20 KHz frequency and 10 % amplitude for 45 s. Microscopy observation confirmed this process did not affect cell integrity.

In the microcosm experiment, cyanobacteria cell abundances were estimated through cell counts in Utermöhl chambers, using an inverted microscope and 1 mL culture samples.

2.5. Cyanobacteria inhibition assays with plant extracts

To screen the allelopathic activity of the different plants against the cyanobacteria, assays using the previously prepared extracts, were performed. The methodology was adapted from Tazart, et al. [55]. The experiments were prepared in 24 well plates. The wells were inoculated with the necessary volume of cyanobacteria culture in exponential phase, to obtain 7×10^6 cells mL^{-1} as final concentration, in a volume of 2,5 mL. Different plant extract concentrations (0,125 mg mL^{-1}, 0,25 mg mL^{-1}, 0,5 mg mL^{-1}, 1 mg mL^{-1} and 2 mg mL^{-1}) were added. The treatment solutions were obtained by diluting the previously prepared plant extracts (Section 2.2) in Z8 medium and subsequently centrifuging them to remove any insoluble particles that might affect light absorbance measurements. Due to possible loss of mass during centrifugation, the final solution concentrations were estimates. Each stock solution had a different concentration due to differences in vial volume and extract mass. The treatments and a negative control with no extract added, were replicated 3 times ($n = 3$). Finally, the absorbance of each plant extract and the absorbance of Z8 medium were also monitored. This provided a baseline allowing us to calculate the absorbance by cyanobacterial cells. The assays were carried out under the same growth conditions as described above, and had a total duration of 72 h. The estimates of cell density were taken only at initial and final time points.

2.6. Microcosm assay

This experiment was carried out with *L. punctata* which showed promising allelopathic activity towards *C. ovalisporum* in previous inhibition assays the screening phase. Furthermore, this duckweed was deemed more advantageous over the other potentially allelopathic species, due to its easy of handling and maintenance in the laboratory and fast growth [56].

The methodology employed consisted in the co-culture of the cyanobacteria with the aquatic plants in 250 mL beakers in a greenhouse. The plants were tested against 2 different densities of *C. ovalisporum*: 1×10^3 cells mL^{-1} (Lower Inoculation Density, LID) and 1×10^6 cells mL^{-1} (Higher Inoculation Density, HID). 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 [57]. For the experiments 1 g (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 ($n = 5$).

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 estimate cyanobacteria abundances. 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.

2.7. Nutrient quantification

Nitrate ion (NO^{3-}) and Phosphate ion (PO_4^{3-}) quantification 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-}.

2.8. Statistical analysis

In the microcosm experiments, a general linear model was performed in order to test the effect of *L. punctata* on the development of *C. ovalisporum*. The abundance of *C. ovalisporum* was the dependent variable and as independent variables were employed the treatments ‘plant’ (control versus presence of *L. punctata*), ‘inoculum’ (higher versus lower inoculation density) and the covariates ‘days’ (log-transformed) and ‘presence of contaminant microalgae’ during the course of the experiment. For this variable, the biovolumes of the species were approximated to the volume of the most similar geometrical shape, and the biovolumes of all the species detected were summed up to conform a single variable. The model was fit with the functions *lm*, from R package *stats*, and the assumption of residual normality with the function *shapiro*.

test, from the same package. Outliers were check with the function *outlierTest* from the *car* package. It was necessary to transform the response variable with the Box-Cox transformation, implemented with the *boxcox* function from package *MASS*. An analysis of variance was performed with the function *Anova*, from *car* package.

3. Results

3.1. Allelopathy screening - extract bioassay

Growth of *C. ovalisporum*, in the plant extract experiments, was analyzed 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 assays using extract of *N. officinale* leaves and extract of *L. punctata*. Table 1 shows the slopes of each linear regression and their significance for the data obtained from the plant extract assays.

In these initial allelopathy assays, 3 extracts showed significant inhibitory effects on growth of the cyanobacteria: *I. pseudacoros* root extract, *L. punctata* extract and *T. latifolia* leaves extract (Table 1, Fig. 1). Otherwise, with the exception of the non-statistically significant effect of *S. erectum* roots extract, every other extract had a positive effect on *C. ovalisporum* growth.

