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Nutrient conditions influence allelopathic capabilities of *Ludwigia adscendens* and other tropical macrophytes against *Microcystis aeruginosa*

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Abstract

1. Macrophytes may release allelochemicals that suppress competing phytoplankton, although it is unclear how their effectiveness could be influenced by environmental nutrient conditions. To approach this, we conducted a series of bioassays against the bloom-forming cyanobacterium *Microcystis aeruginosa* using extracts and exudates from potentially allelopathic macrophytes under varying nutrient conditions.
2. We screened six different tropical macrophytes and identified antialgal effects in both extracts and exudates of *Ludwigia adscendens*, *Persicaria barbata*, *Pontederia crassipes* and *Vallisneria spiralis*. Growth assays indicated that extract inhibitory effectiveness decreased as nutrient concentration increased from 5% to 50% strength.
3. Focusing on the potent antialgal extract of *L. adscendens*, we determined that its inhibitory effects were enhanced by specifically reducing the availability of either micronutrient or bicarbonate availability, indicating a role of these nutrients in protecting algal cells from allelochemicals.
4. Bioactivity-guided fractionation and ultra-performance liquid chromatography – mass spectrometry analyses of *L. adscendens* extract indicated that ellagitannins were the major constituent of bioactive extract fractions, suggesting an antialgal mechanism common to other tannic compounds. Novel allelopathic alkaloids and terpenoid compounds also were described, although their mechanisms of action are unknown.
5. Our findings provide evidence that the availability of carbon and micronutrients to cyanobacteria could influence the effects of macrophyte allelochemicals. This interaction between the nutrient environment and allelochemical sensitivity of cyanobacteria can be substantial, and studies or applications involving allelopathic interactions between macrophytes and phytoplankton should consider this.

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KEYWORDS
allelochemicals, allelopathy, antialgal activity, bioassays, cyanobacteria, extract, exudate, nutrient limitation, phytoplankton

1 | INTRODUCTION

Aquatic macrophytes are important components of freshwater shallow lakes and are involved in many trophic interactions and other ecological processes (Søndergaard & Moss, 1998). Dense macrophyte cover can heavily influence lake ecosystem dynamics by stabilising clear water conditions and structuring biological assemblages (Scheffer et al., 1993; Thomaz et al., 2008). The ability of macrophytes to increase water transparency is largely attributed to the suppression of phytoplankton biomass via mechanisms such as direct nutrient competition (Ozimek et al., 1990), the provision of refugia for phytoplankton-grazing zooplankton (Timms & Moss, 1984), and the release of allelochemicals to inhibit competing phytoplankton (van Donk & van de Bund, 2002). The relative contribution of these factors to the inhibition of phytoplankton is highly variable, with substantial allelopathic capabilities observed only in certain macrophyte species (Mulderij et al., 2007).

Recent advances in the understanding of macrophyte allelopathy have fostered renewed interest in its use as a biological tool to manage cyanobacterial blooms in freshwater lakes (Yuan et al., 2020). Allelochemicals secreted by macrophytes include chemical classes such as polyphenols, flavonoids and alkaloids (Mohamed, 2017; Nakai et al., 2012), and are known to inhibit phytoplankton via several physiological effects, such as extracellular alkaline phosphatase inhibition (Gross, 1999), photosystem II inhibition (Leu et al., 2002) and induction of oxidative damage (Zhang et al., 2010). Macrophyte allelochemicals also tend to be more environmentally friendly than chemical agents owing to their high specificity and biodegradability (Yin et al., 2022; Zhang et al., 2013; Zhu et al., 2021). In contrast to nutrient competition and other well-understood mechanisms, however, the role of allelopathic inhibition in enabling macrophytes to suppress phytoplankton remains an area in need of active investigation.

A key consideration when studying allelopathic interactions between macrophytes and phytoplankton is their sensitivity to nutrients and other environmental factors. Interference of allelopathy by nutrient competition effects is acknowledged as a common issue and also can have implications for the ability of macrophytes to allelopathically suppress phytoplankton in natural ecosystems (Gross et al., 2007; Mohamed, 2017). The relationship between allelochemical effectiveness and the nutrient environment, however, is rarely examined explicitly. Nutrient-rich environments typically favour the dominance of phytoplankton over macrophytes, although some studies suggest that allelopathy could still allow macrophytes to compete effectively, or that its impacts on phytoplankton would be more pronounced under nutrient-enriched conditions (Mulderij et al., 2007; van Donk & van de Bund, 2002). The adaptive significance of allelopathy is further supported by the more frequent occurrence of allelopathically-active macrophyte species (e.g., *Myriophyllum*, *Ceratophyllum*, *Elodea*, and *Najas*) than non-active species (e.g., *Potamogeton* spp.) in eutrophic environments (Hilt & Gross, 2008).

Earlier studies have suggested that the nutrient environment also could modulate allelopathic interactions by influencing the physiology of both the donor macrophyte and the target phytoplankton (Gross, 2003; Reigosa et al., 1999). Nutrient limitation has been found to increase the production and release of allelochemicals in macrophytes such as *Myriophyllum spicatum* (Gross, 2003). Under nutrient stress, the target algal cells also may exhibit greater sensitivity to allelochemical inhibition (Fistarol et al., 2005; Reigosa et al., 1999), although the physiological mechanisms involved remain unclear, especially with regards to macrophyte allelochemicals (Gross et al., 2007; Inderjit & Duke, 2003; Mulderij et al., 2007). For instance, phosphorus (P) limitation has been found to enhance the inhibition of cyanobacterial alkaline phosphatase by tellimagrandin II, an allelochemical produced by *Myriophyllum* (Gross et al., 1996). Increased carbon dioxide supply also has previously been found to reduce the antialgal effectiveness of polyphenolic allelochemicals (Laue et al., 2014).

In the present study, we assessed the allelopathic capabilities of six locally-obtained macrophyte species against the bloom-forming cyanobacteria *Microcystis aeruginosa* and evaluated how the nutrient environment may influence the effectiveness of macrophyte-derived allelochemicals. Various macrophytes were firstly screened for allelopathic capabilities by examining the inhibitory effects of their aqueous extracts as well as their exudates, thus providing evidence for both allelochemical production and release (Gross et al., 2007). After confirming allelopathic capabilities in at least one of these plants, using its extract, we evaluated the importance of specific nutrients in mediating the inhibitory effectiveness of macrophyte allelochemicals for any macrophytes found to be active. By conducting two sets of growth assay experiments, we aimed to (i) assess the allelopathic capabilities of six different tropical macrophyte species against the target cyanobacteria under different nutrient concentrations, and (ii) investigate how the availability of specific nutrients (i.e., nitrogen [N], P, bicarbonate and micronutrient trace metals) influences the antialgal effectiveness of macrophyte allelochemicals. We hypothesised that (i) the growth inhibiting effects of macrophyte extracts and exudates on *M. aeruginosa* would increase as nutrient concentrations decreased, and (ii) reducing the concentrations of specific nutrients, particularly N and P, would increase the sensitivity of *M. aeruginosa* to macrophyte allelochemicals.

2 | MATERIALS AND METHODS

2.1 | Experimental macrophyte and phytoplankton cultures

Six tropical macrophyte species were assessed for allelopathic potential: *Ludwigia adscendens*, *Persicaria barbata* and *Pontederia crassipes* (previously known as *Eichhornia crassipes*; Pellegrini et al., 2018) are floating and/or emergent macrophytes that can be found in various water bodies in Singapore and Southeast Asia; *Hygrophila polysperma* and *Persicaria odorata* are amphibious species that may be found growing fully or partially submerged in water; and *Vallisneria spiralis* grows fully submerged. *Hygrophila polysperma*, *P. barbata* and *L. adscendens* were collected from wild populations, whereas *P. odorata*, *P. crassipes* and *V. spiralis* were purchased from local stores. These species have not been assessed for allelopathic capabilities in Singapore. All plants used in experiments were sustained in fertilised grow-out tanks for at least 2 weeks before each experiment.

A strain of *M. aeruginosa* (PA20130405 MA), originally isolated from Pandan Reservoir in Singapore in 2013 (Mowe et al., 2015), was used as the target organism in growth assays. This strain was maintained in MLA medium, which was derived from ASM-1 medium reported in Gorham et al. (1964), and supplemented with 20 mg/L cycloheximide to inhibit eukaryotic contaminants and incubated in a PERCIVAL Plant Growth Chamber (model I-36LL) at 25–27°C under a 12 h:12 h, light:dark photoperiod illuminated with cool-white fluorescent lamps (mean light intensity ~40 µmol m^{-2} s^{-1}). For the experiments, inoculated cultures were taken from a 21-day culture at the late log-phase, prepared in a 225 cm^2 Corning cell culture flask containing 400 ml of MLA medium. The composition of MLA nutrient media is described in Table 1.

TABLE 1 Composition of full-strength MLA nutrient medium used in algal cultures and assay experiments.

Constituent	Final concentration (mg/L)
Nutrients	
$NaNO_3$	85
K_2HPO_4	6.96
$MgSO_4{\cdot}7H_2O$	49.4
H_3BO_3	2.47
H_2SeO_3	0.0012
$NaHCO_3$	18
$CaCl{\cdot}2H_2O$	29.4
Micronutrients (trace metals)	
Na_2EDTA (chelating agent)	4.56
$FeCl_3{\cdot}6H_2O$	1.58
$MnCl_2{\cdot}4H_2O$	0.36
$CuSO_4{\cdot}5H_2O$	0.01
$ZnSO_4{\cdot}7H_2O$	0.022
$CoCl2{\cdot}6H_2O$	0.01
$Na_2MoO_4{\cdot}2H_2O$	0.006
Vitamins	
Thiamine HCl	0.1
Biotin	0.0005
B12	0.0005

Source: Adapted from Bolch and Blackburn (1996).

