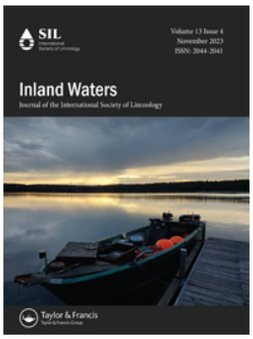

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Hormesis and Antagonism in Low-Dose *Phalaris* Allelochemicals during *Microcystis* Control

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Abstract

Aquatic ecosystems face significant challenges globally from cyanobacterial blooms. *Phalaris arundinacea* (Reed Canary Grass) is used in artificial wetlands and found in natural wetlands. We investigated whether allelochemicals released from *Phalaris* root exudates inhibit *Microcystis aeruginosa* growth. We conducted experiments to disentangle the effect of the root exudates from living plants on resource competition and the potential role of microbiota in controlling *Microcystis* growth. We found that allelochemicals from root exudates and their inhibitory effect decayed over time. Results from filtration experiments and microscopic observations indicated that the removal of microorganisms (≥ 0.22 µm) allowed for the growth of *Microcystis,* suggesting that protists and rotifers may control *Microcystis* growth. We also tested commercial allelochemicals at environmentally relevant concentrations (≤ 1000 µg L^{-1}) against *Microcystis*. Concentrations of 1000 µg L^{-1} of anthraquinone, gallic acid, gramine, hordenine, linoleic acid, naringuin, stigmasterol, tannic acid, 4-nitroindol-5-carboxaldehyde and the mixture of the nine allelochemicals inhibit *Microcystis* ($\geq 87\%$). The minimum effective concentration was determined to be 100 µg L^{-1} for most allelochemicals, except for anthraquinone, which had a hormetic effect stimulating *Microcystis* growth by up to 70% compared to the controls. Our findings indicate that allelochemicals could be used to control *Microcystis,* but it is essential to establish the effective allelochemical minimum inhibitory concentrations from biofilters, wetlands, or macrophytes to assess their potential for managing *Microcystis*, other cyanobacteria, and microalgae. The increasing global use of artificial wetlands to control cyanobacterial blooms justifies further investigation into allelochemical concentrations, including decaying trends over time and hormetic effects.

Keywords: Cyanobacteria management, Macrophytes, Constructed wetlands, Root exudates, Anthraquinone, Aquatic ecosystems.

Introduction

Cyanobacterial blooms in aquatic ecosystems present severe ecological and environmental challenges, driving the implementation of nature-based solutions like floating wetlands. Macrophytes within wetland systems—encompassing emergent, submerged, and floating aquatic plants across various vascular families (e.g., Poaceae)—are key in sustaining clear and healthy freshwater environments. While the precise mechanisms remain under investigation, potential explanations for the mechanisms that macrophytes exert control over cyanobacterial blooms include resource competition for nutrients and light (Mohamed, 2017; West *et al*., 2017), the provision of refuges that bolster zooplankton populations (Lürling and De Senerpont Domis, 2013), and allelopathy, which emerges as an effective strategy when compared to resource competition (Phillips *et al*., 1978; 2016; Donk and van de Bund, 2002; Erhard and Gross, 2006; Hilt *et al*., 2008; Castro-Castellon *et al*., 2016; Bi *et al*, 2019; Mowe *et al*., 2019; He *et al*., 2023).

Allelochemicals are produced in all macrophyte organs and can be released to the aquatic environment from leaves, shoots, roots and flowers as exudates or leachates, and there is no exclusivity that the producing organ is the one releasing them (Shanchez-Moreiras *et al*., 2003; Chang *et al*., 2012; Dhaouadi *et al*., 2015). Understanding allelopathic interactions between primary producers remains challenging, regardless of the scale of investigation, whether conducted in the field or laboratory. (Bauer *et al*., 2009; Vanderstukken *et al*., 2011, 2014; Svanys *et al*., 2014). The efficiency of the allelopathic effect depends on different factors, including the concentration of the allelochemical, the distance to the target cell, and the environmental conditions (Gross *et al*., 2007). Laboratory settings often use extracts or exudates with unrealistically high environmental allelochemical concentrations, e.g.1, 2, and 4 mg L^{-1} (Techer *et al.,* 2016, Wang *et al*., 2017; He *et al.*, 2023) while allelopathic interactions in natural environments are likely to occur within low concentration ranges (Erhard and Gross, 2006; Hilt *et al*., 2008) and the extended exposure of cyanobacteria cells to these compounds can alter the dynamics of such interactions (Chen, *et al*., 2012; Wang and Liu, 2023).

Allelochemicals have the potential to play a role in controlling *Microcystis* growth, but it is essential to understand their interactions within a broader ecological context. Cyanobacterial blooms are often too large for direct consumption by cladocerans and copepods, and rely on their breakdown by rotifers, protozoans, and fungi (Gerphagnon *et al.*, 2015; Gilbert *et al*., 2022). This process makes the blooms accessible for zooplankton ingestion, facilitating nutrient recycling (Haraldsson *et al*., 2018). These blooms are 'hot spots' for ameboid diversity (Weger, *et al.,* 2024), suggesting these microorganisms could play a role in bloom reduction.

A previous study by Castro-Castellon *et al*. (2021) using continuous flow experiments showed that the release of allelochemicals by *Phalaris arundinacea* (hereafter *Phalaris*) roots into aqueous media exhibited

algistatic/algicidal activity. There was a significant decrease in *Microcystis aeruginosa* (hereafter *Microcystis*) cell numbers and complete depigmentation after 96 hours (h), coinciding with identifying allelochemicals in the water. There remained, however, a question about whether allelochemicals alone were responsible for this effect or whether a combination of other factors, such as nutrient competition and biological interactions, also contributed to the observed reduction of *Microcystis* cells.

This study aims to elucidate the inhibitory effects of allelochemicals, at environmentally relevant low concentrations ($\leq 1000\ \mu g\ L^{-1}$), released from the root exudates of *Phalaris* on *Microcystis*. The investigation seeks to isolate these effects from confounding factors such as nutrient competition by living plants and potential microbiological interactions, providing clearer insight into the direct chemical interactions between species. We hypothesize that the inhibitory effect of root exudates on the growth of *Microcystis* will vary over time primarily due to the decay of allelochemicals rather than nutrient competition. We also expect the inhibitory effect to differ between filtered and unfiltered exudates, as the presence or absence of microorganisms influences allelochemical and *Microcystis* interactions. We pose the following research questions. 1) How does unfiltered versus filtered root exudate influence *Microcystis* growth? 2) Is *Microcystis* growth affected by the allelochemicals from *Phalaris* root exudates obtained at different intervals? To test the hypothesis and address the research questions, we conducted an experiment where *Microcystis* cells in the exponential growth phase were exposed to filtered and unfiltered aqueous root exudates obtained from *Phalaris* biofilters after One-Day and Five-Day-Immersion.