Exposure to *L. punctata* extract had a strong effect on *C. ovalisporum* growth (Fig. 1). Cyanobacterial inhibition was observed at all tested extract concentrations, although only statistically significantly so, at the highest concentration of 2 mg mL^{-1}, in which the treatment led to a complete decline in the cyanobacterial population. 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.

Despite the significantly overall negative effect shown by the regression model, exposure to the *I. pseudacoros* root and *T. latifolia* leave extracts showed a bi-phasic pattern (Fig. 1). At the lowest concentrations, there was a positive effect of these extracts on the cyanobacteria cell abundance, and a clear inhibition of *C. ovalisporum* was only observed at the highest concentration (2 mg mL^{-1}).

3.2. Allelopathy bioassay in microcosm

The total number of data for this analysis was 197, after removal of 3 outliers. Cell quantification showed both test groups had negative effect on C. ovalisporum abundance compared to the control groups (Fig. 2). The analysis of variance and linear model showed a significant negative effect of the presence of *L. punctata* plants on the growth of *C. ovalisporum* (Table 2) whereas the inoculum size did not have a significant effect and the presence of contaminant microalgae had a significant positive correlation, as their populations were developed at the same time as *C. ovalisporum*, and were also repressed by the presence of *L. punctata*. This result indicates that the contaminant microalgae did not inhibit the growth of *C. ovalisporum*.

Table 1
Results of the linear regression fits for the slopes for the plant extract bioassays against *C. ovalisporum*.

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

3.3. Microcosm 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 (Fig. 3). 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 (Fig. 3) but not in the controls. This was probably due to the inhibition effect of plants on cyanobacterial growth, which reduced consumption of this nutrient. In the groups with plants (LID and HID) phosphate concentrations increased from replenishment (day 10) to the end of the experiment (day 35). Overall, the large initial concentrations of phosphate and the slight trend of increase after replenishment (in the presence of plants) indicates that there was no shortage of phosphate for *C. ovalisporum* during any period of the experiment. Therefore, low or negative growth of this cyanobacteria cannot be attributed to limitation by this nutrient.

Regarding nitrate, its availability was not restricted at any point of the experiment (Fig. 3). Analysis shows an increase in nitrate levels through the whole experiment. 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 nitrate that could impair *C. ovalisporum* growth and survival.

4. Discussion

The main objective of this work was to survey aquatic macrophyte species with potential to develop biocontrol methods of cyanobacteria growth. With this in mind, a screening in 7 aquatic macrophyte species was first carried out, to test allelopathic properties, using plant extracts. The results obtained do not account for intra-species genetic variations, environmental factors, or seasonal variations in the synthesis and accumulation of bioactive/allelopathic compounds in the plant tissues, since all plant extracts were prepared from a small number of plant material collected one time in October 2021. 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. Notwithstanding all these considerations, the results obtained from extract bioassays showed significant inhibitory effects on the growth of *C. ovalisporum* when exposed to *L. punctata* extracts. Besides the dramatic algicidal effect at the highest concentration, the increased water contact and fast propagation of this small macrophyte make this species ideal for the purpose of bloom control. As such, investigation proceeded with this species. Contrarily, extracts from other macrophytes studied, e.g. *I. pseudacoros, L. minor, N. officinale, S. erectum, T. latifolia and W. arrhiza* showed a stimulatory effect on the growth of the cyanobacteria. These extracts, most likely, do not comprise allelopathic substances against cyanobacteria furthermore, provided an additional input of nutrients, nitrogen or phosphorus in organic form (easier to assimilate by the cyanobacteria than the inorganic forms) and other biomolecules including growth promoters, that overall contributed to stimulate growth of *C. ovalisporum*. The pattern of growth shown with exposure to *T. latifolia* and *I. pseudacoros* extracts is consistent with a hormetic effect (Fig. 1). Extracts of these species hold a mixture of substances that, at low concentrations stimulate *C. ovalisporum* cultures but at higher concentrations inhibit the growth of cyanobacteria.