2.2 | Aqueous extraction and exudate collection

In order to obtain macrophyte exudates containing potential allelochemicals, macrophyte cuttings were obtained from growing tanks and rinsed with 0.3% sodium hypochlorite solution to eliminate possible algal contaminants (e.g., epiphytes and phytoplankton). For each species, cuttings were placed into separate containers filled with sterile ultra-pure water from Milli-Q® water purification system (henceforth referred to as Milli-Q water) to simulate an aquatic environment that allelochemicals would be exuded into (Figure 1). For the smaller submerged and amphibious species (*H. polysperma*, *P. barbata* and *V. spiralis*), 25 g of shoots were bundled together with inert polyester material and submerged entirely into separate 1-L Schott bottles filled with Milli-Q water. For the larger emergent and floating species (*L. adscendens*, *P. barbata* and *P. crassipes*), 100 g of plant material each were put into separate plastic aquaria (32 × 18 × 21 cm, L × W × H) containing 4 L of Milli-Q water, leaving only stems and leaves above water or partially submerged. Macrophytes were kept under a 12 h:12 h, light:dark photoperiod illuminated with a cool white fluorescent lamp for 7 days to allow exuded compounds to accumulate. All containers were placed within a larger holding container to reduce illumination of the container from indirect light sources.

Aqueous extraction was carried out in this study, as water-soluble components were expected to be most relevant to allelopathy (Erhard & Gross, 2006; Gao et al., 2014; Mangao et al., 2020). Up to 100 g of fresh shoots per species were used. Whole shoots were rinsed with Milli-Q water, blotted dry and macerated using an electric grinder. Plant material was then suspended with Milli-Q water (5 mL per g plant biomass) in darkness for 1 h. The mixture was then passed through a fine sieve and the filtrate was centrifuged at 10,000 ***g*** for 15 min at 4°C. The supernatant was collected, and vacuum-filtered through 0.45-µm cellulose acetate filters. Water from each tank, containing potential exudates, was collected, and then subjected to identical centrifugation and filtration procedures to remove contaminant microorganisms and solid material.

All extract and exudate samples were stored at −30°C. To estimate the concentrations of macrophyte extracts, 1 mL extract aliquots were dried using a CentriVap vacuum concentrator and the residue was weighed to estimate the solute concentration ($N = 5$; refer to Table S1). All exudate concentrations were too low to assess via this method and were instead applied in treatments relative to their neat concentrations.

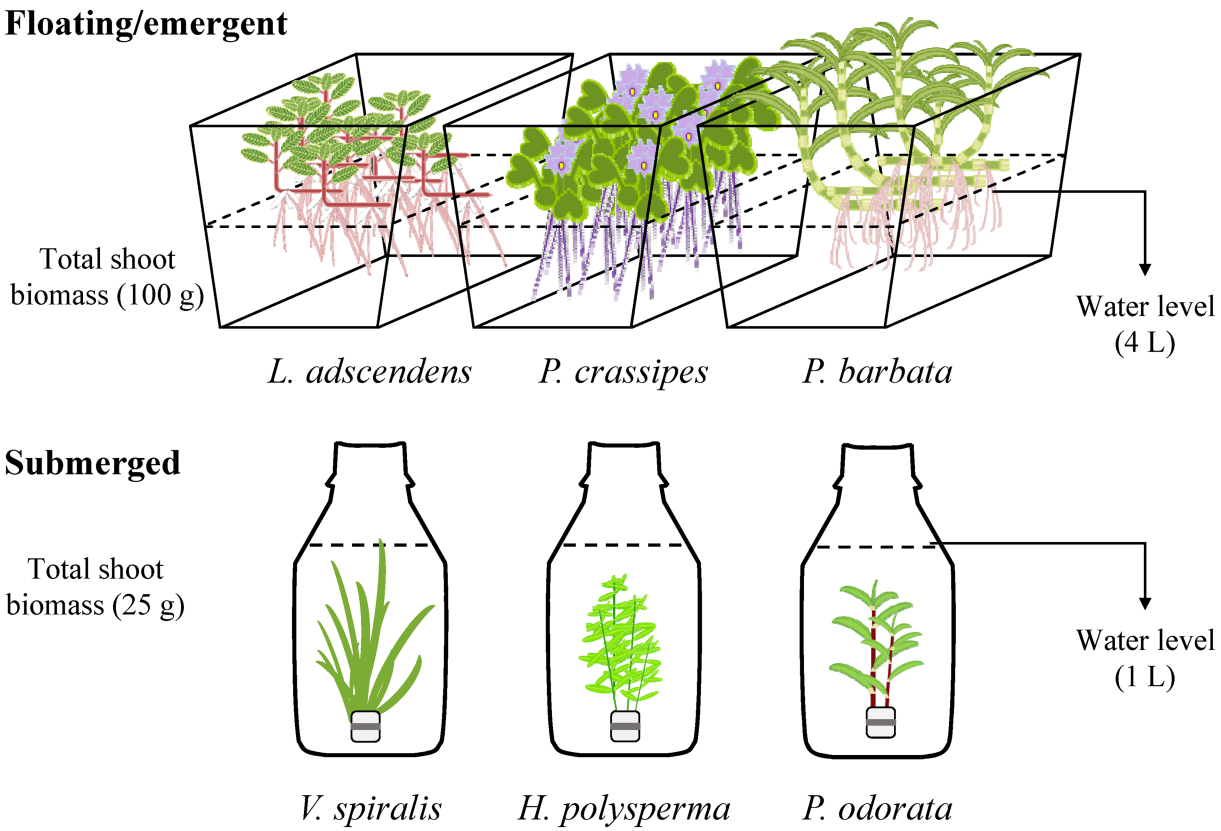


FIGURE 1 Set-ups used to collect exudates from the studied macrophytes. Floating/emergent: shoots of *Ludwigia adscendens*, *Persicaria barbata* and *Pontederia crassipes* were placed into containers containing 4 L of Milli-Q water. Submerged: shoots of *Hygrophila polysperma*, *Persicaria odorata* and *Vallisneria spiralis* were placed into Schott bottles containing 1 L of autoclaved deionised water.

2.3 | Study design

2.3.1 | Experiment 1: Screening macrophytes for allelopathic capabilities under a range of nutrient levels

The potential allelopathic capabilities of six macrophyte species, via their extracts and exudates, at varying nutrient conditions were established via a growth curve assay. Test cultures of *M. aeruginosa* were prepared at 5%, 10%, 25% and 50% strength MLA nutrient media and then treated with fixed concentrations of aqueous extract or exudate from each macrophyte species. Under these conditions, the concentrations of nitrate and phosphate in media were ecologically relevant, being analogous to moderately eutrophic to hypereutrophic lake environments (i.e., 5%–50% MLA is equivalent to 0.7–7.0 mg NO_3-N/L and 0.062–0.62 mg PO_4-P/L).

Extract and exudate treatments were applied at concentrations that allowed for exponential growth to be observed during the 14 days of incubation (Table 2). Most extracts were applied at 50 mg/L, except for *L. adscendens* and *P. barbata*, which completely inhibited growth for at least 14 days and were thus diluted to 10 mg/L for the experiment. Likewise, most exudates were applied at 90% strength relative to the raw exudate sample except for *L. adscendens*, which was diluted to 20% strength owing to its inhibitory potency. For positive controls, tannic acid (TA) was applied at two concentrations (1 and 5 mg/L), with the higher concentration exhibiting complete inhibition of *M. aeruginosa*. TA was used to represent a generic polyphenol allelochemical, which inhibits algal cells via reactive oxygen species (ROS) induction (see Chang et al., 2012; Eigemann et al., 2013). For the negative control, Milli-Q water was used in place of any antialgal treatment. Cycloheximide was added to all experimental cultures to inhibit fungal contaminants to a final concentration of 40 mg/L.

TABLE 2 Minimally effective treatment doses for extracts and exudates of each macrophyte species tested.

Species	Treatment dosage	
	Extract (mg/L)	Exudate (% strength)
Hygrophila polysperma	50	90
Ludwigia adscendens	10[a]	20[a]
Persicaria barbata	50	90
Persicaria odorata	10[a]	90
Pontederia crassipes	50	90
Vallisneria spiralis	50	90

[a]In a preliminary experiment, concentrations above the listed values for these treatments completely inhibited *M. aeruginosa* growth over 14 days.

After preparation, experimental cultures for each treatment combination were aliquoted to sterile 24-well microtitre plates (volume per well 2.7 mL; $N = 4$). The lids were then replaced, and plates were sealed with parafilm to limit evaporation and maintained at 25–27°C under continuous illumination with cool-white fluorescent lighting for 14 days (mean light intensity ~45 μmol m^{-2} s^{-1}). Each day, cultures were resuspended using a pipette and 100-μL samples were taken to estimate cell-density by measuring absorbance at 680 nm using a TECAN microplate reader, according to the linear relationship between optical density and cell density.

2.3.2 | Experiment 2: Effects of selective nutrient limitation on allelopathic efficacy

Ludwigia adscendens extract exhibited the most potent antialgal activity and was selected for subsequent experiments. In this assay, different MLA media components were selectively adjusted to varying concentrations to evaluate their importance in mediating allelochemical sensitivity in *M. aeruginosa*: NO_3^-, PO_4^{-3}, HCO_3^- (inorganic carbon [C] source) and micronutrients. Batches of test cultures were prepared according to differing combinations of five nutrient conditions (–N, –P, –HCO_3^-, -micronutrients, all four adjusted together) that were selectively adjusted to four concentration levels relative to stock MLA media (5%, 10%, 25%, 50%) (Table 3). All other media components otherwise not mentioned were fixed at 50% strength relative to stock MLA for all nutrient conditions. Subsequently, each of five treatment types were then applied: a negative control using sterile Milli-Q water, 1 mg/L TA, 5 mg/L TA, 10 mg/L LA extract, and 25 mg/L LA extract.

