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Allelopathic potential of Microcystin-RR at environmentally relevant concentrations: Species-specific growth responses of phytoplankton

Running title: Species-Specific Phytoplankton Responses to Microcystin-RR

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ABSTRACT

Microcystin-RR (MC-RR), a common cyanotoxin frequently detected in freshwater ecosystems, can influence phytoplankton dynamics by altering the growth patterns of coexisting species. While a large body of work has examined the allelopathic effects of microcystin-RR (MC-LR) and other microcystin variants, there are relatively few studies that specifically address the impact of pure MC-RR on phytoplankton species, particularly those that themselves produce this toxin, under environmentally relevant concentrations. This study investigated the effects of an environmentally relevant concentrations of MC-RR (1, 5, and 10 μg L^{-1}) on the growth of five phytoplankton species: three cyanobacteria (*Trichormus variabilis*, *Nostoc* sp., *Microcystis* sp.) and two green microalgae (*Chlorella* sp., *Scenedesmus* sp.), under laboratory conditions. Growth responses were monitored spectrophotometrically to determine chlorophyll *a* concentration over a 14-day period. Additionally, MC-RR was identified and quantified in the treated cyanobacterial cultures using the HPLC-DAD technique. The results demonstrated species-specific responses. MC-RR exhibited a stimulatory effect on both green algae species. In contrast, *T. variabilis* showed progressive growth inhibition, which became statistically significant after day 5. *Nostoc* sp. displayed slight, non-significant inhibition, while *Microcystis* sp. showed tolerance to MC-RR exposure. These findings demonstrate the allelopathic potential of MC-RR, with species-specific effects on growth that reflect differential sensitivity among phytoplankton taxa. The results underscore the ecological relevance of this toxin in shaping phytoplankton community structure and species interactions in freshwater ecosystems.

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Introduction

Allelopathy is a biological phenomenon in which biochemicals produced by one organism have positive or negative effects on other organisms (Cheng and Cheng, 2015). A wide range of organisms, including plants, cyanobacteria, algae, fungi, and soil microorganisms, can synthesize and release bioactive molecules that act as allelochemicals and thereby exert allelopathic effects (Polyak & Sukharevich, 2025; Leao et al., 2010; Revillini et al., 2023). Effects of allelopathy can be either inhibitory or stimulatory, and they can shape species composition and diversity by conferring competitive advantages to certain organisms (Willis, 2007). Allelopathy plays a significant role in shaping the structure and dynamics of aquatic microbial communities (Gross, 2003).

Phytoplankton is a diverse group of photosynthetic prokaryotic and eukaryotic microorganisms which are widely distributed in both aquatic (freshwater and marine) and terrestrial environments, where they contribute significantly to primary production and global carbon cycling (Falkowski, 1994, Kosek et al., 2016). Ecologically, phytoplankton serve as a foundational component of the aquatic food chain (Yarnold et al., 2019), providing essential nutrients for zooplankton and other consumers. As eukaryotic unicellular or colonial organisms, green microalgae (phylum *Chlorophyta*) are characterized by the presence of chlorophylls *a* and *b*, giving them their distinctive green color, and by storing energy in the form of starch within their chloroplasts (Graham et al., 2009). In addition to their ecological roles, microalgae have attracted growing interest for their potential in biotechnology, particularly in the fields of biofuel production, wastewater treatment, and as sources of valuable biomolecules such as antioxidants, pigments, and fatty acids (Srimongkol et al., 2022). Cyanobacteria represent a diverse and widespread group of photosynthetic prokaryotes belonging to the domain Bacteria, that can thrive in a wide range of environmental conditions, from freshwater and marine systems to extreme habitats such as hot springs and deserts (Schirrmeister et al., 2015; Whitton & Potts, 2012). Ecologically, cyanobacteria as well as green microalgae, are primary producers and form the base of many aquatic food webs (Saleem et al., 2025). However, under favorable conditions such as high nutrient availability, warm temperatures, and stable water columns, some cyanobacteria and green microalgae can proliferate excessively, leading to harmful algal blooms (HABs) (Paerl & Otten, 2013). These blooms not only disrupt aquatic ecosystems but can also produce a variety of bioactive and toxic compounds, including microcystins, cylindrospermopsins, and anatoxins, which pose serious risks to animal and human health (Zanchett & Oliveira-Filho, 2013).