In the microcosm assays, the difficulty in creating an environment more protected from biocontaminants was the major challenge. Ideally,

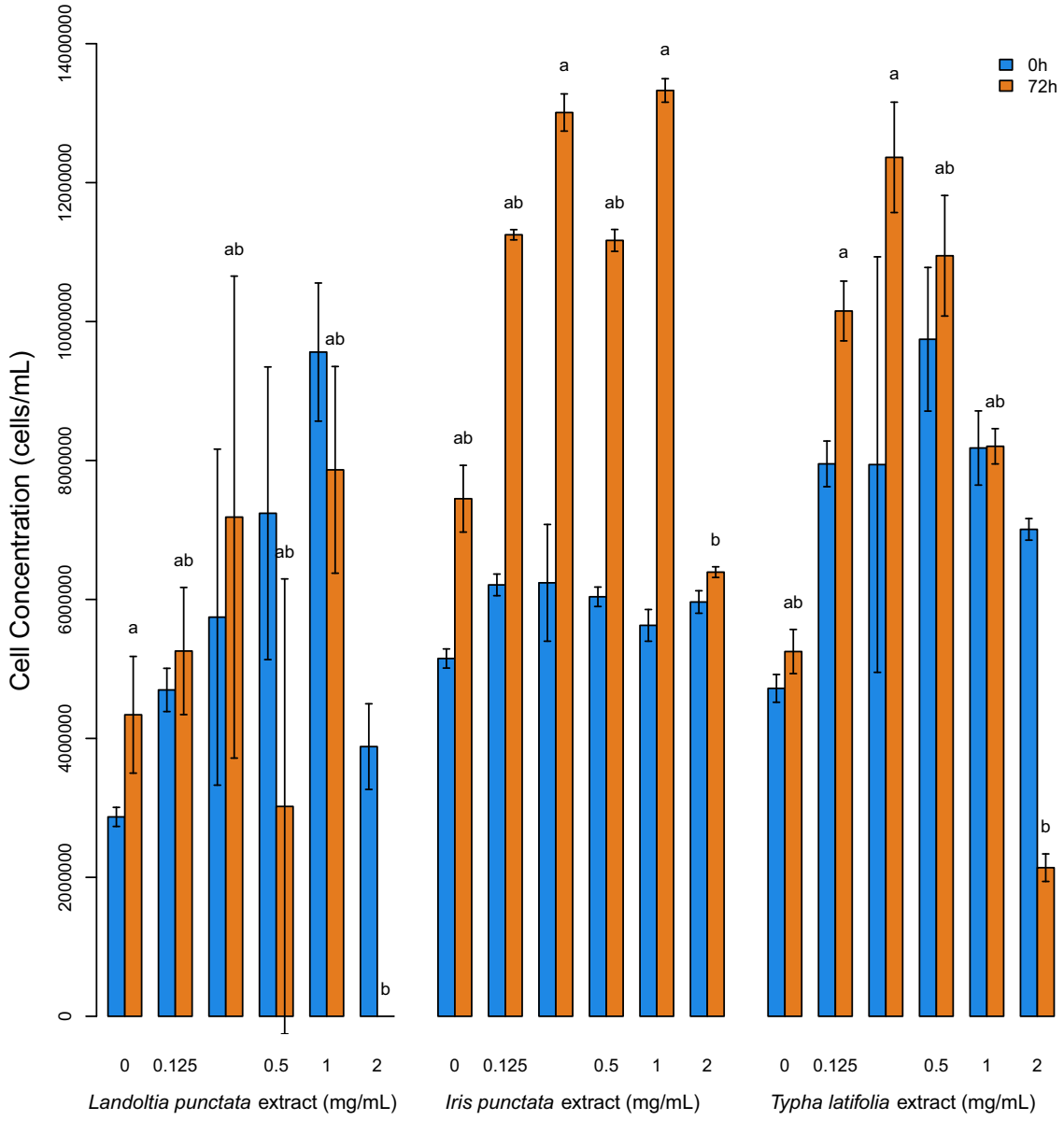

Fig. 1. Barplots of the results of the bioassays performed with *Landoltia punctata* extract, *Iris punctata* root extract and *Typha latifolia* leaves extract showing means ± standard deviation of *Chrysosporum ovalisporum* cell concentrations. 0 h –denotes cell concentrations estimated at the beginning of the experiment. 72 h – denotes cell concentrations estimated at the end of the experiment. Letters on top of deviation bars indicate homogeneous subsets (groups showing the same pattern of significant differences in the pairwise comparisons). Data points represent the average values of 3 replicates.