Test cultures for each treatment combination were aliquoted to the internal 60 wells of sterile 96-well microtitre plates (volume per well 200 μl; $N = 6$). Peripheral wells were filled with Milli-Q water, and the lids replaced and sealed with parafilm to limit evaporation. The microplate cultures were maintained at 25–27°C under continuous illumination with cool-white fluorescent lighting for 14 days (mean light intensity ~45 μmol m^{-2} s^{-1}). Each day, cultures were resuspended using a multi-channel pipette and the cell-density was estimated by measuring absorbance at 680 nm using a TECAN microplate reader.

2.3.3 | Experiment 3: Determination of candidate antialgal compounds in *Ludwigia adscendens* extract

In order to evaluate the composition of active antialgal compounds in *L. adscendens* extract, an activity-guided fractionation was carried out using reversed-phase high-performance liquid chromatography (RP-HPLC), detailed below. Fractions were collected every 1.5 min for a total of 60 fractions (F1–60) at 0.9 ml/min. The inhibitory efficacy of each HPLC fraction was then evaluated at different dilutions via growth assay, using 96-well microplates prepared in a similar manner as in Experiment 2. The following experimental treatments were then applied to entire plates ($N = 3$):

(i) Negative control (Deionised water)
(ii) Positive control (Final concentration 5 μg/mL TA)
(iii) 4× diluted LA fractions
(iv) 10× diluted LA fractions
(v) 25× diluted LA fractions

In each of the 60 interior wells, test cultures of *M. aeruginosa* were prepared and subjected to their respective treatments such that the final *M. aeruginosa* cell density was 10,000 cells/ml and MLA strength was 50% of stock. Microplates were covered, sealed and incubated at 25–27°C under continuous illumination with cool-white fluorescent lamps for 8 days.

Microplate cultures were then resuspended in their wells using a multichannel pipette and cell-density was estimated via optical density at 680 nm using a TECAN microplate reader. For treatment plates, the inhibition efficacy in each fraction well was calculated as percentage inhibition relative to the negative control plate after 8 days. Fractions with high inhibition efficacy analysed using ultra-performance liquid chromatography-mass spectrometry (UPLC–MS) to identify their constituents.

2.4 | Reversed-phase HPLC fractionation

The RP-HPLC was carried out on an Agilent 1200 series HPLC system using a Zorbax RP C18 column (3.5 μm, 4.6 × 150 mm). Solvent A was water (0.1% formic acid) and solvent B was methanol (0.1%

TABLE 3 Nutrient components used to prepare each nutrient limitation condition: 5%, 10%, 25% and 50% strength.

	Nutrient concentration (% strength)			
Limiting condition	**$NaNO_3$**	**K_2HPO_4**	**$NaHCO_3$**	**Micronutrients**
All nutrients	5/10/25/50	5/10/25/50	5/10/25/50	5/10/25/50
Nitrogen	5/10/25/50	50	50	50
Phosphorus	50	5/10/25/50	50	50
Bicarbonate	50	50	5/10/25/50	50
Micronutrient	50	50	50	5/10/25/50

Note: Not adjusted (50% strength): $MgSO_4 \cdot 7H_2O$, H_3BO_3, H_2SeO_3, $CaCl \cdot 2H_2O$, Thiamine, HCl, Biotin B12. See Table 1 for stock nutrient concentrations.

formic acid). For each extract, the injection volume was 1 ml, flow rate was 0.9 ml/min and the following dynamic gradient was applied: 0–10 min, 0% B; 10–78 min, 0–45% B; 78–79 min, 45–95% B; 79–84 min, 95% B; 84–85 min, 95–0% B; 85–90 min, 0% B. The detector was set up with the deuterium lamp for highest intensity and absorbance wavelengths of 215, 254 and 280 nm were used to screen for the absorbance of certain terpenoids (Cheung et al., 2001), phenolics (Realini, 1981) and flavonoids (Abourashed et al., 2002), respectively. Separated fractions were collected every 1.5 min for the duration of each run for a total of 60 fractions. Four HPLC runs were performed, and the respective fractions were consolidated with a combined volume of ~5.4 ml. Each fraction sample was frozen at −80°C, lyophilised, and then reconstituted in 2 mL Milli-Q water. Samples were stored at −30°C until use.

2.5 | UPLC QToF-MS identification

The most bioactive extract fractions at 100× concentration were analysed by LC–MS. Chromatography was carried out on a Waters ACQUITY™ UPLC system comprising an automated sample manager maintained at 4°C. 5 µL of each fraction/sample were sequentially injected onto BEH C18 column 1.7 µM, 2.1 × 100 mm (Waters) maintained at 35°C. Reverse-phase chromatography was performed using a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) mobile phases pumped at 0.25 ml/min by a binary solvent manager. The elution gradient used was as follows: 0–1 min, 5% B; 1–5 min, 5–30% B; 5–7 min, 30–32% B; 7–14 min, 32–50% B; 14–18 min, 50–90% B; 18–19 min, 90% B; 19–22 min, 90–5% B. Each sample was injected in triplicates (5 µl each) followed by a blank wash.

Eluted compounds were subjected to quantitative time-of-floght (QToF)-MS identification by Synapt G2-Si mass spectrometer (Waters), equipped with electrospray ionisation source and operated in positive mode. Data were acquired for 22 min with full scan in the 501,200 Da mass range using MS^E mode with 4 V and 10 V as low and high collision energy values, respectively. Samples were ionised at a capillary voltage of 3 kV and source temperature 120°C. To ensure accurate mass analysis, 200 pg/mL leucine-enkephalin (m/z 556.24 Da) was used as an external lockspray reference with a flow rate of 10 µl/min. All data were obtained in centroid mode and acquired using MASSLYNX software (Waters).

MS data analysis for all samples was processed on UNIFI version 1.9.2 software platform (Waters). A non-targeted screening approach was adopted to identify compounds present in the various extract samples. For data analysis, the following processing parameters were used for peak identification: automatic chromatographic 2D peak detection, with a peak detection limit of 1,000 per chromatogram. Targets were detected by mass with an error of ±10 ppm for identified compounds and five best matches were generated. We selected positive adducts including H^+, Na^+ and negative adducts containing $HCOO^-$ and H^- for any transformations and changes during the LC–MS analysis. Compound elucidation was performed by referencing the measured m/z of ions against a search database comprising the m/z of >6500 components, compiled from the Metabolite Profiling library (ESI positive ions) and the Traditional Chinese Medicine library (Waters). As the focus of this study is on metabolite discovery, identified compounds were only qualitatively analysed and not compared quantitatively.

Sample processing generated lists of identified compounds, which were then further processed to remove artefacts and less relevant compounds (e.g., carbohydrates, amino acids). A minimum detector response of 500 counts (100 counts for exudate samples owing to their much lower concentration of constituents) and a mass error threshold of ±5 mDa were used to exclude compounds with high signal/noise ratio and large mass differences compared to the theoretical mass. Compounds eluted during the last 3 min of the gradient (the wash step) for each fraction were excluded from analysis. False positives from possible column contamination or carryover between were excluded through comparison with sample blanks. Fits were identified if they were present in at least two of the three replicates. Compounds detected multiple times at different retention times were treated as structural isomers, as has been reported for castalagin and its isomer vescalagin, and for pedunculagin (Dincheva et al., 2013; Salminen et al., 2004). This filtering process was adapted from the natural products solution application provided by Waters Corporation.

2.6 | Data analysis

Using the R package *growthrates*, we fitted logistic growth curves to the estimated cell density of *M. aeruginosa* over time for each replicate. The Baranyi growth model (Baranyi & Roberts, 1994) provided the best fit for the experimental data and was used to estimate the following growth parameters: maximum growth rate (μ_{max}), carrying capacity (K) (i.e., the maximum cell density the nutrient medium can support) and physiological state (h_0) for each treatment. Initial abundance (y_0) was set to 100,000 cells/ml. The lag phase was defined as the period before the exponential growth phase, during which cell numbers remain nearly constant. This was determined from growth parameters by dividing h_0 by μ_{max} and was used as the main indicator of growth inhibition in *M. aeruginosa*.

During the experiment, the optical density of test cultures tended to gradually increase up to around Day 2, independent of actual cell density. This phenomenon was observed in a previous study testing plant extracts (Chen et al., 2012) and can be attributed to colorimetric reactions between the nutrient medium and polyphenols in extracts/exudates. To account for this inflation of optical density, estimated cell densities for all treatments were normalised to 100,000 cells/ml at Day 2 for analytical purposes. Because some test cultures began to either decline shortly after reaching the stationary phase

or increase owing to concentration from evaporation, the number of time points used for models was arbitrarily limited to exclude any deviant data points.

Differences in growth parameters were compared by (i) two-way analysis of variance (ANOVA) in Experiment 1, with "Extract treatment" and "Nutrient concentration" as interacting main effects, and (ii) three-way ANOVA in Experiment 2, with "Extract treatment", "Limiting nutrient" and "Nutrient concentration" as interacting main effects, followed by Tukey's honestly significant differences as the *post hoc* test. In replicates exhibiting no obvious algal growth, μ_{max} and *K* were excluded from analysis and the duration of the lag phase was assigned a value of 14 days for statistical analyses. For Experiment 1, comparisons were made for the growth parameters μ_{max}, *K*, and lag between treatments and the negative control. For Experiment 2, statistical comparisons were only made for lag time between nutrient concentrations for each treatment type, since μ_{max} and *K* were expected to increase proportionately with nutrient availability. The assumptions of the ANOVA, approximate normal distribution, and variance homogeneity were checked using Shapiro–Wilk's and Levene's tests, respectively.

All statistical analyses were performed in the R version 3.5.1 (R Development Core Team, 2018) and using the *growthrates* package (Petzoldt, 2017). Differences were considered statistically significant at $p < 0.05$.