In freshwater ecosystems, cyanobacteria are known not only for their primary productivity and ecological plasticity but also for their ability to produce a wide array of secondary metabolites with allelopathic potential. Cyanobacterial allelopathy is widespread and occurs in almost all aquatic habitats (Śliwińska-Wilczewska et al., 2022). Production of allelopathic compounds represents an

adaptation performed by some cyanobacteria to get a competitive advantage over other primary producers (Śliwińska-Wilczewska et al., 2021). Cyanobacteria produce potent compounds known as cyanotoxins, the ecological and allelopathic roles of which remain only partly understood (Teneva et al., 2023). Among these, microcystins (MCs), a group of cyclic heptapeptide hepatotoxins, are predominantly recognized for their toxic effects on animals and humans. The cytotoxic effects of cyanotoxins have been well studied in animals and humans, but many questions remain about their effects on coexisting phytoplankton communities (Omidi et al., 2021). However, beyond their toxicological significance, MCs have increasingly been investigated for their ecological roles, including potential allelopathic functions within phytoplankton communities (Wei et al., 2024; Polyak & Sukharevich, 2025).

MCs are heptapeptides formed by L-amino acids, regularly distributed in a cyclic structure based on the general structure: cyclo-[(1)-alanine-(2)-X-(3)- methyl aspartic acid-(4)-*Z*-(5)-Adda-(6)-glutamic acid-(7)-methyl dehydroalanine] (Martínez-Piernas et al., 2025). Since 2010, MC-RR has been found in water bodies and cyanobacterial blooms in Africa, America, Asia, and Europe, and is the most frequent MC congener in lakes, rivers, and reservoirs in China (Junfeng et al., 2010). Microcystin-RR (MC-RR) is one of the most commonly detected microcystin variants in freshwater systems and, as this congener is the second most common cyanotoxin in the environment (Díez-Quijada et al., 2019), it merits particular attention in ecological-toxicological studies. Its occurrence at environmentally relevant concentrations, particularly during and after cyanobacterial blooms, raises important questions regarding its impact on coexisting microorganisms. While MC-RR has been extensively studied in terms of its mode of toxicity and accumulation in higher trophic levels, its effects on aquatic primary producers, especially in the context of interspecific interactions, remain underexplored.

Cyanobacteria and green microalgae often coexist and compete for limited resources in eutrophic waters (Suikkanen et al., 2004). The ability of some cyanobacterial species to influence competitors' growth via allelochemical release may confer a competitive advantage, contributing to bloom dominance and persistence. However, responses to such chemical cues can vary widely depending on the target species, their physiological state, and environmental conditions (Li et al., 2023). Shifts in community structure, driven by group-specific environmental sensitivities, can alter elemental cycling at both local and global scales (Litchman et al., 2015).

This study aims to assess the allelopathic potential of MC-RR at environmentally relevant concentrations by evaluating species-specific growth responses in selected cyanobacteria and green microalgae. The findings are expected to contribute to a better understanding of the ecological functions of MCs in shaping the structure of the phytoplankton community.

We hypothesize that MC-RR, at concentrations commonly detected in natural waters, exerts species-specific allelopathic effects on the growth of freshwater phytoplankton. Specifically, exposure to environmentally relevant concentrations of MC-RR will affect the growth rates of

cyanobacteria and green microalgae, reflecting species-specific sensitivity. Additionally, we are particularly interested in how cyanobacterial strains that produce MC-RR will respond to the presence of that toxin under experimental conditions. We further posit that cyanobacterial strains will exhibit greater tolerance to MC-RR than green microalgae, suggesting a potential competitive advantage during bloom events.