We also tested the effect of commercially obtained allelochemicals against *Microcystis* to evaluate their influence on the growth of *Microcystis*. We pose the specific research question: 3) How does *Microcystis* growth vary with three concentrations of nine allelochemicals, namely gallic acid, hordenine, gramine, naringin, 4-nitroindol-5-carboxaldehyde, anthraquinone, tannic acid, linoleic acid, stigmasterol and a mix with all allelochemicals? The second experiment focuses on exposing *Microcystis* cells to environmentally relevant concentrations of nine individual allelochemicals and an allelochemical mix, measuring cell concentration and quantifying the percentage inhibition achieved by these allelochemicals.

These data will provide a greater understanding of the interactions between allelochemicals and *Microcystis* growth, particularly growth inhibition that is isolated from nutrient competition between macrophytes and *Microcystis*.

Materials and Methods

Experimental set-up

The following experiments were carried out to investigate: a) if there are differences between the effects of unfiltered and filtered root exudates from immersed biofilters on the reduction of *Microcystis* growth over time and b) if the observed reduction in *Microcystis* growth is due solely to allelochemicals, nutrient competition or if there is a potential biological mechanism.

Biofilters were built using seeds of *Phalaris arundinacea* from British Wildflower Plants (Norfolk, UK) cultivated in sterile coconut coir pellets. After two weeks, the plantlet roots' were rinsed, and the stems above the roots were wrapped in 1 cm width foam strips and placed in 50 ml tubes (Figure 1.a). The plantlets were grown hydroponically in 50% Hoagland's solution to enhance hairy root production. Plastic straws were inserted in the tubes for gas exchange and to facilitate solution replenishment every 3-4 days. To prevent damage from light, the tubes were wrapped in aluminium foil. The plants were kept in a growing tent with a 10:14 h light/dark cycle (Conn *et al*., 2013) under blue compact fluorescent lamps (2 x 125-watt) providing 40 µmol photons m^{-2} s^{-1} under photosynthetic active radiation measured with a terrestrial quantum sensor LI-190SA and light meter LI-250A (Li-Cor International, Ltd., UK). After 10 weeks, 48 plants with roots 8-10 cm long were placed in galvanized stainless-steel cages and transferred to 3 L units (Castro-Castellon *et al.,* 2021).

Biofilters were immersed in the units when 18 weeks old. Three rectangular units (3 L capacity) labelled A, B, and C were filled with Oxfordshire Tap Water (UK)with a biofilter immersed in each (Figure 1.a-c). Tap water was selected as a suitable aqueous media for this experiment because of the nutrients and limited microbiology. This tap water has the following average *(n*=4) nutrient composition: nitrate as nitrogen (NO_3^-, 4.36 mg L^{-1}), nitrite as nitrogen (NO_2^-, <0.016 mg L^{-1}), ammonium as nitrogen (NH_4^+, <0.03 mg L^{-1}), soluble reactive phosphorus (SRP, 0.313 mg L^{-1}), and pH (7.43).

Biofilters were immersed for 24 h, 72 h and 120 h (named the One-Day-Immersion, Three-Day-Immersion and Five-Day-Immersion experiments respectively) (Figure 2). The water was changed, and the roots were rinsed after each immersion period. Duration of immersions was based on observations from a previous experiment, named "low flow experiments", where the units with biofilter showed significant cell reduction, and complete cell depigmentation was evident after 96 h (Castro-Castellon *et al*., 2021). For these experiments, three replicates of 100 mL (one from each biofilter) were extracted and analyzed with the simultaneous method developed by high-performance liquid chromatography with ultraviolet and diode-array detectors (HPLC- UV-DAD) as described in Castro-Castellon *et al*. (2021). Two subsets (unfiltered and filtered) from each biofilter were tested with *Microcystis* for the One-Day and Five-Day-Immersion.

Microcystis growth response to unfiltered and filtered root exudate

This experiment used replicates of unfiltered and filtered root exudates from biofilters A, B and C incubated with *Microcystis* cells. Two controls were used: a) *Microcystis* cells in the standard media (BG11), used to culture cyanobacteria (blue-green algae), to determine cell fitness and *Microcystis* growth to ideal conditions, compared with the treatments, and b) using Oxfordshire tap water to determine *Microcystis* growth response in the same medium (tap water) as the filtered and unfiltered biofilter roots.

Microcystis was obtained from the Culture Collection of Algae and Protozoa (CCAP, SAMS Limited, UK) (*Microcystis aeruginosa* 1450/3) and cultured in the standard media for cyanobacteria BG11 (modified by Ripka et al., 1979). Aseptic techniques and materials were used throughout the experiment, and every four days, the culture flasks were shaken and tested for contamination before adding 10 ml of fresh media, maintaining *Microcystis* at the exponential growth phase. Cultures were kept at photosynthetic active radiation (PAR) of 10 µmol photons m^{-2} s^{-1} (recommended by the CCAP) and measured with a terrestrial quantum sensor LI-190SA and light meter LI-250A (Li-Cor International, Ltd., UK) under a constant temperature at 21-23 °C. For these experiments, from a 250 ml flask, *Microcystis* cells were diluted to (1:10) with an Optical Density (O.D.) measured in a Shimadzu spectrophotometer at 620 nm 0.605 ±0.005 equating to approximately (~4.5×10^5 cells mL^{-1}) and kept in a light cycle (12:12) h at the recommended PAR.

Root exudate samples (1 L) from biofilters A, B, and C were taken separately from the One-Day and Five-Day-Immersion. Each 500 mL subsample from A, B, and C was halved, producing two subsamples each (filtered and unfiltered). Subsamples (250 ml) were dispensed in aliquots of 22.5 mL to a set of five 50 mL sterile tubes for A, B and C. All tubes were prefilled with 2.5 mL BG11 and 2.5 mL *Microcystis* at the exponential phase. The other 250 mL subsample root exudate sample was filtered using a 0.22 µm pore size (nominal value given by the manufacturer) and 30 mm diameter filter (Fisherbrand, UK) and dispensed to another set of five 50 mL tubes (A, B and C). The filter will remove microorganisms from the root exudate if present. The controls contained either 22.5 mL of tap water or 22.5 mL of BG11. All tubes were exposed to the recommended PAR by the CCPA.

Samples were taken at the following time points: (*t1*) which corresponds to 24 h, (*t3*) 72 h, and (*t5*) 120 h after *Microcystis* incubation with root exudates from the One-Day-Immersion. Aliquots of 0.5 mL with a calibrated pipette were taken from the tubes and transferred to a 96-deep well plate (ThermoScientific, UK). The data were analysed by flow cytometry using the BD Accuri C6 software. This procedure was followed for the Five-Day-Immersion experiment.

Samples from *t5* were collected to assess whether variations in *Microcystis* growth were attributable to allelochemicals or potentially influenced by biological organisms. The response was quantified by comparing the average *Microcystis* cell growth to the initial cell counts on the first day, under conditions with either filtered or unfiltered root exudates.