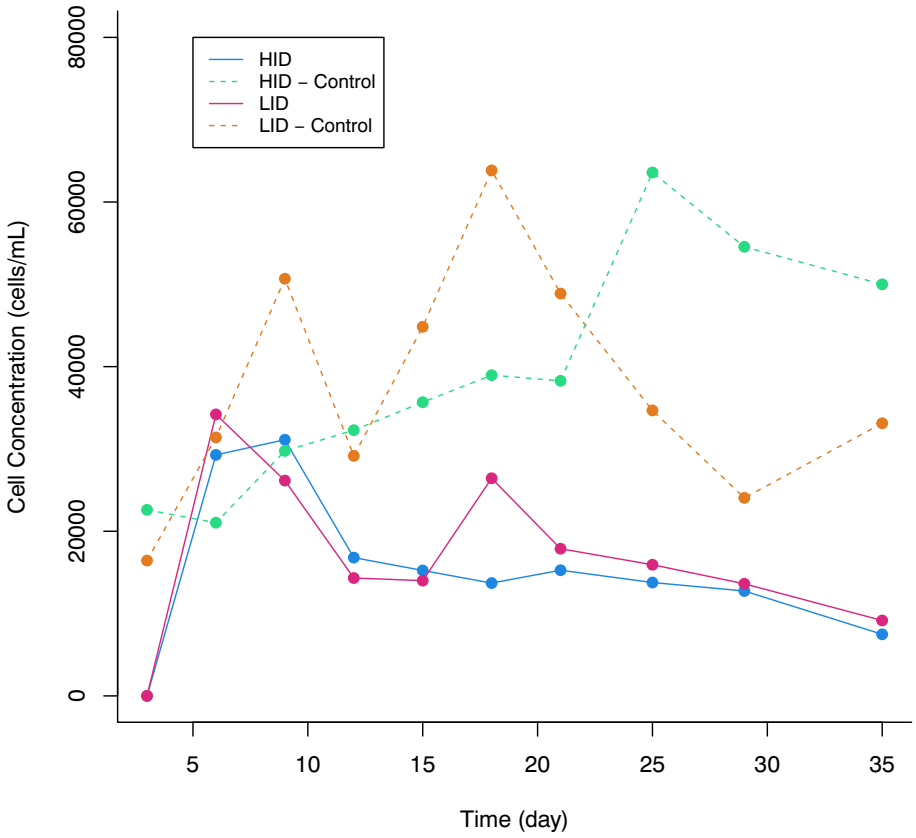

Fig. 2. *C. ovalisporum* cell abundance variation throughout the experiment duration. HID – Higher Inoculation Density – denotes groups inoculated with 1×10^6 cells/mL. LID – Lower Inoculation Density – denotes groups inoculated with 1×10^3 cells/mL. Control denotes groups where *L. punctata* was absent. Data points represent the average values of 6 replicates.