Experimental

Phytoplankton cultures and cultivation conditions

Chlorella sp., *Scenedesmus* sp., *Nostoc* sp. and *Microcystis* sp. were isolated from a freshwater pond (43°17'51.9"N 21°47'40.8" E) in Southeast Serbia and were cultivated at the Department of Biology and Ecology, Faculty of Science and Mathematics in Niš. They are identified using an identification key to the genus level (Burchadt, 2014). *Trichormus variabilis* 0441 (Kützing ex Bornet & Flahault) (Komárek & Anagnostidis, 1989) (heterotypic synonym *Anabaena variabilis*) was isolated from the Danube River in the Vojvodina region (Serbia). It was cultivated in the Department of Biology and Ecology laboratory in Novi Sad (NSCCC). *T. variabilis* was identified using molecular methods described in the previous study (Stankovic et al., 2022).

All cultures were prepared in 250 ml Erlenmeyer flasks at 24 °C under cool LED lighting (15 W, 6400 K, 1500 lm) with a 16:8 h light: dark cycle and constant aeration. Standard BG11 (Rippka et al., 1979) liquid medium was used to cultivate all species except *A. variabilis* and *Nostoc* sp. These species were cultivated using modified BG11 medium without a nitrogen source.

Exposure conditions to MC-RR and growth influence test

To examine the influence of MC-RR on several phytoplankton species, the effect of different concentrations of MC-RR (1, 5, 10 µg/L) on the growth rate of *A. variabilis*, *Microcystis* sp., *Nostoc* sp., *Chlorella* sp., *Scenedesmus* sp., within two weeks was monitored. Investigated concentrations of MC-RR are based on their ecological relevance: they span from the low-level World Health Organization drinking-water guideline (~1 µg L^{-1}, set for MC-LR) through concentrations commonly encountered during cyanobacterial blooms (5–10 µg L^{-1}). This range allows a clear characterization of dose–response effects, while noting that MC-RR is generally considered to be less toxic than MC-LR. In the experiment, in the sterile glass test tubes 10 ml of liquid medium BG11 were added, 2 ml of a culture of each strain individually, and a specific concentration of MC-RR. Each concentration for each tested strain was set up in triplicate, as were the controls (9 replicates for each species). Controls (without MC-RR) were monitored for assay validity. All tubes were incubated at 21°C under cool LED lighting for a 16-h photoperiod for 14 days. Phytoplankton growth was monitored spectrophotometrically to determine chlorophyll concentration. In the first seven days the measurements were taken daily to capture the rapid initial growth and transition into the exponential phase; thereafter a single measurement was taken at day

14 to characterize the stationary/plateau phase of the growth curve. During the two weeks of the experiment, 1 ml of the culture, previously vortexed, was placed in cuvettes, and the optical density at 678, 720, and 750 nm was determined spectrophotometrically (Shimadzu UV-1650PC, double-beam). The concentration of chlorophyll *a* was calculated according to the following equation (Stankovic, 2020):

$$Chl\ (\mathrm{m}g\times ml\text{-}1) = 14.96 \cdot (OD678 - OD750) - 0{,}616 \cdot (OD720 - OD750) \qquad \text{(Eq. 1)}$$

MC-RR detection and quantification

To assess whether *T. variabilis*, *Nostoc* sp., and *Microcystis* sp. produce the cyanotoxin MC-RR, 500 mg of lyophilized biomass were extracted with 5 mL of a solvent mixture (75% methanol, 25% water). The suspension was sonicated in an ultrasonic bath for 30 minutes to ensure efficient cell disruption. Following ultrasonication, the samples were centrifuged at 4000 rpm for 10 minutes at 20 °C, and the obtained supernatant was filtered through 0.22 µm membrane filters (Agilent), according to the method described by Minasyan et al. (2018).