Microscopic observations of filtered and unfiltered root exudate samples

An aliquot of 1 mL was taken at *t5* from each unfiltered and filtered tubes from the One-Day and Five-Day-Immersion. The samples were mounted on a Sedgewick-Rafter slide and observed under a light microscope (Eclipse E-200 Nikkon, UK) ×20. Then, the chamber was scanned from left to right, then down from right to left successively to identify the presence of microorganisms. Semi-quantitative measurement can be provided at the request of the reviewers. This measurement is an average of the microscopic annotations from the five unfiltered tubes at *t5*, where single microorganisms were noted, but if there were several, these were ≥5 but <10.

It would have been noted if there had been "too many," which the authors considered greater than 10. Following these notes, a rough estimate of protozoans and rotifers could be average as follows: 5 ml (all observations) exudate – 2 flagellate protozoan, 8 rotifer, 2 amoebae (heliozoan), 21 ciliates, debris. The average for 27.5 ml in the tubes was calculated.

Percentage inhibition experiment with commercially sourced allelochemicals

The experiment involving commercially sourced allelochemicals determined the inhibition of *Microcystis* growth by the following selected allelochemicals: anthraquinone, gallic acid, gramine, hordenine, linoleic acid, naringuin, stigmasterol, tannic acid and 4-nitroindole-5-carboxaldehyde and a mix of an equal percentage of these compounds. The range of allelochemical concentrations used in the *Microcystis* growth experiments was 1000, 100 and 10 µg L^{-1}. Three concentrations are the minimum recommended by the OECD (2011) for cyanobacteria growth response in dose-test experiments. These concentrations were selected based on the work of Techer *et al.,* (2016), who applied commercial allelochemicals to mesocosms containing *Microcystis*, achieving final dilutions of 1, 2, and 4 mg L^{-1}. We chose lower concentrations to approximate potential natural scenarios, considering factors like continuous water flow and variable macrophyte density. This approach reflects findings like the EC50 of gallic acid for *Microcystis aeruginosa* (1.0 mg L^{-1}) (Nakai *et al.,* 2000). *Microcystis* cells from a culture at the exponential growth phase were used in the experiments. The response of *Microcystis* growth was evaluated as a function of the exposure concentration and compared with replicates of a control with no allelochemicals, BG11 and *Microcystis*, named negative control and in methanol as a positive control.

The concentration of *Microcystis* cells was measured using 1 mL aliquots from the culture flasks transferred to quartz cuvettes (Sigma-Aldrich, UK) at 620 nm wavelength. Measurements were performed with 1:1 culture dilution to reproduce the final dilution in the microtubes. From the microtubes, aliquots were taken for the measurements in the 96-well plates. Calibration and validation were conducted by using an improved Neubauer haemocytometer (AC 2000 standard two chambers 1/400 Hawksley, UK) and flow cytometry using a BD Accuri C6 instrument (BD Biosciences, UK) as described in Castro-Castellon *et al.,* (2021).

The experimental procedure was as follows: two sets of clear 1.6 mL microcentrifuge tubes (Fisherbrand, UK) were autoclaved and dried in an oven at 40 °C (Genlab 100 litres, UK). Aliquots of 0.675 mL and 0.735 mL of both BG11 and *Microcystis* cells in BG11, were transferred to each set of microtubes (1000 µg L^{-1}, 100 µg L^{-1}). Another set was prepared by dilution of the 100 µg L^{-1} with a final concentration of 10 µg L^{-1}. Aliquots of 0.3 mL were taken from these microtubes and transferred to 96-well plates (Thermo Scientific™ clear flat bottom), and their O.D. was measured at a wavelength of 595 nm excitation with 615 nm emission in a Tecan Infinite F200 plate reader. Each treatment set consisted of 10 allelochemicals (nine individual allelochemicals and an allelochemical-mix made up using the same nine allelochemicals) at each target concentration, with a positive control. This procedure was carried out at time zero (here it corresponds to the beginning of incubation), 72 h and 120 h, denoted respectively as *t0*, *t3* and *t5*.

The inhibitory effect of the allelochemicals on the *Microcystis* growth was estimated by calculating the percentage of inhibition over the control, following Hong *et al*., (2009) as in equation (1):

$$\text{Inhibition (\%)} = ((Ci - Cf)/ Ci) \times 100 \quad (1)$$

where Ci is the positive control, Cf is *Microcystis* cell numbers in the microtubes treated with each of the allelochemicals.

Data processing and statistical analysis

Exploratory and statistical analyses were undertaken using Excel (Microsoft © 2013) and SPSS v. 22.0 (IBM statistic software, UK). Data were checked to meet the parametric assumptions: a normal distribution and equal variances. Because most data were repeated measurements, no checks for independence were conducted, and the repeated measure analysis of the variance (RM-ANOVA) test was used (Dytham, 2011). Maulchy's test of sphericity measures the differences between pairs of scores computed for each tested element and the variance for each set of differences is calculated (Field, 2009). The assumption of sphericity is a condition that needs to be met in repeated-measure designs, with the test statistic being significant ($p < 0.05$). The Bonferroni correction was applied to the standard errors.

A repeated measures analysis of variance (RM-ANOVA), with three levels, mixed model with replication was applied to the data. The measured dependent variable is *Microcystis* growth (cell counts) and the independent factors were: biofilter units, treatment and time. The design had three levels: 1) The biofilter with three biofilter units being tested (a=3); 2) Two types of treatment (b=2); unfiltered exudate and the filtrate of the exudate sample, each with five replicates (c=5) per unit; and 3) Time was the repeated fixed factor in this design (d=3). Hence, *Microcystis* measurements were carried out as follows: *t1, t3* and *t5* as explained in the materials and methods. Each replicate (n=5 per treatment) was measured once at the specified time points (n=3) for each replicate, constituting the repeated measurements.

Parametric assumptions (normal distribution and equal variances) could not be met for the Five-Day-Immersion, and the non-parametric Friedman's test was used (Dytham, 2011). This test used two-factor levels (treatment and the biofilter units) with a repeated measure (time), and there was more than one sample (n=5 replicates). A Kruskal-Wallis test was used to test for differences between the time groups (*t1*, *t3* and *t5*) for unfiltered and filtered root exudate samples from biofilters A, B and C.

Results

Allelochemicals present in root exudates

Two allelochemicals were identified and semi-quantified from this experiment: anthraquinone and 4-nitroindol-5-carboxaldehide. Anthraquinone for the One-Day-Immersion (24 h) showed concentrations (1300 ± 270 µg L^{-1}) which were higher than the highest non-extracted standard (900 µg L^{-1}), where the Limit of Quantification (LOQ) was 88.48 µg L^{-1} and Limit of Detection (LOD) was 29.20 µg L^{-1}. The LOQ is the lowest analyte

concentration that can be quantitatively measured with precision, while the LOD is the lowest concentration that can be reliably detected but not necessarily quantified. The LOD represents the point at which the signal produced by the analyte is distinguishable from the baseline noise. Anthraquinone results were at or below 80 µg L^{-1} for the Three-Day-Immersion (72 h) but above LOD; and for the Five-Day-Immersion (120 h) were below the LOD. Results for 4-nitroindol-5-carboxaldehyde were semi-quantitative for Five-Day-Immersion, as they were between 44.93 µg L^{-1} (LOQ) and 14.83 µg L^{-1} (LOD).