3 | RESULTS

3.1 | Allelopathic capabilities under varying nutrient conditions

Growth curves for each treatment under different nutrient conditions are shown in Figure 2. Pairwise comparisons for different growth parameters found that only lag time was significantly affected by the treatment type. μ_{max} was similar across treatments except at low nutrient concentrations, and *K* was proportionate to nutrient concentration, unless algal growth was completely inhibited. Growth inhibition in treatments was expressed as a right-shift of the original growth curve which can be expressed as increases in lag phase duration, with stronger effects observed at lower MLA concentrations (Figure 3). In some cases, treatments appeared to completely inhibit growth during the experiment, possibly indicating that the lag phase extended beyond the 14-day experimental period (Figure 2). Pairwise comparisons against the negative control showed that most macrophyte extracts and exudates significantly inhibited the growth of *Microcystis* cultures ($p < 0.05$), except for exudates from *H. polysperma*, *P. odorata* and *P. crassipes* ($p = 0.99$–1.00), which did not induce any inhibitory effect regardless of nutrient condition. There were significant effects of nutrient concentration, extract treatment, and their interaction on the lag time of *M. aeruginosa* growth ($p < 0.05$; Table 4).

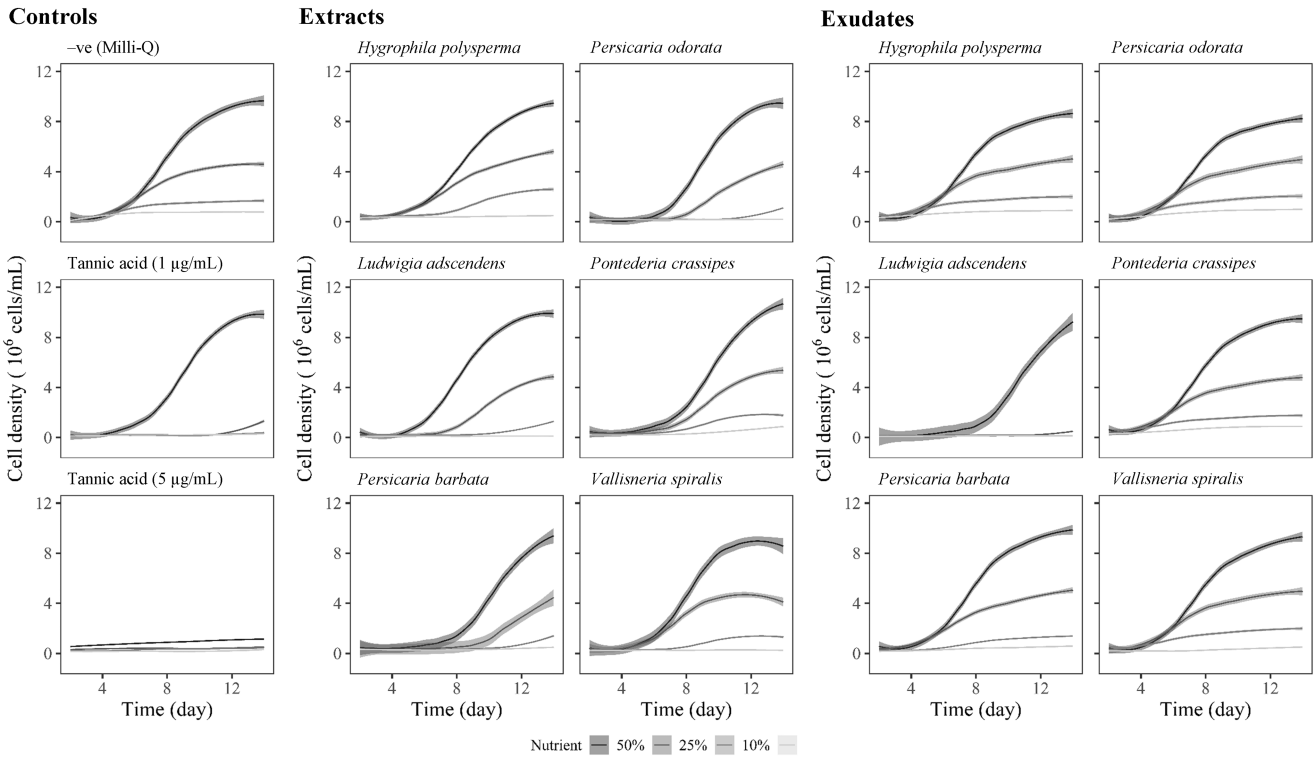


FIGURE 2 *Microcystis aeruginosa* growth curves following exposure to macrophyte extracts and exudates at different nutrient treatments (50%, 25%, 10%, 5% strength MLA). Extract concentrations used were 10 µg/mL for *Ludwigia adscendens* and Persicaria odorata, and 25 µg/mL for all other species. Exudates were applied at 20% strength for *L. adscendens* and 90% for all other species. Milli-Q water was used as a negative control, and tannic acid 1 µg/mL and 5 µg/mL were used as positive controls. Data from Days 2–14 are shown after correcting for optical discoloration.

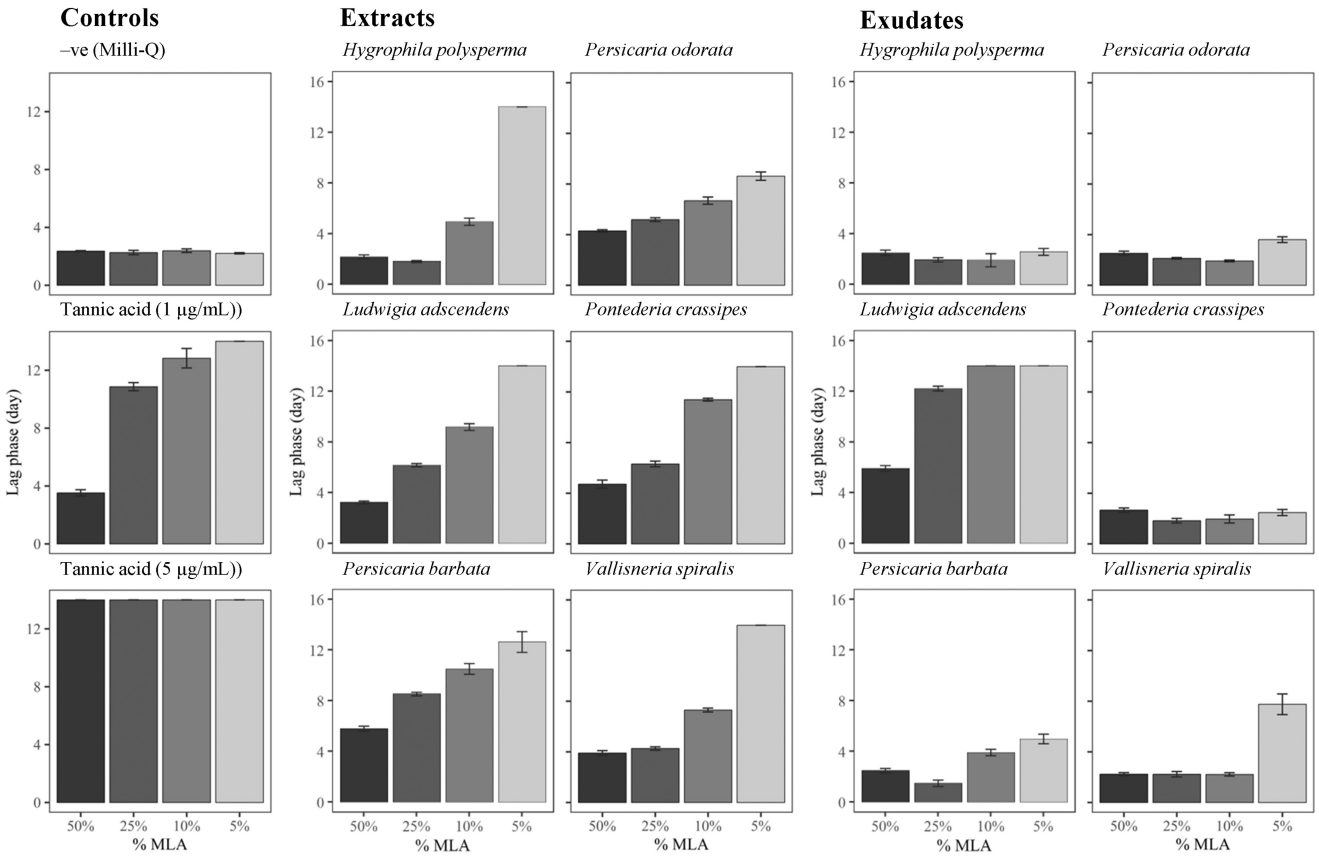


FIGURE 3 Estimated lag phase durations obtained from growth curve models for each macrophyte and nutrient combination. For the tannic acid (5 µg/mL) treatment, no algal growth was observed in all concentrations of MLA media. Treatments that completely inhibited *Microcystis aeruginosa* growth in experiments were assumed to have lag phases greater than 14 days and are depicted as such in the chart. Error bars denote standard error ($N = 4$).

TABLE 4 Summary of two-way ANOVA results for effects of extract treatment and nutrient concentration on the lag time parameter of *Microcystis aeruginosa* growth.

Explanatory variables	*df*	Sum of squares	*F*-value	*p*-value
Concentration	3	2.045	4.352	<0.01
Treatment	14	9.883	4.507	<0.001
Concentration × Limitation	42	19.121	2.907	<0.001

In the negative control, reducing nutrient concentrations alone had no significant effect on lag time, averaging 2.21–2.39 days across different nutrient conditions (Figure 3). For TA 1 mg/L and macrophyte extract treatments, successive reductions of nutrient concentrations resulted in significant increases in lag time, indicating an increase in the inhibitory effectiveness. Extracts from *L. adscendens* and *P. odorata* exhibited the strongest inhibitory effects despite being applied at lower concentrations, with lag times of 3.22 ± 0.11 days and 4.72 ± 0.32 days, respectively, at 50% MLA strength. No growth was observed in the TA 5 mg/L treatment.