Toxin detection and characterization were performed using an Agilent 1200 Series HPLC system (Agilent Technologies, USA), equipped with a photodiode array (DAD) detector, an autosampler, a binary pump, and ChemStation software. Chromatographic separation was achieved on a Supelcosil ABZ Plus analytical column (Supelco, 150 × 4.6 mm, 5 µm). The mobile phases consisted of water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% trifluoroacetic acid (TFA). A linear gradient from 20% to 80% of solvent B was applied over 30 minutes, at a flow rate of 1 mL min^{-1}, with a column temperature of 40 °C and an injection volume of 10 µL. UV absorbance spectra were recorded in the 190–300 nm range using the DAD detector. Toxin identification and quantification were carried out by comparison with certified MC-RR standards (LGC, Germany).

Statistical Analyses

Statistical data processing was performed by STATISTICA 8 software (Statsoft, Inc., Tulsa, OK, USA). An independent t-test was used to determine the significance of the difference in mean values between groups, with statistical significance set at $p < 0.05$.

Results and Discussion

In this study, we observed that exposure to low concentrations of MC-RR (1 µg/L and 5 µg/L) did not lead to any measurable changes in the growth rates of the five tested phytoplankton strains, three cyanobacteria and two green microalgae, compared to control groups. On the other hand, exposure to a concentration of 10 µg/L of the same toxin resulted in a detectable alteration in the growth rate of certain tested strains. Accordingly, only the effect of the 10 µg/L concentration was graphically analysed in the subsequent sections.

Cyanobacterial strains and their responses

The growth dynamics of the three cyanobacterial strains were then investigated in detail to assess the effect of the toxin treatment (Figure 1).

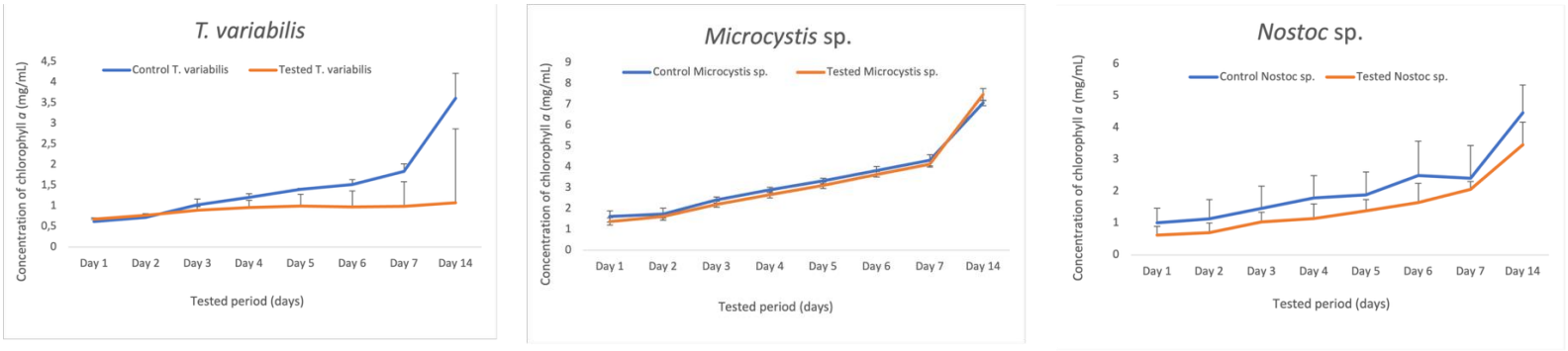

Figure 1. Spectrophotometric comparison of the growth of tested cyanobacteria exposed to MC-RR (10 µg/L), compared to the growth of the same strains in control conditions. Mean chlorophyll-a values (mg/mL) are shown for eight consecutive days (n = 3). Positive error bars represent +SD above the mean