Determining the effect of root exudate on *Microcystis* growth

Significant differences were found in the number of *Microcystis* cells from the treatments (unfiltered and filtered) compared to both controls (BG11 and tap water). The control in BG11 showed the response of *Microcystis* growth in ideal conditions, and the control in tap water demonstrated the growth response within the same media in which the treatments were carried out. After 72 h (*t3)* of exposure, *Microcystis* growth decreases in the controls and the treatments for the One-Day-Immersion (Figure 3). Results for *Microcystis* growth as a function of time are significant for both controls, showing exponential growth at *t5*. *Microcystis* growth is strongly inhibited with the unfiltered and weakly inhibited with the filtered treatments at *t5*.

Five-Day-Immersion results for *Microcystis* growth as a function of time significantly increase for both controls and filtered treatment, showing exponential growth at *t5*. *Microcystis* growth for the unfiltered treatment does not change, indicating a strong inhibition of *Microcystis* growth (Figure 4). Also, in the Five-Day-Immersion, the standard error at *t3* is large for the unfiltered treatment.

Statistical analysis One-Day root exudate treatment on *Microcystis* growth

The data fits the assumptions of a normal distribution and equal variances. The result was that Maulchy's test of sphericity was non-significant ($p>0.05$) for the main effect biofilter ($p=0.084$), Maulchy's test of sphericity was non-significant ($p>0.05$) for the main effect biofilter ($p=0.084$) nor was it significant for the interaction biofilter*treatment ($p=0.75$), hence meeting the assumptions.

Repeated measures ANOVA (F and *p*-values) in Table 1 showed the effects of biofilters, unfiltered and filtered root exudate treatment and their interaction as a function of time (*t1*, *t3* and *t5*) on *Microcystis* growth (cells mL^{-1}). The RM-ANOVA (Table 1) showed that the biofilter (root exudate source) and the interaction biofilter*time were non-significant ($p>0.05$). However, significant differences ($p<0.05$) were found for the interaction biofilter*treatment. These results indicate differences in the effect of the treatment (unfiltered and filtered) on *Microcystis* growth response and that there is no difference between the three biofilters, the source of the root exudate samples.

Inhibition of *Microcystis* growth response (cells mL^{-1}) to treatment and time was significant ($p<0.05$), indicating that *Microcystis* growth decreased with the unfiltered treatment at *t3* compared to *t1*. The results of the effect of the interaction between treatment and time were significant ($p<0.05$), with the greatest positive effect on the growth of *Microcystis* observed at *t5* with the filtered treatment (Figure 5).

Statistical analysis Five-Day-Immersion experiment on *Microcystis* growth

Results for the Five-Day-Immersion, using the non-parametric Friedman's test, showed there were significant differences between the treatments (unfiltered and filtered) ($X^2= 46.7$, $df=5$, $p<0.05$, n=6). The Kruskal Wallis test allowed for testing paired differences of the medians. Significant differences ($p<0.05$) were found over time (*t1* and *t3*) for unfiltered samples from biofilters B and C (n=10) and for the filtered samples between A, B, and C. Some falcon tubes were not properly closed for biofilter A and the data was lost. Therefore, data from (t5) could not be used in the test (Table 2).

Microscopic observations of unfiltered and filtered root exudate samples

Protozoans and invertebrates were present in the unfiltered root exudate tubes. A semi-quantitative measurement is provided as an average of the five tubes as follows: heliozoans (11 organisms mL^{-1}), zooflagellates (11 organisms mL^{-1}), ciliates (116 organisms mL^{-1}), and rotifers (44 organisms mL^{-1}). Debris was present in all tubes. Although some green micro-algae were present, the quantitative analysis by flow cytometry (Castro-Castellon *et al*., 2021) of the filtered root exudates showed only one population of *Microcystis*, indicating these were in comparatively low numbers.

Effect of commercial allelochemical on inhibiting *Microcystis* growth

Microcystis is a slow-growing cyanobacterium, and the results for this type of experiment were those for *t5* (OECD, 2011). The inhibition of *Microcystis* growth in the presence of most allelochemicals at 1000 µg L^{-1} was, on average >68% compared to the controls (orange bars, Figure 6). The percentage inhibition of *Microcystis* by seven of the nine compounds was above 92% compared to the controls. Apart from linoleic and tannic acid, for which responses were 70% and 68%, with large standard deviations, all the other allelochemicals tested showed small standard deviations. The inhibition of *Microcystis* growth in the presence of most allelochemicals at 100 µg L^{-1} was >50% compared to the controls. However, we found that anthraquinone stimulated *Microcystis* growth by up to 70% compared to controls (dark bars, Figure 6). No inhibition of *Microcystis* at 10 µg L^{-1} was found in commercial allelochemicals (not shown). *Microcystis* O.D. (615 nm Tecan) and microtubes (620 nm) were 0.103 and 0.105, corresponding to a cell concentration of approximately 35,000 cells mL^{-1}.

Discussion

Differences in the effect of the unfiltered and the filtered aqueous root exudate on *Microcystis* growth as a function of time.

In these experiments, we exposed *Microcystis* solely to root exudate. Therefore, it is reasonable to state that the observed mechanism suppressing *Microcystis* growth is primarily attributed to allelochemicals rather than to competition for nutrients between the roots and *Microcystis*. At the same time, we acknowledge the potential for nutrient competition from other microorganisms, such as bacteria and fungi, in the unfiltered samples. Also, we acknowledge that there is a strong suggestion that microorganisms play a role in controlling *Microcystis* growth.

It should be noted that our experiments used aqueous root exudate samples without prior root extraction to assess their effects on *Microcystis* cells. These experiments are some of the first to investigate the allelopathic impact of aqueous root exudate on *Microcystis* cells. Nakai *et al.* (2008) also employed aqueous root exudate but conducted their experiments differently, raising two significant points. Firstly, Nakai *et al.* (2008) extracted and concentrated their aqueous root exudate in methanol, potentially introducing inhibitory effects of methanol on *Microcystis*. Secondly, their reported bioassay inhibition results extended over 25 days, considerably longer than the typical duration of growth inhibition tests documented in the literature, which range from 24 to 336 hours (up to 14 days) for single-species studies (Nakai *et al.*, 2000; Hilt and Gross, 2008; Lürling and Beekman, 2010).