Macrophyte exudates generally exhibited weaker inhibitory effects compared to their respective extracts (Figures 2 and 3). Only the exudates from *L. adscendens* exhibited inhibitory effects on the *Microcystis* test cultures with lag times of 5.91 ± 0.21 days at 50% MLA, with increasing effectiveness as nutrient concentrations decreased, with 12.2 ± 0.19 days at 25% MLA, and completely inhibiting algal growth at 5%–10% MLA. By contrast, other macrophyte exudates only inhibited *M. aeruginosa* growth at much lower nutrient concentrations: For *P. barbata* exudates, lag time increased to 3.92 ± 0.26 days at 10% MLA and 4.98 ± 0.38 days at 5% MLA; for *P. crassipes* exudates, lag time increased to 3.6 ± 0.23 days at 5% MLA; for *V. spiralis* exudates, lag time increased to 7.76 ± 0.81 days at 5% strength MLA. Exudates from *H. polysperma* and *P. odorata* were ineffective against *M. aeruginosa*.

3.2 | Effects of specific nutrient limitation

Reducing the concentrations of all nutrient components of MLA and, individually, bicarbonate and micronutrients, significantly enhanced the inhibitory effects of the allelochemical treatments, TA and LA extract (Table 5), by prolonging the lag phase of *M. aeruginosa* (Figure 4) and shifting growth curves right. Reducing the concentrations of all nutrients or specifically N also resulted in proportionate decreases to the carrying capacity of *M. aeruginosa* cultures in all treatments (Figure 4).

TABLE 5 Summary of three-way ANOVA results for effects of extract treatment and nutrient concentration on the lag time parameter of *Microcystis aeruginosa* growth.

Explanatory variables	*df*	Sum of squares	*F*-value	*p*-value
Concentration	3	538	1627.4	<0.001
Limitation	4	1019	2310.9	<0.001
Treatment	4	11,148	25,293.5	<0.001
Concentration × Limitation	12	702	530.8	<0.001
Concentration × Treatment	12	923	698.4	<0.001
Limitation × Treatment	16	1,507	854.8	<0.001
Concentration × Limitation × Treatment	48	1,101	208.2	<0.001

Three-way ANOVA found that extract treatment, limiting nutrient and nutrient concentration, as well as all interaction terms, significantly affected the lag time of *M. aeruginosa* ($p < 0.05$; Table 5). Estimated lag times for all combinations of treatment and nutrient conditions are shown in Figure 5. Several treatments prolonged the lag phase to the extent that complete growth curves could not be obtained (see Figure 4), thus the lag times for these treatments were assumed to be either less than or greater than 14 days, depending on whether any growth was observed. For the TA (5 mg/L) treatment, reducing the concentrations of all nutrient components, bicarbonate or micronutrients below 50% strength completely inhibited *M. aeruginosa* growth. At 5% P, the effect on lag time was significant relative to higher P concentrations ($p < 0.05$), although the effect was weak and inconsistent compared to other limiting nutrient conditions.

Pairwise comparisons found that, lag times were insensitive to nutrient conditions in the negative control, whereas in extract treatments, lag times were similarly insensitive to N or P concentrations, but increased when all nutrient, C and micronutrient concentrations were reduced. These comparisons indicate that nutrient reduction alone did not significantly affect lag time, except when extract treatments also were present, in the case that all nutrient, C and micronutrient concentrations were reduced.

For other limiting nutrients, reducing the concentration resulted in more substantial increases in lag time. Reducing bicarbonate concentrations enhanced the inhibitory effects of TA (1 mg/L) and LA extract (10 mg/L) treatments slightly, with significantly ($p < 0.05$) longer lag times at 5% and 10% compared to 25% and 50% strength. A stronger effect of bicarbonate availability on lag time was observed with a higher dose of LA extract (25 mg/L), evidenced by the growth curve shifting further right at decreasing bicarbonate concentrations (Figures 4 and 5).

Reducing micronutrient concentrations also had a comparable effect on inhibitory effectiveness, even at lower allelochemical doses. In both TA (1 mg/L) and LA extract (10 mg/L) treatments, *M. aeruginosa* lag time significantly increased from 2.29 ± 0.04 days at 50% strength micronutrients to 3.67 ± 0.09 days and 6.11 ± 0.28 at 10% and 5% strength, respectively (Figure 4). In the LA extract (25 mg/L) treatment, the gradual right-shift of the growth-curve indicated that lag time increased from 5.05 ± 0.27 days at 50% strength micronutrients to <14 days at 25% strength and >14 days at both 5% and 10% strength (Figures 4 and 5).

When all nutrient components were concurrently limited, the effects on allelochemical effectiveness were significantly stronger than when individual nutrients were limited. In the TA (1 mg/L) treatment, *M. aeruginosa* lag time significantly increased from 1.95 ± 0.18 days at 50% strength nutrients to 2.98 ± 0.17 days and < 14 days at 10% and 5% strength, respectively (Figure 5). In the LA (10 mg/L) treatment, the lag time significantly increased from 2.30 ± 0.09 days at 50% strength nutrients to 4.00 ± 0.14 days at 25% strength, <14 days at 10% strength, and >14 days at 5% strength. In the LA (25 mg/L) treatment, the right-shift of the growth-curve indicated an increase in lag time from 3.44 ± 0.42 days at 50% strength to <14 days at 25% strength and >14 days at 10% and 5% strength (Figures 4 and 5). The N and P concentrations did not substantially affect the inhibitory effectiveness of allelochemical treatments.

3.3 | Determination of candidate antialgal compounds in *Ludwigia adscendens* extract

The HPLC chromatographic profile of LA aqueous extract is shown in Figure 6. A broad chromatographic band was observed shortly after the start of the run, between ~0 and 12 min (fractions F1–9), indicating the presence of unbound compounds eluting from the column. The bulk of extract constituents was eluted between 14 and 62 min (F10–42); in this region, a broad band was present, indicating numerous unresolved compound peaks. From 80 to 88 min (F54–58), a broad peak/band was observed, caused by remnant compounds eluting from the column.

From the growth inhibition assay, the percentage inhibition of extract fractions relative to the negative control after 8 days was up to 99.4%. Inhibitory efficacy generally decreased with increasing dilution factor (i.e., 4×, 10×, 20×), allowing for the relative inhibitory strength of each fraction to be compared. At 4× dilution, most fractions from F2 to 42 strongly inhibited algal growth except for F9, which did not exhibit any inhibitory effect. Increasing to 10× dilution, the antialgal efficacy for several fractions was reduced and separate groups of more active fractions were noted: F2–4, F11–12, F16–36 and F38–41. Finally, at 20× dilution, the inhibitory efficacy of most fractions was substantially diminished, except for a small number of more active fractions: F22, F24–28, F32–34 and F39. Of these, fractions containing the most distinct peaks and inhibitory strength were selected for UPLC-MS analysis: F22, 24, 27, 28, 32, 33, 34 and 39. For the positive control, growth inhibition was always above 90%.