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 short, 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 and presence of some debris. This condition did not have a significant impact in nutrient consumption although it may have been responsible for a slight increase in nitrogen levels at the beginning of the experiment, before nutrient replenishment. Additionally, our statistical analysis showed that contaminant species were similarly inhibited by the presence of *L. punctata*. Overall, the results obtained corroborate the existence of inhibitory effects already observed with extracts bioassays. Nutrient quantification validates the allelopathic inhibition hypothesis, having ensured no nutrient depletion occurred during the experiment. Also, the artificial shading implemented in the controls ensured that reduced light incidence had no effect on inhibition of cyanobacterial growth. Besides *C. ovalisporum*, other cyanobacteria and microalgae present showed inhibition by the presence of *L. punctata*. It has also been reported that treatments with petroleum ether, dichloromethane, and ethyl acetate extracts of *L. punctata* have shown significant inhibitory effects on the growth rate of cyanobacteria *M. aeruginosa*, [58] . This suggests that the effects of this macrophyte may be of wide spectrum against autotrophic microorganisms. We must consider that our experimental design did not account for possible losses of *C. ovalisporum* through adsorption to *L. punctata* roots. However, based on the lack of bibliographic evidence about this aspect with filamentous species, we believe that this aspect might have had minimal if any impact. The mechanisms through which the allelochemicals act upon these cyanobacteria have yet to be described. However, in the same study Dan, et al. [58] observed decreased levels in chlorophyll *a* and phycobiliproteins, which indicates the hindering of the photosynthetic process in the cyanobacteria treated with ethyl acetate extracts from *L. punctata*. Additional research is thus required to isolate, purify and characterize the allelochemicals responsible for the observed inhibition effects. Several plant metabolites have been shown to inhibit the cyanobacteria *M. aeruginosa*, many are polyphenolic substances. Among cyanobacteria allelochemicals known are artemisinin extracted from *Artemisia annua*, nonanoic acid produced by *Myriophyllum spicatum* or Lactic acid (2-hydroxypropionic acid) present in the culture medium of submerged macrophytes such as *Ceratophyllum demersum* and *Vallisneria spiralis* [59]. Ouyang, et al. [59] showed some of these allelochemicals can be combined to reduce even more the growth of the cyanobacteria *M. aeruginosa* (inhibition rates of 80 %). Salicylic acid from *Oryza sativa*, showed also to be a potent inhibitor of *M. aeruginosa* growth [60]. Unfortunately, to our knowledge no allelochemicals have been identified with activity against the toxic

Table 2
Results for the fit of a general linear model and the analysis of variance performed with abundance of *C. ovalisporum* in the microcosm experiments.

Variable	Model fit		ANOVA		
	coefficient	*p*-value	*F*	df	*p*-value
Presence of *L. punctata*	−71.06 (presence)	< 0.001	76.66	1	< 0.001
Log(day)	14.99	< 0.01	7.46	1	< 0.01
Inoculum size	2.56	0.73	0.12	1	0.73
Microalgae contaminant	0.17	< 0.001	16.33	1	< 0.001