Statistical analysis revealed a significant inhibition of growth in *T. variabilis* from day 5 until the end of the 14-day experiment when exposed to the cyanotoxin MC-RR at a concentration of 10 µg/L (Figure 2). Lower concentrations (1 µg/L and 5 µg/L) produced no measurable changes compared with the control. In contrast, for *Nostoc sp.* and *Microcystis sp.*, the presence of dissolved MC-RR at all three tested concentrations (1, 5, and 10 µg/L) had no significant effect on growth throughout the experimental period. Both strains exhibited growth trajectories similar to their controls, indicating high tolerance to the applied toxin.

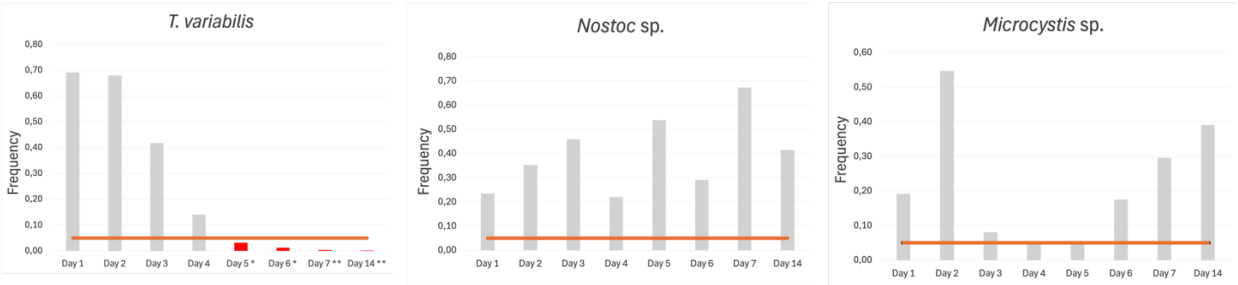

Figure 2. Bars represent the values for daily comparisons between the Control and MC-RR treatments, with significant inhibition highlighted in red. The horizontal line denotes the significance threshold at $p = 0.05$. A single asterisk (*) indicates $p < 0.05$, two asterisks (**) indicate $p < 0.01$, and three asterisks (***) indicate $p < 0.001$

Quantification of intracellular MC-RR by HPLC-DAD confirmed intracellular toxin production in all three cyanobacteria (Figure 3). The measured intracellular concentrations were 16.62 µg/g in *T. variabilis*, 8.13 µg/g in *Nostoc sp.*, and 4.95 µg/g in *Microcystis sp.* These findings suggest that the strains naturally produce MC-RR, reflecting their underlying genetic capacity for its biosynthesis.

The inhibitory response of *T. variabilis* to 10 µg/L MC-RR contrasts with the tolerance observed in *Nostoc sp.* and *Microcystis sp.*, suggesting species-specific sensitivity and possibly differences in detoxification capacity or cellular uptake of the toxin. Previous studies have indicated that cyanobacteria capable of producing MCs often exhibit self-protection mechanisms, including regulated export via ABC transporters, sequestration within specific compartments, and glutathione-dependent conjugation that prevent intracellular accumulation of harmful concentrations (Zilliges et al., 2011; Wei et al., 2024).