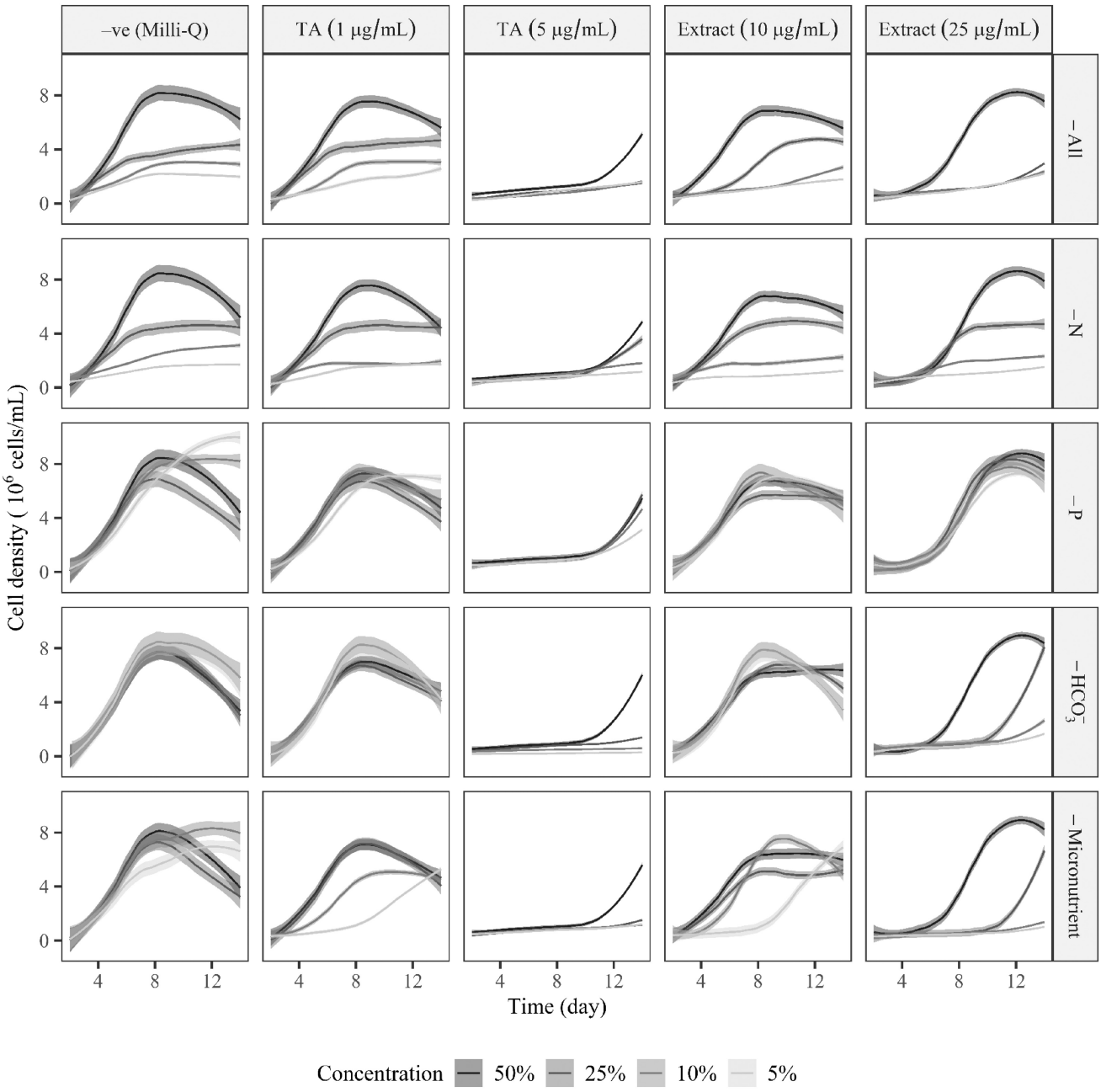


FIGURE 4 Growth curves of *Microcystis aeruginosa* test cultures for the –ve control, TA (1 µg/mL) and *Ludwigia adscendens* extract (25 mg/L) treatments, under combined, bicarbonate and micronutrient limitation. For each limiting nutrient, the concentration of the specific nutrient was adjusted to 5%, 10%, 25%, or 50%, while other components were adjusted to 50% relative to stock MLA. For combined limitation, the concentration of the entire MLA medium was adjusted to 5%, 10%, 25% or 50%.

3.4 | Chemical constituents of selected extract fractions and exudates from *Ludwigia adscendens*

With our selection criteria, we identified a total of 95 constituents across the selected extract fractions, comprising five coumarins, 18 ellagitannins, five flavonoids, three proanthocyanidins, 15 other phenolic compounds, 14 alkaloids, 29 terpenoids and five other miscellaneous compounds were identified. Relative abundances for each chemical class are reported for each sample in Table 6. The complete list of identified constituents is provided in Table S2. Chemical compositions of earlier-eluted fractions (F11, 12, 16) were distinct from those of later fractions (F22, 24, 27, 28, 32–34, 39). Early fractions contained mostly alkaloids, coumarins and terpenoids. Intermediate fractions (F22, 24, 27, 28, 32–34) contained mostly ellagitannins, with alkaloids and terpenoids being moderately abundant, while coumarins were absent. In the later-eluting F39, alkaloids were the most abundant class although ellagitannins were absent.

Overall, ellagitannins were the most abundant compounds in active fractions. Isomers of castalagin (RT: 3.9, 4.7 and 5.6 min)

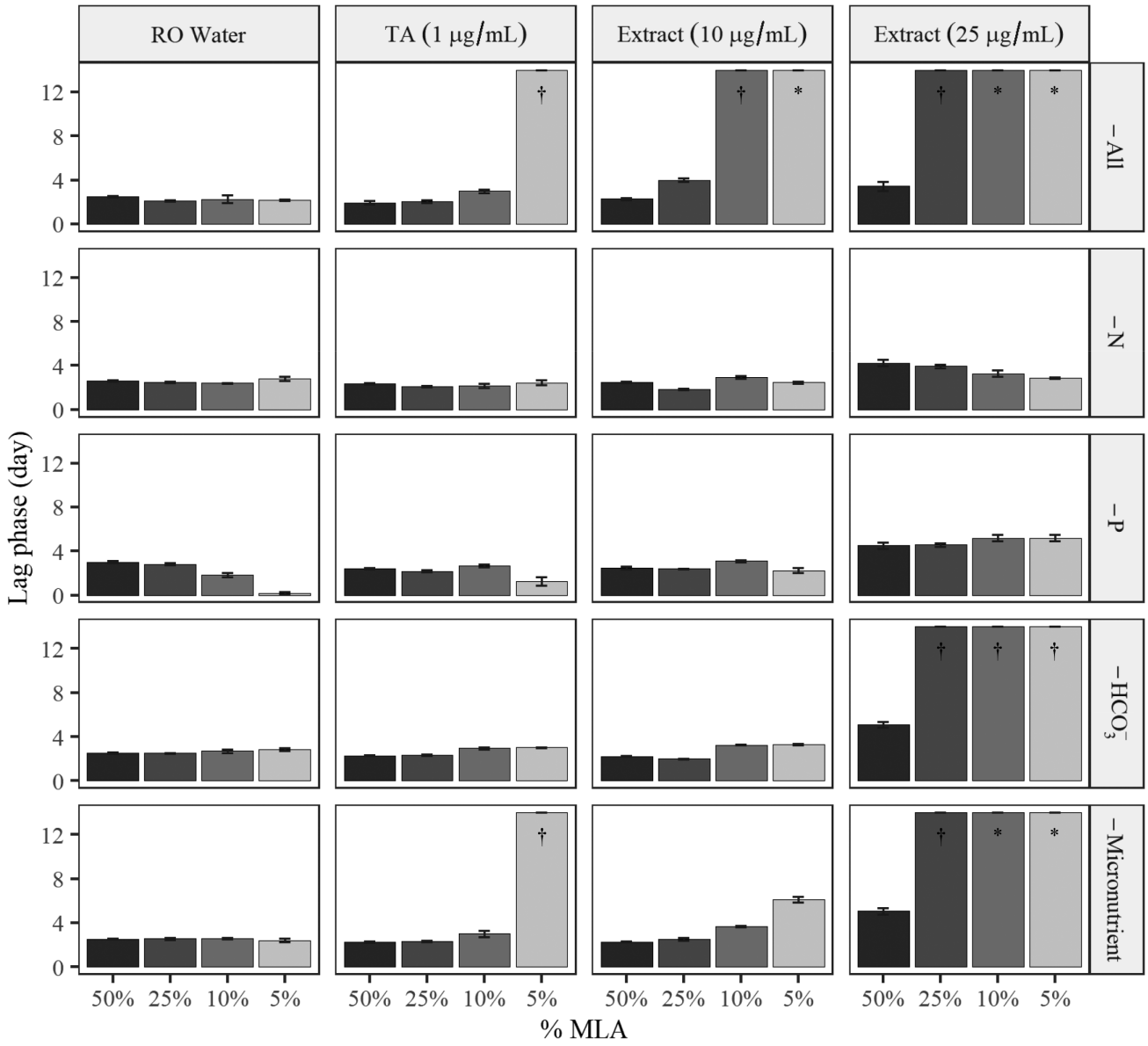


FIGURE 5 Estimated lag phases obtained from growth curve models for each combination of treatment type and nutrient limitation. For each limiting nutrient, the concentration of the specific nutrient was adjusted to 5%, 10%, 25% or 50%, while other components were adjusted to 50% relative to stock MLA. For combined limitation, the concentration of the entire MLA medium was adjusted to 5%, 10%, 25% or 50%. The TA (5 mg/L) treatment was omitted because algal growth was completely inhibited under most nutrient conditions. Treatments that completely inhibited *Microcystis aeruginosa* growth in experiments were assumed to have lag phases greater 14 days and depicted as such. Error bars denote standard error ($N = 6$). *indicates that no growth was observed throughout the experiment (lag time >14 days). †indicates that exponential growth was observed, but the lag time could not be estimated owing to the incomplete growth curve (lag time <14 days).

constituted the most abundant group of compounds; geraniin (RT: 3.9, 4.8 and 5.6 min) and pedunculagin (RT: 3.2, 3.8 and 4.9 min) and also were relatively abundant. A small number of coumarins were identified, mainly within fractions F11–12, 16 and 39, with phellopterin (RT: 3.1 and 18.5 min) being exceptionally abundant in F16, comprising 95.9% of coumarins (74.29% of all constituents). Several alkaloid constituents were relatively abundant, with pseudostrychnine (RT: 17.5 and 18.0 min) the most abundant. Terpenoids comprised the largest number of unique compounds identified from extracts, most of which were not exceptionally abundant. Xiongterpene (RT: 16.6, 17.6, 17.9 min) was the most abundant terpenoid, followed by shizukanolide A (RT: 11.7 min), ganolactone (RT: 18.4 min), eucommioside (RT: 17.7 min) and numerous other less abundant compounds.

4 | DISCUSSION

This study demonstrated that the efficacy of macrophyte allelochemicals against cyanobacteria targets such as *M. aeruginosa* can be moderated by the prevailing nutrient conditions. Of the six