Our findings from the One-Day-Immersion reveal a discernible inhibitory effect on *Microcystis* growth after *t5* compared to the controls. Particularly, when comparing the unfiltered and filtered treatments, significant differences emerge with an increase in *Microcystis* growth response to the filtered treatment at *t5* in both the One-Day and Five-Day-Immersion experiments. These results strongly support the hypothesis that the reduction of *Microcystis* cells is not attributable to nutrient competition, as living roots were absent. Moreover, the samples that appeared "bleached",' in which *Microcystis* cells are devoid of pigmentation, indicate oxidative processes, a characteristic manifestation of the allelochemical impact on cyanobacteria.

Our research demonstrates that anthraquinone concentration is high at 24 h (1300 ± 270 µg L^{-1}) and exhibits strong inhibition of *Microcystis* growth at concentrations ≥1000 µg L^{-1}. Despite this, allelochemical degradation is evident at 72 h with concentrations at 80 µg L^{-1} (below LOQ = 88.48 µg L^{-1}) and with, consequently, low anthraquinone concentrations at or below LOD (≤29.20 µg L^{-1}) at 120 h may have induced a hormetic effect in the absence of microorganisms (Figure 5). In natural settings, even with a continuous release of allelochemicals into the water environment, *Microcystis* exposure to initially high concentrations and subsequent continuous exposure may cause irreversible damage and inhibit growth (Shao *et al.*, 2013; Laue *et al.*, 2014). Conversely, continuous low concentrations may stimulate growth. Future research should investigate the interactions of a range of understudied allelochemicals with cyanobacteria.

Biological mechanisms involved in the inhibition of *Microcystis* growth

Our results suggest that allelochemicals released from the roots of *Phalaris* biofilters can inhibit *Microcystis*'s growth. However, a biological mechanism also contributes to the further reduction of *Microcystis*'s growth.

In the One-Day-Immersion experiment, all three biofilters (A, B, and C) had a similar effect on inhibiting *Microcystis* growth. In the Five-Day-Immersion, the anthraquinone concentration was much lower than in the One-Day-Immersion. However, *Microcystis* growth was still suppressed in the unfiltered samples. This suggests there is another mechanism suppressing *Microcystis* growth in the unfiltered samples.

One possible explanation for this mechanism is the presence of protozoa and invertebrates in the root exudates. Microscopic observations and the identification of protozoa and invertebrates in the root exudate samples suggest that these organisms may be grazing on *Microcystis* cells. Another possible explanation is the production of antimicrobial compounds by the microbiota, which could suppress *Microcystis* cell growth. Other studies have reviewed the role of protozoan and rotifer grazing and infection by fungi and prokaryotes (Gerphagon et al., 2015; Lishke *et al*., 2016; Gilbert *et al*., 2022). It is worth noting, that not identifying the observed protozoa (heliozoan, ciliates and flagellates) and rotifer to lower taxonomic levels nor that these were quantified is a limitation of the present study.

Further research is needed to determine what mechanisms are at play to further reduce Microcystis growth in the unfiltered samples. However, the results of this study suggest that a combination of allelochemicals and biological factors may be responsible for suppressing *Microcystis* growth.

Effect of the different commercially obtained allelochemicals on *Microcystis*

The highest concentration of allelochemicals (1000 µg L^{-1}) used in this experiment suppressed the growth of *Microcystis* by at least 87% (anthraquinone, gallic acid, gramine, hordenine, naringuin, stigmasterol, 4-nitroindol-5-carboxaldehyde and the mix of the nine allelochemicals) and 68% for linoleic and tannic acid. However, the minimum effective concentration was 100 µg L^{-1} for most allelochemicals, except for anthraquinone (Figure 6).

Our study highlighted the synergistic effect of anthraquinone (68%) on *Microcystis* growth at a concentration of 100 µg L^{-1}. This effect is known as hormesis, a phenomenon in which a substance harmful at high doses can be beneficial at low doses. The hormetic effect has been demonstrated for various organisms, including microalgae, cyanobacteria, plants, and zooplankton. (Forbes, 2000, Calabrese and Blain, 2009; Cerbin *et al*, 2010; OECD 2011; Zhang *et al*, 2020). The hormetic effect has been demonstrated on *Microcystis* growth when exposed to other substances, such as the allelochemical ethyl-2-methylacetoacetate isolated from *Phragmites communis*, pentachlorophenol (pesticide), amoxicillin, flame retardants and lubricants (Hong *et al*., 2008; Liu *et al*., 2015, 2016; Zhang *et al*., 2022). However, the stimulatory effect of anthraquinone, a common allelochemical from macrophytes, on *Microcystis* growth has not been described in the literature before.

The results for linoleic and tannic acids were unexpected. The inhibition response of *Microcystis* growth to these two allelochemicals was very similar at the 1000 and 100 µg L^{-1} concentration treatments. This could be because linoleic and tannic acids are fatty acids that can cause cell aggregation. This aggregation could prevent some cells from directly contacting the allelochemicals.

The initial concentration of *Microcystis* cells ($3.5x10^4$ mL^{-1}) employed in this study falls within the recommended range established by the OECD (2011). This concentration is substantially lower by an order of magnitude compared to concentrations ranging from $1x10^5$-$1x10^6$ mL^{-1} used in similar investigations (Lürling and Beekman, 2010). Furthermore, when examining allelochemical concentrations, prior experiments have generally employed levels of ≥1 mg L^{-1} derived from dried root extracts or commercially available allelochemicals to illustrate their algastatic or algaecidal effects (Lürling *et al.*, 2016; Mohamed, 2017). In contrast, our research was conducted with a final sample volume of 1.5 mL, representing an order of magnitude increase compared to typical laboratory experiments where final volumes are measured in microliters and with a low allelochemical concentration, mimicking interactions in natural aquatic environments.

Further work should be done to investigate the minimum inhibitory concentrations (MIC) of other allelochemicals on *Microcystis* (below 1000 µg L^{-1}), and the hormetic effect of a range of allelochemicals that are likely to occur in natural settings at low doses. This must include understanding allelochemical concentration changes in dynamic water conditions where the distance from the roots to the target cells is in constant flux. The results of this study suggest that allelochemicals have the potential to be used to manage *Microcystis* blooms. Determining the optimal allelochemical (MIC) from biofilters, wetlands or macrophytes is crucial for assessing their potential in managing *Microcystis*, other cyanobacteria, and microalgae.

Conclusions

In this study, we postulated that the inhibition of *Microcystis* growth resulting from macrophyte root exudate was attributable to allelochemicals rather than nutrient competition. With the unfiltered and filtered root exudate experiment, we also showed that despite allelochemical decay over time, the presence of microorganisms in the unfiltered exudate suggests their potential for controlling *Microcystis*. The following evidence supports our hypothesis:

Firstly, the experiments were intentionally designed without biofilters, eliminating any direct nutrient competition between the root uptake of nutrients and *Microcystis*. This deliberate omission reinforces that nutrient competition is not a significant factor in the observed growth inhibition.

Secondly, by using unfiltered root exudate samples, we ensured that any potential allelochemicals remained unaltered but also opened the possibility that microbiota might be involved.