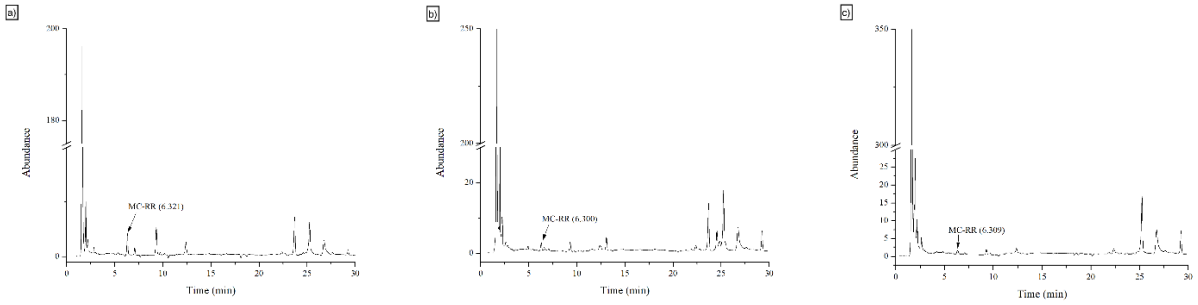

Figure 3. Chromatograms of detected and quantified MC-RR in tested Cyanobacteria (a- *Microcystis* sp., b-*Nostoc* sp., c-*T. variabilis*)

The growth inhibition detected in *T. variabilis* at the highest concentration may indicate that exogenous MC-RR exceeded its physiological tolerance threshold, disrupting protein phosphatase activity, photosynthetic efficiency, or redox homeostasis. MCs, including MC-RR, are known to bind and inhibit serine/threonine protein phosphatases 1 and 2A (MacKintosh et al., 1990; Žegura et al., 2011), thereby impairing signal transduction and increasing reactive oxygen species (ROS) formation. Such stress responses could explain the statistically significant suppression of growth observed after day 5.

By contrast, *Nostoc* sp. and *Microcystis* sp. showed no measurable inhibition, likely reflecting a higher basal tolerance or more efficient detoxification of MC-RR. Both genera are well-known microcystin producers and may sustain growth even in environments containing dissolved toxins (Kaplan et al., 2012; Hu & Rzymski, 2019). This insensitivity to environmentally relevant concentrations (~ 10 µg/L) supports the hypothesis that cyanobacteria are generally resilient to their own toxins, while they possess a genetic background to produce specific toxins. The quantification of intracellular MC-RR in tested cyanobacteria further supports this interpretation. Our findings of strain-specific MC-RR production are consistent with earlier studies indicating

that cell quotas may vary by more than an order of magnitude depending on nutrient status, light intensity, and growth phase (Christiansen et al., 2003, Wood et al., 2021).

Taken together, our findings indicate that *T. variabilis* is moderately sensitive to externally applied MC-RR at 10 µg/L, whereas *Nostoc* sp. and *Microcystis* sp. are largely unaffected. All three strains are confirmed MC-RR producers, which implies that physiological regulation of intracellular and extracellular toxin pools plays a central role in determining their tolerance. Similarly, a previous study (Li et al., 2009) showed that MCs can trigger stress responses even in their producer cyanobacteria, as MC-RR induces antioxidant enzyme genes (*sodB*, *katG*) and the heat-shock protein gene *dnaK2* (encoded for Hsp70) in *Synechocystis*. Also, proteomic analyses in *Microcystis aeruginosa* reveal changes in protein profiles linked to toxin production, suggesting a multifaceted regulation of intracellular toxin pools (Alexova et al., 2011; Tonietto et al., 2012). These results highlight the complexity of cyanobacterial interactions with dissolved MCs and underscore that toxin effects are both strain and concentration dependent.

It should be noted that the applied MC-RR concentration in our experiment was measured independently of the live cultures, and we did not monitor real-time synthesis, uptake, efflux, or degradation of MC-RR by the cells during the experiment. Therefore, we cannot exclude the possibility that extracellular and intracellular MC-RR levels changed over the course of the experiment in ways that influenced our observations. For example, cyanobacteria may retain, sequester, or re-bind MCs, or conversely release them under stress or via lysis — dynamics that could modulate toxicity and physiological responses. Indeed, previous studies suggest that microcystin export and protein-bound pools can vary across the growth cycle, indicating complex regulation of toxin partitioning (Wei et al., 2016; Greenstein et al., 2020).