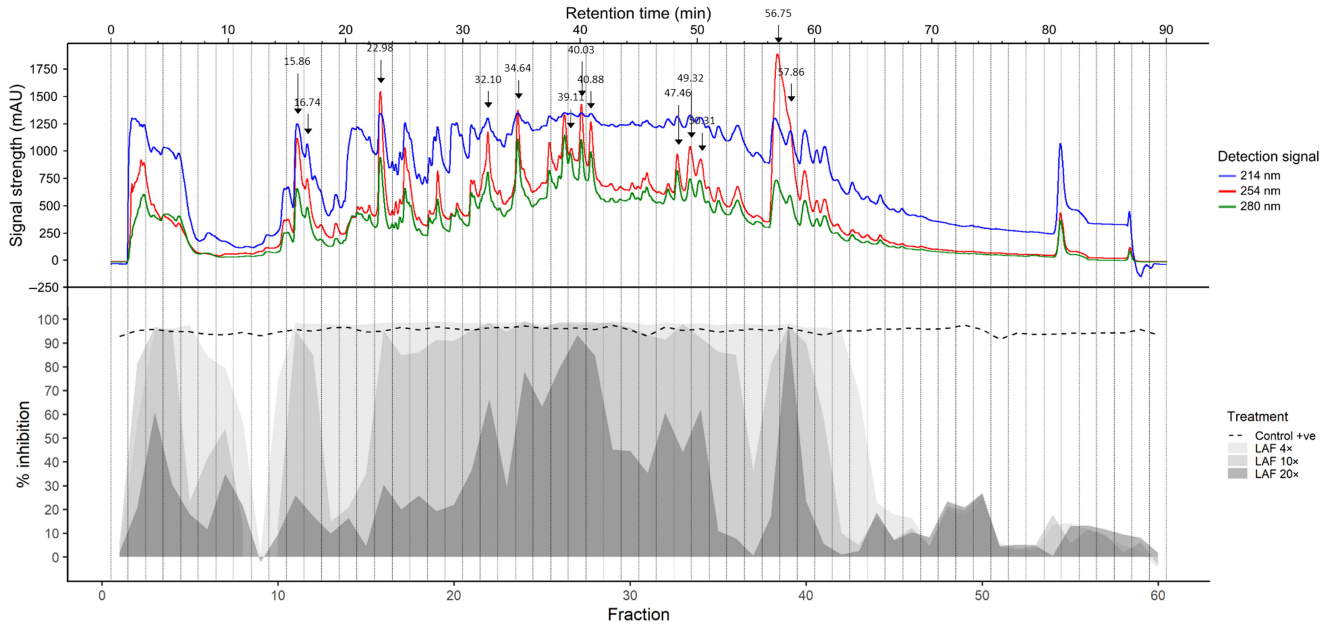


FIGURE 6 Reverse-phased high-performance liquid chromatography chromatogram of *Ludwigia adscendens* aqueous extract at detection wavelengths 214, 254 and 280 nm, fractionated into 60 fractions based on time (denoted by vertical lines). Corresponding inhibition efficacy against *Microcystis aeruginosa* after 8 days, relative to the negative control, is described in the chart below for three dilutions (4×, 10× and 20×). The dashed line indicates the percentage inhibition of the TA positive control. Retention times (min) for major peaks corresponding with antialgal activity in fractions are indicated.

TABLE 6 Relative abundance (%) of the major chemical classes as constituents of the selected extract fractions and of the concentrated exudate sample.

	Selected extract fractions										
Chemical class	**F11**	**F12**	**F16**	**F22**	**F24**	**F27**	**F28**	**F32**	**F33**	**F34**	**F39**
Phenolics											
Coumarins	11.6%		77.5%								9.3%
Ellagitannins				53.0%	89.8%	56.8%	51.9%	71.8%	31.1%	51.2%	
Flavonoids				1.6%				3.0%		4.2%	10.3%
Proanthocyanidin				3.2%	1.5%	10.8%	25.5%		1.0%		
Other phenolics	20.5%		5.5%	1.3%		2.2%		3.0%	56.7%	1.7%	
Alkaloids	14.7%		11.1%	12.6%	4.3%	14.9%	11.7%		5.1%	31.5%	48.0%
Terpenoids	48.3%	100.00	5.9%	24.8%	1.6%	12.0%	10.9%	11.8%	3.0%	9.1%	20.1%
Miscellaneous	4.9%			3.6%	2.8%	3.3%		10.4%	3.1%	2.3%	12.3%

macrophytes examined, four species yielded both extracts and exudates that exhibited antialgal inhibitory effects on *M. aeruginosa*, providing evidence of allelopathic capabilities in these species. Extracts and exudates of *L. adscendens* were notably potent and represent the first report of allelopathic capabilities against cyanobacteria in this species. The effectiveness of macrophyte-derived extracts was found to be enhanced when nutrient levels were reduced, suggesting that the nutrient status could mediate the allelochemical sensitivity of algal cells. Subsequent bioassay-guided fractionation indicated that the major inhibitory compounds present in *L. adscendens* extract were phenolic compounds, particularly elagitannins. Alkaloids and terpenoids also were abundant in some bioactive fractions. The identity of these constituent compounds in *L. adscendens* extract would offer clues as to the primary inhibitory mechanism observed here, as well as possible explanations for the interaction effect with nutrient conditions.

4.1 | Allelopathic potentials of various macrophyte extracts and exudates on the cyanobacterium *Microcystis aeruginosa*

In our experimental approach, the inhibitory effects of antialgal treatments on *M. aeruginosa* were expressed as a prolonged lag phase/delayed log growth. A similar algistatic effect has previously been reported by Mulderij et al. (2005) for *M. aeruginosa* exposed to exudates from *Stratiotes aloides*. It is important to note that the effects of nutrient concentrations and extract/exudate treatment

on *M. aeruginosa* growth dynamics were largely independent, the former determining the carrying capacity and the latter influencing growth lag. Although effective macrophytes initially arrested algal growth, the eventual resumption of exponential growth in some treatments matches earlier findings that allelochemicals have a limited lifespan in the environment (Gao, Dong, et al., 2017; Hilt & Gross, 2008; Nakai et al., 2000).

Experiment 1 offered evidence of allelopathic capabilities against cyanobacteria in *L. adscendens*, *P. barbata*, *P. crassipes* and *V. spiralis*. This finding corroborates earlier reports of allelopathy in *P. crassipes* (Shanab et al., 2010; Wu et al., 2012) and *V. spiralis* (Gao, Liu, Wang, et al., 2011), and demonstrates for the first time that *L. adscendens* and *P. barbata* can use allelopathy to reduce phytoplankton biomass as suggested in previous *ex situ* and *in situ* experiments (Mowe et al., 2019; Sim et al., 2021). While all extracts effectively inhibited *M. aeruginosa*, the potency of the exudates varied considerably between species: *L. adscendens* yielded the most potent extracts and exudates, whereas exudates from other species were only effective when MLA concentrations were reduced to 5%–10% strength. Notably, *Ludwigia* species have been reported to synthesise phytochemicals with diverse bioactive effects, including antibacterial, phytotoxic and repellent effects (Dandelot et al., 2008; Shilpi et al., 2010), although their antialgal capabilities have not yet been demonstrated in prior studies. The effectiveness of macrophyte exudates also was largely incongruent with that of their respective extracts, suggesting that the presence of antialgal compounds in a macrophyte's extracts may not accurately predict its allelopathic potential.

4.2 | Limiting availability of selected nutrients enhances the efficacy of macrophyte-derived antialgal compounds

In aquatic environments, algal cells can be exposed to a plethora of compounds with deleterious effects on cell viability, including allelochemicals from macrophytes, and thus must possess physiological means to neutralise harmful substances that they encounter. However, the efficacy of these mechanisms can be affected by extrinsic factors. Previous researchers have proposed that nutrient limitation could increase the sensitivity of the target phytoplankton to allelochemicals by altering their physiological state (Fistarol et al., 2005; Reigosa et al., 1999). This was broadly attributed to the essential role of nutrients in cellular functions such as protein synthesis, gene expression and DNA repair (Grossman et al., 1994), although their precise influence on allelochemical sensitivity in cyanobacteria is unclear. Other environmental factors, such as pollutant levels, also could impact allelochemical effectiveness (Zuo et al., 2016). Here, we demonstrated that reducing the availability of certain nutrients in the environment can significantly enhance the inhibitory effect of macrophyte allelochemicals against *M. aeruginosa*. Conversely, higher nutrient concentrations could negate any inhibitory effects from weaker antialgal treatments, indicated by the ineffectiveness of exudates of *P. barbata*, *P. crassipes* and *V. spiralis* above 5% strength MLA media (Figure 2).

Focusing on extracts from *L. adscendens*, which exhibited the most potent antialgal activity, we found that specifically reducing the availability of either bicarbonate (i.e., C source) or trace metal micronutrients could enhance inhibitory effects on *M. aeruginosa*. This suggests that these nutrients may be important for sustaining growth or maintaining protective cellular mechanisms while under allelochemical exposure. We also note that the growth inhibition effect was more potent when all nutrient components were reduced (i.e., N, P, bicarbonate, and micronutrients), compared to when only individual nutrients were adjusted, indicating synergistic roles of multiple nutrients in mitigating allelochemical stress. Nitrogen was determined to be the main limiting nutrient in our study since the carrying capacity was proportionate to the concentration of N in the treatment, likely indicating resource-limitation effects. However, N availability did not appear to influence the sensitivity of algal cells to growth inhibition by macrophyte allelochemicals, despite its significance as a macronutrient for the growth and proliferation of algal cells. Bicarbonate, as a vital C source within the nutrient medium, may also have been temporarily limiting at 5% and 10% strength, indicated by the biphasic growth curve in those treatments. We surmise that this indicated a physiological adaptation to sustain growth in a low-C environment, prior to reaching the carrying capacity imposed by a different limiting nutrient, which is likely to be N. Unlike the macronutrient N or C source of bicarbonate, micronutrient concentrations did not have an obvious effect on *M. aeruginosa* growth in the negative control, hence whether it was limiting growth was difficult to assess. Nevertheless, reducing the availability of either micronutrients or bicarbonate enhanced the potency of *L. adscendens* extracts to a comparable degree, underpinning the significance of these nutrients in interactions with some macrophyte allelochemicals.

Trends in the growth responses of *M. aeruginosa* under various nutrient conditions to nutrient limitation were generally similar between both tannic acid and *L. adscendens* extract treatments. On the one hand, tannic acid is a polyphenolic compound with antialgal properties that was chosen as a positive control to broadly represent macrophyte allelochemicals, many of which also are polyphenols and tannins (Gao, Liu, Zhou, et al., 2011), with common toxicological mechanisms, such as the induction of oxidative stress (Chang et al., 2012; Eigemann et al., 2013). On the other, the extract is a representative sample of the entire secondary metabolite repertoire of *L. adscendens* and also may include bioactive compounds with differing antialgal mechanisms.

4.3 | Tannins, alkaloids and terpenoids are major constituents of antialgal fractions of *Ludwigia adscendens* extract—insight into bioactive mechanisms

Moderately polar phenolic compounds and their derivatives were highly abundant in the most potent fractions of *L. adscendens* extract.