Thirdly, unfiltered root exudate effectively suppressed *Microcystis* growth, whereas filtered root exudate did not. This divergence strongly implies that microbiota, more than allelochemicals (which also decay with time and could stimulate growth), may be the primary agents responsible for this inhibitory effect.

Lastly, observing "bleached" samples as a result of depigmented *Microcystis* cells following exposure to root exudate indicates oxidative processes. This aligns with the characteristic impact of allelochemicals on cyanobacteria cells, further reinforcing their role in growth inhibition.

We present evidence supporting the role of allelochemicals and, potentially, microbiota in inhibiting Microcystis growth. This novel study has advanced our understanding of these complex interactions in aquatic ecosystems, suggesting that nature-based solutions, such as floating treatment wetlands or the restoration of wetlands, could be beneficial for controlling *Microcystis*, other cyanobacteria, and algal blooms.

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Statements and Declaration

The authors declare no conflict of interest.

My passion for water quality research began during my MSc in Microbiology and Parasitology at the Complutense University of Madrid, where I studied the environmental impacts of ostrich farms. I continued this focus in the UK, holding various water industry roles. While overseeing reservoirs for Wessex Water, I completed an MSc by Research in Aquatic Ecology and Ecosystem Change at the University of Bristol, examining eutrophication and cyanobacterial blooms.

My PhD at the University of Oxford focused on floating treatment wetlands to combat algal loading onto a supply water treatment works, sponsored by Thames Water, later, I served as Field/Process Scientist. As a Research Technician, I took part in the international project RESPiRES investigating urban water systems for sustainable smart cities. My diverse experience includes roles as a Visiting Research Fellow and Associate Lecturer at Bath Spa University, Chemistry Team Leader at Welsh Water, and Senior Scientist at Industrial Phycology focusing on phosphorus removal using microalgae.

I returned to academia as a Research Associate on the METAL_SoLVER (Mine Effluent Treatment At Low-cost using Sustainable Vertical Flow Reactors) project at the Geoenvironmental Research Centre within the School of Engineering at Cardiff University. Currently, I am a Research Associate in Biogeochemistry for the QUANTUM project investigating the impact of livestock on freshwater ecosystems at the School of Geographical Sciences, University of Bristol.

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Table 1. Interactions between biofilters, treatment, and time as analyzed using repeated measures ANOVA. Significance ($p \leq 0.005$) was found between the treatments (filtered and unfiltered) and all interactions as a function of time.

	SS	***df***	**MS**	***F***	***p*-value**
Biofilters	$8.23*10^{8}$	2	$4.12*10^{8}$	0.35	0.709
Biofilters*Time	$4.69*10^{10}$	4	$1.17*10^{10}$	0.994	0.430
Error (biofilters)	$2.8*10^{11}$	24	$1.17*10^{10}$		
Treatment	$1.37*10^{11}$	1	$1.37*10^{11}$	11.58	**0.005**
Treatment*Time (interaction)	$3.59*10^{11}$	2	$1.79*10^{11}$	15.19	**0.001**
Error (treatment)	$1.42*10^{11}$	12	$1.18*10^{10}$		
Biofilters*Treatment	$2.81*10^{11}$	2	$1.40*10^{11}$	10.98	**0.001**
Biofilters*Treatment*Time (interaction)	$5.17*10^{11}$	4	$1.28*10^{10}$	10.13	**0.001**
Error (Treatment*Time)	$3.07*10^{11}$	24	$1.28*10^{10}$		

SS= Sum of squares; *df*= degrees of freedom; *MS*=mean square, *F*= F distribution

Table 2 Kruskal Wallis test for unfiltered and filtered samples from biofilters A, B and C with overtime (*t1* and *t3*). The test statistic (X^2), degrees of freedom (*df*) and *p*-value are given.

Biofilters – Treatment

	A Unfiltered	B Unfiltered	C Unfiltered	A Filtered	B Filtered	C Filtered
(X^2)	2.455	6.818	6.902	6.860	4.811	5.771
df	1	1	1	1	1	1
p-value	0.117	**0.009**	**0.009**	**0.009**	**0.028**	**0.016**

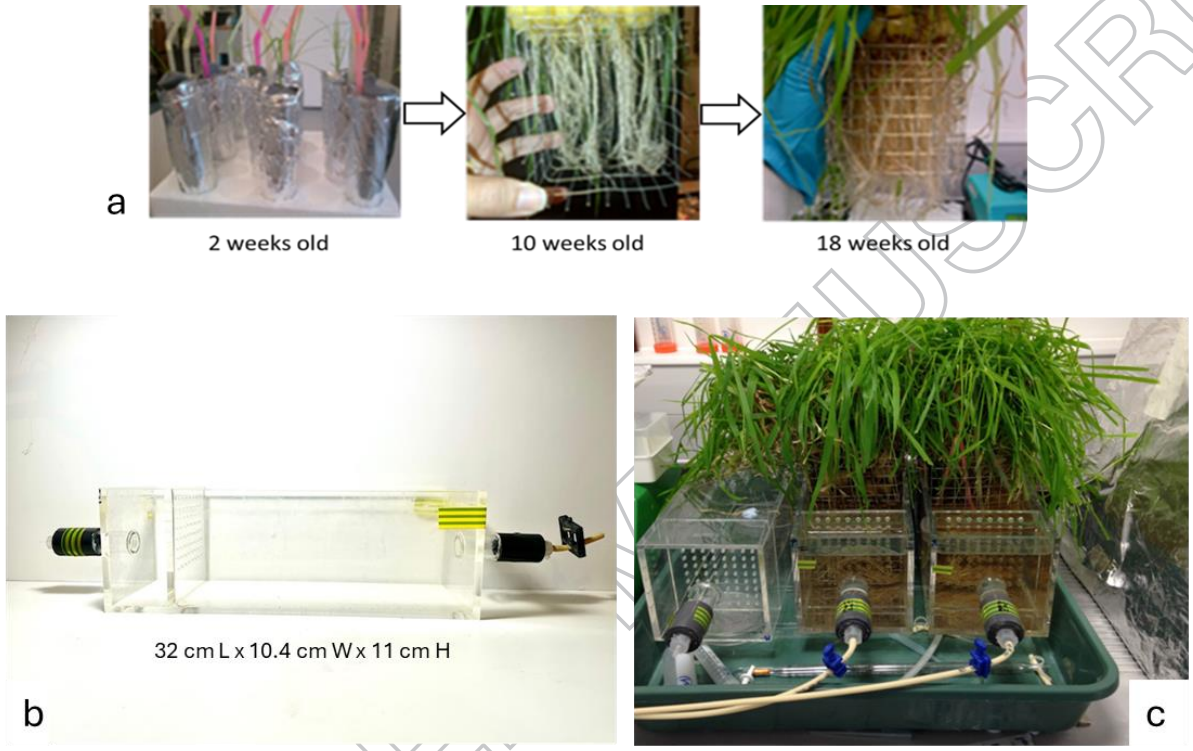
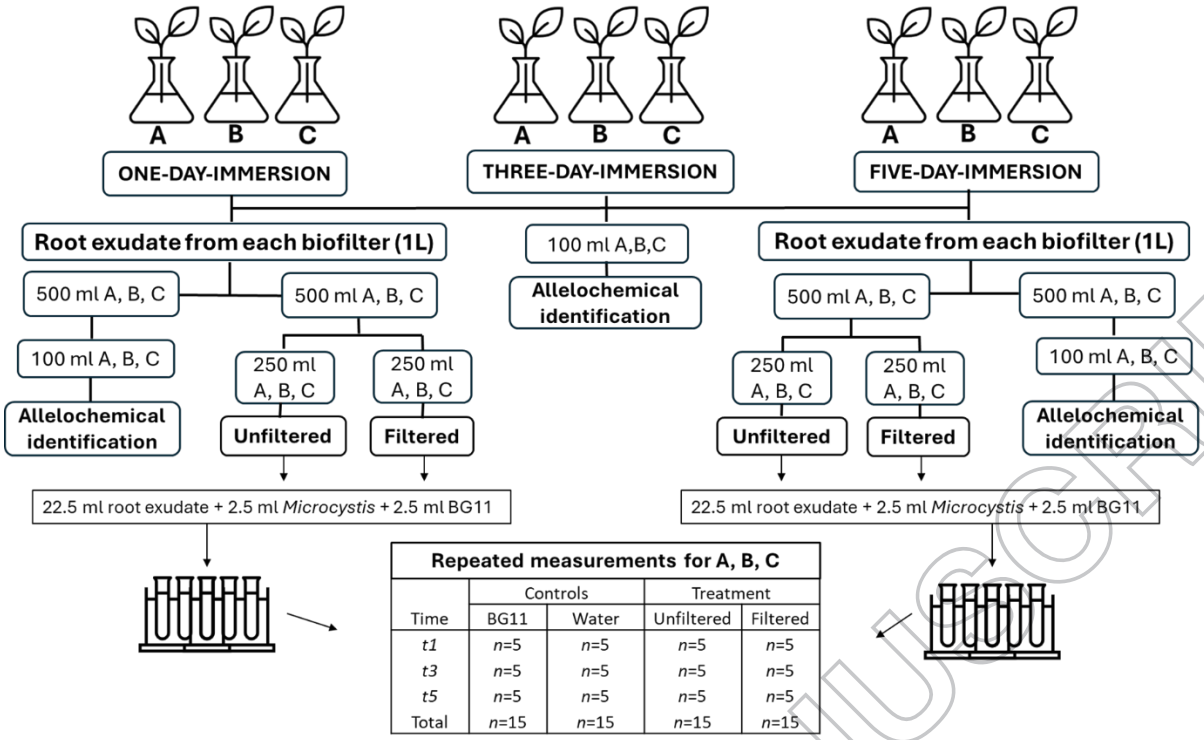