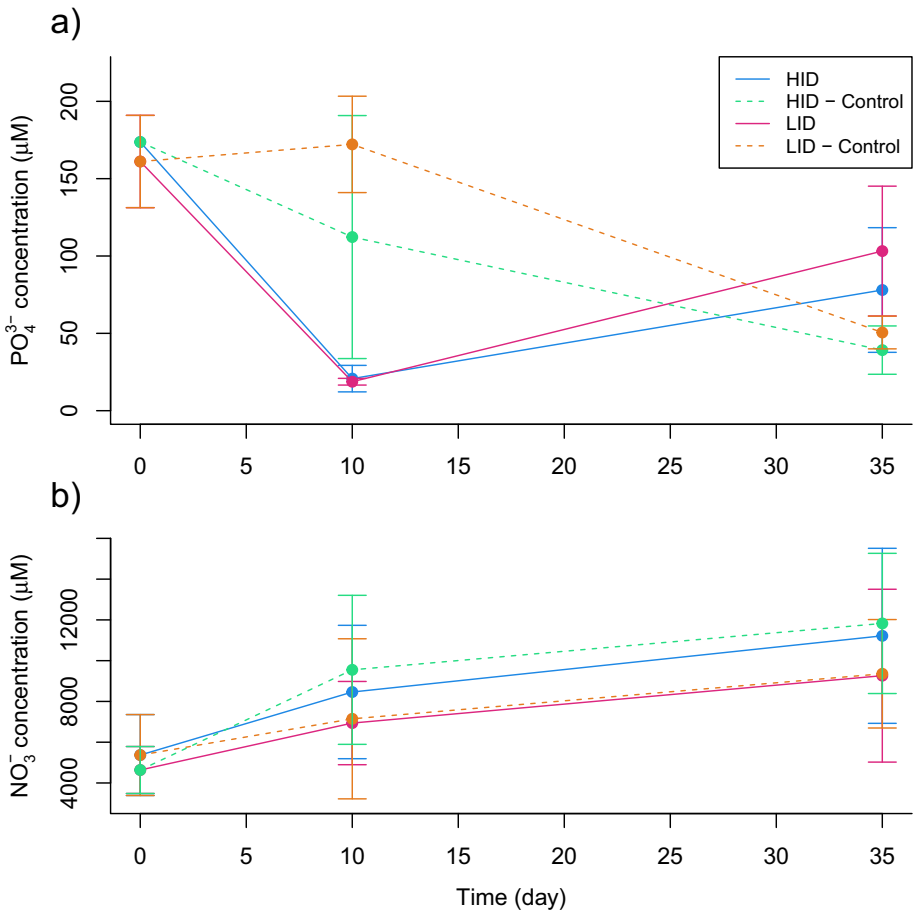

Fig. 3. Nitrate (a) and Phosphate (b) concentration in the medium by treatment during the microcosm experiment. HID – Higher Inoculation Density – denotes groups inoculated with 1×10^6 cells/mL. LID – Lower Inoculation Density – denotes groups inoculated with 1×10^3 cells/mL. Control denotes groups where *L. punctata* was absent. Data points represent the average values of 6 replicates. Nutrient replenishment occurred on the 10th day of the experiment.
P.N. performed research, analyzed data and wrote the manuscript, A.C. and A.B. designed research, help analyzing data, revised and edited the manuscript. V·V supervised research, revised and edited the manuscript.

cyanobacteria *C. ovalisporum*.

We foresee as future research the scale-up of the microcosm experiment, to confirm and validate the methodology and the potential application in the field of *L. punctata*, such as EFBs or CWs. There is the need to test the performance of the microcosm for the control of other cyanobacteria species, during longer periods of time. Toxicological assays are also required to evaluate the effect *L. punctata* and *C. ovalisporum* co-culture has on the production and release of cyanotoxins. Although supplementary studies are required, a previous study of *L. punctata* exposure to whole cell extracts of *Cylindrospermopsis raciborskii*, a cylindrospermopsin producer, suggests that the bioaccumulation of this toxin in the plant biomass is very limited, if existent [61].

In conclusion, this study demonstrates that *L. punctata* is a promising plant candidate to integrate EFBs and CWs, designed specifically to reduce and/or prevent the outgrowth of cyanobacteria and, while at the same time, having limited detrimental effects to the local environment.

CRediT authorship contribution statement

P. Nascimento: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **V. Vasconcelos:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **A. Campos:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **A. Barreiro:** Conceptualization and design, supervision, colaboration in experimental work, data analysis, manuscript writing and review.

Declaration of competing interest

The authors, we declare no conflicts of interest.

Data availability

Data will be made available on request.

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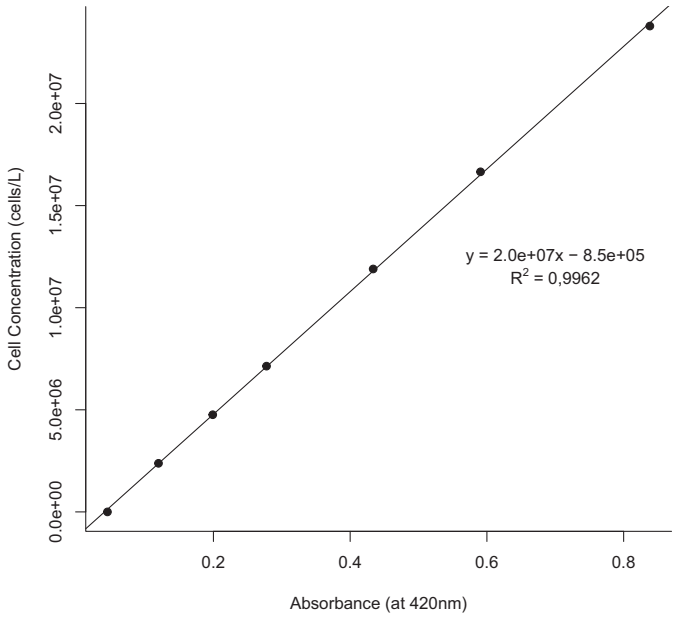
Annex 1.



Calibration curve determined for *C. ovalisporum* cellular concentration through absorbance measurements. Letters on top of deviation bars indicate homogeneous subsets (groups showing the same pattern of significant differences in the pairwise comparisons).

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