These compounds are commonly associated with macrophyte allelopathy (Gross et al., 1996; Hu & Hong, 2008; Laue et al., 2014) and are likely to have been the major contributors to its inhibitory effects against *M. aeruginosa* here. Most of the identified compounds are novel and have not been implicated in allelopathic phenomena previously. Notably, the allelopathic effects of *L. adscendens* could be attributed to a diverse range of different compounds, rather than a particular metabolite produced in abundance. Of these, ellagitannins were the most abundant class of compounds identified in *L. adscendens* extract, with tellimagrandin II, a known ellagitannin allelochemical produced by *Myriophyllum spicatum* also being present (Gross et al., 1996). Tannic compounds, such as ellagitannins and proanthocyanidins, are reported to exert a range of deleterious effects on algal cells, including by reducing photosystem II activity, lowering Chlorophyll-*a* content and inducing oxidative damage (Laue et al., 2014). As the antialgal activities of tannins are attributed to their hydrolysable subunits (Nakai et al., 2000), we expect that most tannic compounds, including tannic acid used in the control, would share similar mechanisms of action. While tannins are common throughout the plant kingdom, *L. adscendens* may be notable for the substantial quantities it produces and releases to the surroundings.

Other compounds were detected at much lower quantities. Alkaloids are another important class of allelochemicals and include gramine, a potent antialgal compound found in many grasses (Semenov & Granik, 2004). In bioactive fractions, we detected the indole alkaloid pseudostrychnine and several β-carboline alkaloids (i.e., picrasidine O and crenatidine), compounds that may possess antimicrobial activities including membrane disruption, inhibiting protein synthesis, and impeding DNA function. (e.g., Alamri & Mohamed, 2013; Cao et al., 2007; Kodani et al., 2002; Song et al., 2014; Tandon et al., 2014). Terpenoids and coumarins were the main components in less bioactive fractions and are thus expected to be less effective allelochemicals, especially compared to phenolics and alkaloids (Tamokou et al., 2017). Nevertheless, some coumarins have been determined to be allelochemicals in *Alternathera philoxeroides* (Zuo et al., 2016), and many others, including phellopterin, could exhibit antimicrobial activity (Walasek et al., 2015) or bacterial DNA gyrase inhibition (Qin et al., 2020; Torres et al., 2014).

Although the exact toxicological mechanisms of the *L. adscendens* extract on *M. aeruginosa* cannot be ascertained from our study, its composition allows us to infer how nutrient availability could have influenced algal sensitivity. For example, cyanobacteria are known secrete extracellular polymeric substances (EPS) to interfere with allelochemical activity (Ni et al., 2017), particularly against tannic compounds (Yin et al., 2022). The quantity and composition of the EPS determines its protective efficacy and can be positively influenced by the availability of iron (Jiang & Zheng, 2018), bicarbonate (Vergnes et al., 2019) and, although not observed here, nitrate and phosphate (Jiang & Zheng, 2018). Hence, reducing the availability of these nutrients could have impaired EPS formation, and increased the sensitivity of *M. aeruginosa* to allelochemicals, more-so if multiple nutrients are limited. Limited nutrient availability can also exacerbate allelochemical stress through the inability to supply essential cofactors, particularly iron or manganese, for oxidative stress proteins (Ferreira & Straus, 1994; Kaushik et al., 2015; Rueter & Petersen, 1987; Saha et al., 2016), exacerbating ROS stress from photosynthesis (Murata et al., 2007; Takahashi & Murata, 2006) and impaired metabolic homeostasis (Laue et al., 2014; Stöckel et al., 2013).

Contrary to expectations, the availability of the major macronutrients N and P did strongly influence allelochemical sensitivity in *M. aeruginosa*. One reason may be because N was always the limiting nutrient at stationary phase, thus obscuring any contrast between replete and limiting conditions. These observations also suggest that the general physiological responses of algal cells to macronutrient limitation, such as reducing biomacromolecule synthesis and increasing membrane permeability, may not strongly influence their sensitivity to the allelochemicals being studied, as previously suggested by Fistarol et al. (2005). Evidently, limiting multiple nutrient components had the strongest effect on allelochemical sensitivity in *M. aeruginosa* (Figure 5), implying a synergistic role of multiple nutrient pathways for cellular protection. This interaction could be attributed to different nutrients being involved in distinct physiological processes (Grossman et al., 1994; Kaushik et al., 2015; Rueter & Petersen, 1987). Further research would be necessary to identify possible synergies between different co-limiting nutrients.

4.4 | Implications and future studies

Notably, the potency of macrophyte allelochemicals varied considerably between species in addition to nutrient concentrations. One possible explanation is that allelochemical effectiveness may depend on the plant's ecology and susceptibility to phytoplankton competition for light or nutrients. For instance, free-floating (*P. crassipes*) and amphibious macrophytes (*H. polysperma* and *P. odorata*) are less susceptible to shading out by phytoplankton, thus lowering the importance of allelopathic capabilities. However, *Vallisneria* spp. are tolerant to low light and are rooted to the sediment, thus making it less susceptible to light and nutrient competition with phytoplankton (Gao, Zhang, et al., 2017). While the allelopathic capability of individual plants was found to be relatively weak, *Vallisneria* spp. usually grow in dense stands in nature where they are known to inhibit algal growth (Joglekar et al., 2001), possibly by the combined accumulation of allelochemicals to threshold concentrations. Like *P. crassipes*, floating creepers such as *L. adscendens* and *P. barbata* are unaffected by shading, although their less compact growth form may make them less able to shade out phytoplankton or compete for nutrients, thus necessitating allelopathy to inhibit competing phytoplankton. Future studies could investigate this plausible relationship between the life history and ecology of macrophytes and their allelopathic capabilities.

The nutrient-dependent sensitivity of algal cells to macrophyte allelochemicals could have important implications for our understanding of macrophyte–phytoplankton interactions. These findings suggest that allelopathic traits of macrophytes may have

diminished potency in environments that are enriched in inorganic C or certain trace metals, which can occur either naturally, such as nutrient release from storm surges, or because of anthropogenic influence, such as runoff from farms or urban developments (Facey et al., 2021). Considering that trace metals are often overlooked when managing nutrient loading in freshwater ecosystems (Facey et al., 2019), their influence on allelopathic interactions between macrophytes and phytoplankton is likely to be unaccounted for. This phenomenon could contribute to the breakdown of macrophyte-dominance in clear lakes under elevated nutrient loads and further emphasises the importance of nutrient control for managing phytoplankton, particularly of specific nutrients that can confer allelopathy resistance.

A warranted concern is that experimental conditions could mask the inhibitory effects of macrophytes, especially since many *ex situ* studies employ nutrient-replete conditions in their methodologies (Lürling et al., 2006; Tazart et al., 2020). Some approaches also set nutrients to non-limiting levels to exclude nutrient competition as a factor in co-existence experiments (Mulderij et al., 2005; Nakai et al., 1999), which could potentially obscure antialgal activity by macrophyte metabolites unless nutrient levels are adjusted accordingly, as demonstrated here. Since such elevated nutrient conditions are rare in nature, many macrophytes may have evolved allelochemicals that are most effective at lower nutrient thresholds. It may even be the case that allelopathy may be more widespread than originally perceived, albeit obscured by the approaches used for assessment.

Highly controlled laboratory assays may not accurately predict natural ecological interactions, and we are aware of certain caveats. Firstly, allelochemical concentrations were tested at static concentrations, whereas live macrophytes are expected to continuously exude and sustain these compounds at a certain level over time (Nakai et al., 2000). Secondly, non-axenic *M. aeruginosa* cultures were used, meaning that algae-associated heterotrophic bacteria are likely to have been present. These bacterial communities could mediate allelochemical effectiveness by accelerating the degradation of allelochemicals (Bauer et al., 2010), as well as by influencing the physiological and nutritional dynamics of algal cells (Shen et al., 2011). Thirdly, since a combination of micronutrients was evaluated in a batch treatment, it was unclear which element was responsible for the effect.

5 | CONCLUSION

Allelopathic interactions play a crucial role in mediating the dynamics of macrophyte and phytoplankton populations in freshwater ecosystems. Our study contributes to the literature by demonstrating using multiple macrophyte species, that the availability of nutrients could play a notable role in mediating the effectiveness of macrophyte allelochemicals against cyanobacteria. *M. aeruginosa* exhibited greater sensitivity to inhibition by antialgal compounds from macrophytes when nutrient concentrations were reduced and can potentially resist these growth-inhibiting effects when nutrients were replete. Of the macrophytes studied, *L. adscendens* exhibited exceptional antialgal activity, and its extracts were subjected to further examination, revealing that the increased allelochemical sensitivity of *M. aeruginosa* could be attributed to the reduction of micronutrient trace metals and bicarbonate availability, with stronger effects when multiple nutrients were limited at once. While the actual toxicological mechanisms of antialgal compounds present in *L. adscendens* extract were not examined, similarities in inhibition dynamics to tannic acid suggest that oxidative stress, which could be exacerbated by deficiencies in certain trace metals and C, may be involved. This relationship between nutrient availability and allelochemical sensitivity in cyanobacteria highlights the importance of accounting for nutrient conditions when examining allelopathic interactions in the field and or in experimental studies.

AUTHOR CONTRIBUTIONS

Conceptualisation: Darren Z. H. Sim, Simon M. Mitrovic. Conducting the research: Darren Z. H. Sim. Developing methods: Darren Z. H. Sim, Maxine A. D. Mowe, Nikhil K. Tulsian. Preparation of figures and tables, data interpretation, data analysis, writing: Darren Z. H. Sim, Maxine A. D. Mowe, Simon M. Mitrovic, Nikhil K. Tulsian, Ganesh S. Anand, Darren C. J. Yeo.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Data are available from the authors (D. Z. H. Sim) upon reasonable request.

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