Figure 1. a. Biofilter-making process left to right, *Phalaris* two-week-old plants, biofilters with 16 plants 8-10 weeks old, and biofilters root density at 18 – 20 weeks ready for immersion. b. Units for the biofilter. c. Immersed biofilters.



Repeated measurements for A, B, C

	Controls		Treatment	
Time	BG11	Water	Unfiltered	Filtered
t1	*n*=5	*n*=5	*n*=5	*n*=5
t3	*n*=5	*n*=5	*n*=5	*n*=5
t5	*n*=5	*n*=5	*n*=5	*n*=5
Total	*n*=15	*n*=15	*n*=15	*n*=15

Figure 2. Experimental setup using root exudates from Biofilters A, B, and C. Biofilters were immersed 24, 72, and 120 h corresponding to One-, Three-, and Five-Day-Immersion. Samples from each biofilter were tested for allelochemical identification. Subsets of root exudates (unfiltered and filtered) from each biofilter were tested with Microcystis and BG11 for the One-Day and Five-Day-Immersion.

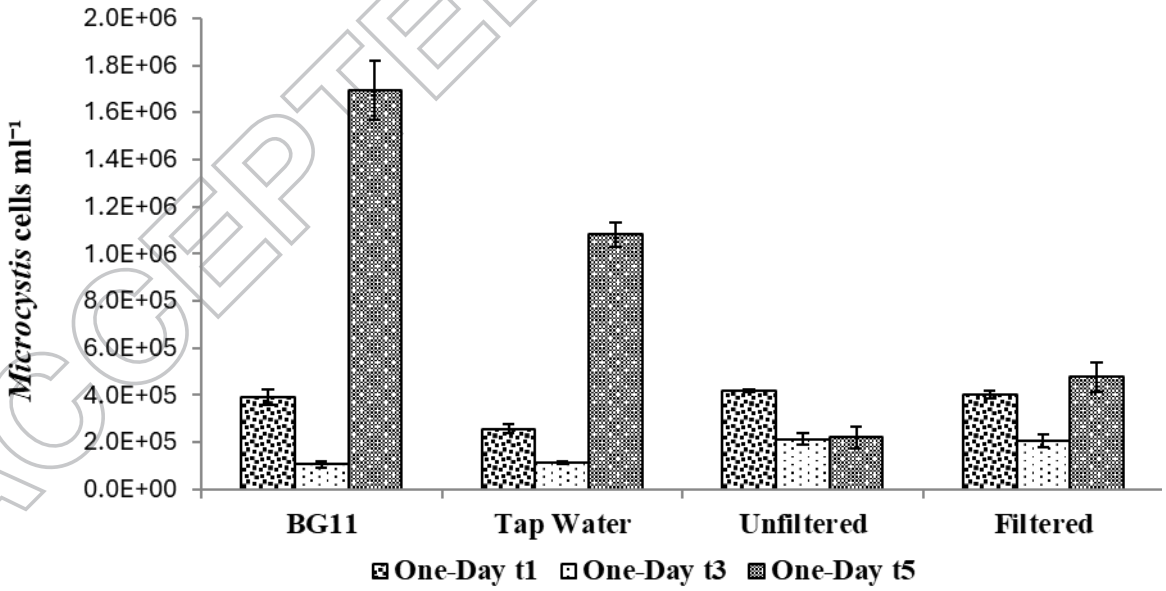


Figure 3. *Microcystis* growth (cells mL^{-1}) (mean±SE) for the controls (BG11 and tap water) and the response to the One-Day-Immersion with treatment (unfiltered and filtered) (*n*=15) on the 24 h (*t1*), 72 h (*t3)* and 120 h (*t5*).

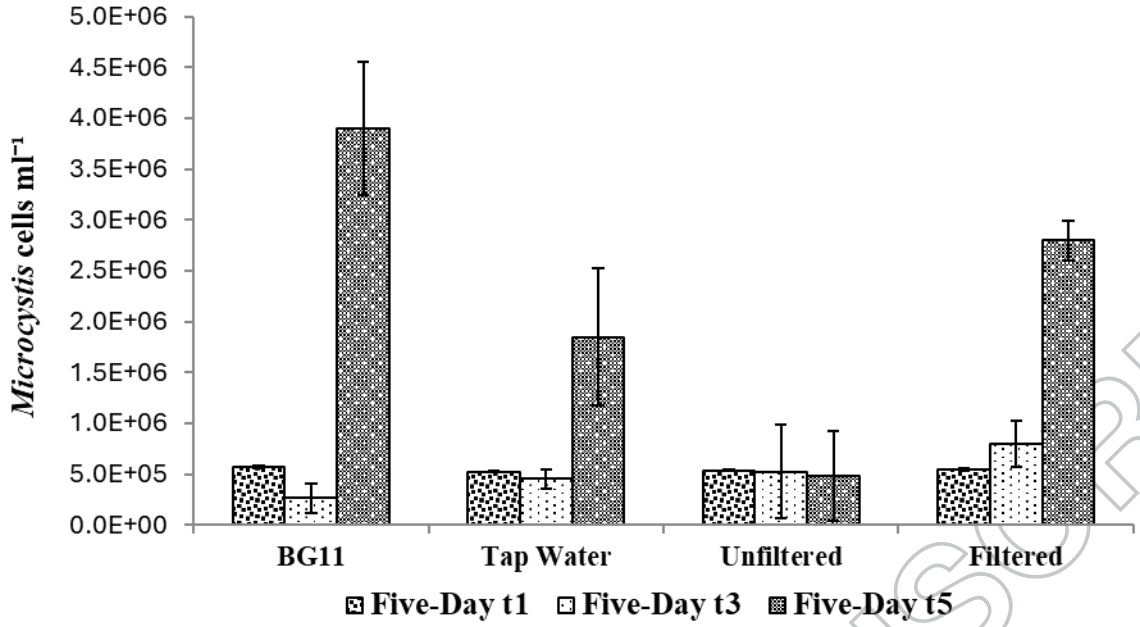


Figure 4. *Microcystis* growth (cells mL^{-1}) (mean±SE) for the controls (BG11 and Tap Water) and the response to the Five-Day-Immersion with treatment (unfiltered and filtered) (*n*=15) on the 24 h (*t1*), 72 h (*t3)* and 120 h (*t5*).

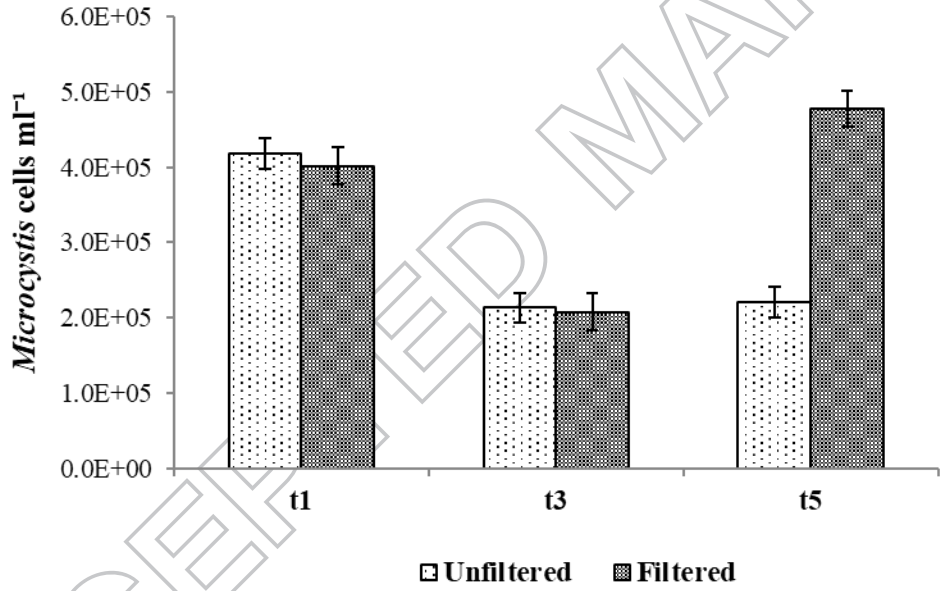


Figure 5. The mean (±SE) of *Microcystis* growth (cells mL^{-1}) in response to unfiltered and filtered treatment of the aqueous root exudate (*n*=15) as a function of time (*t1*, *t3* and *t5*).

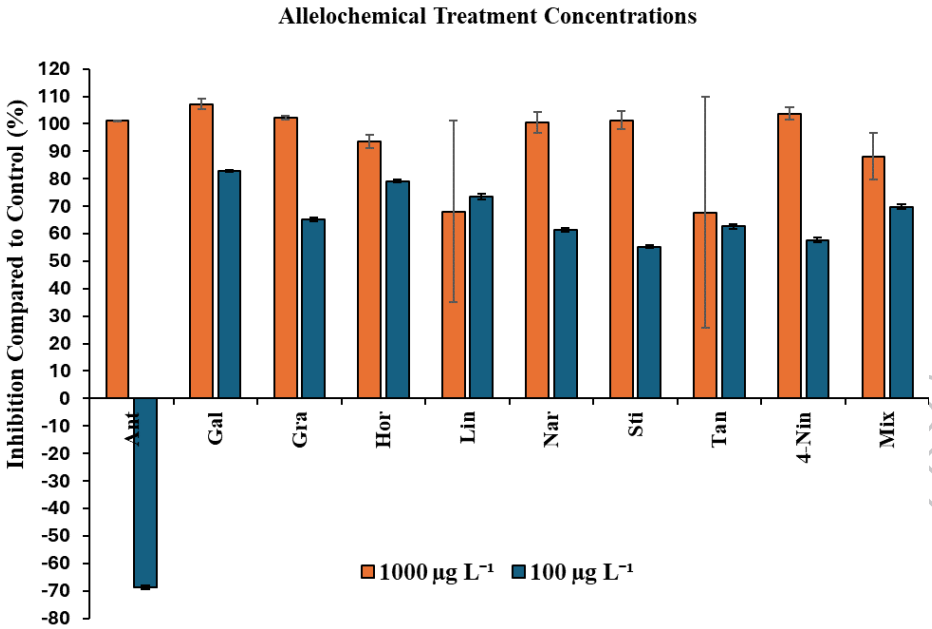


Figure 6. Percentage inhibition of Microcystis by commercial allelochemicals (mean±SD) with a concentration of 1000 µg L^{-1} (orange bars) and of 100 µg L^{-1} (dark bars) compared to a Microcystis control in the absence of allelochemicals (n=6). Tested allelochemicals: anthraquinone (Ant), acid galic (Gal), gramine (Gra), hordenine (Hor), linoleic acid (Lin), naringuin (Nar), stigmasterol (Sti), tannic acid (Tan), 4-nitroindol-5-carboxaldehyde (4-Nin) and *allelochemical*-mix (Mix).

Supplementary material

Supplementary Figure 1. Preparation of the allelochemicals and dilutions for the percentage inhibition experiment.