Microalgal strains and their responses

Over a 14-day period, exposure of the green microalgae *Scenedesmus* sp. and *Chlorella* sp. to MC-RR at three concentrations revealed that only 10 µg/L elicited a stimulatory effect on growth (Figure 4). For both microalgae species, the lower concentrations (1 µg/L and 5 µg/L) did not produce statistically significant differences in growth compared to controls over the 14-day period. In contrast, at 10 µg/L, the response diverged. For Scenedesmus sp., a statistically significant stimulation of growth was observed from day 5 onward, and for *Chlorella* sp., stimulation reached significance only at day 14 (Figure 5). Thus, MC-RR at 10 µg/L triggered enhanced biomass production in both tested green microalgae under the culture conditions used.

The stimulatory effect of MC-RR on these green microalgae at 10 µg/L, and the absence of effect at 1 and 5 µg/L, indicate a threshold effect and a non-linear dose-response. That is, below about 10 µg/L, no detectable response occurred, but at this level, the microalgae responded by increasing growth. This pattern is consistent with the phenomenon of hormesis, where a low dose of some environmental agent can cause low stimulation or have a beneficial effect on certain cells, while a high dose can cause inhibitory or toxic effects (Mattson, 2007).

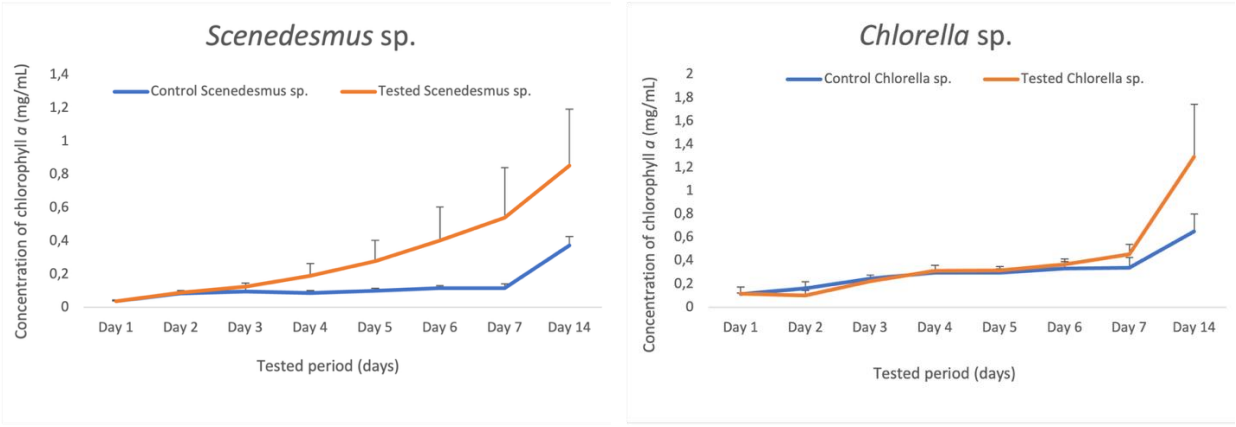

Figure 4. Spectrophotometric comparison of the growth of tested green microalgae exposed to MC-RR (10 µg/L), compared to the growth of the same strains in control conditions. Mean chlorophyll a values (mg/mL) are shown for eight consecutive days (n = 3). Positive error bars represent +SD above the mean. The control culture shows a continuous increase in chlorophyll a content, whereas the MC-RR treatment exhibits reduced growth throughout the experiment

In the literature, the effects of MCs on green algae are variable and often inhibitory. Most previous work on green algae and MCs (especially the variant Microcystin-LR, MC-LR) has focused on inhibitory responses at high doses. For example, exposure of *Chlorella vulgaris* to MC-LR at hundreds of µg/L caused growth inhibition and oxidative stress (Campos et al., 2013; Teneva et al., 2023; Li et al., 2024). However, a review found that some green algae respond to pure cyanotoxins (MC-LR at 5 g/L) or allelopathic compounds under certain conditions with stimulation rather than inhibition (Teneva et al., 2013). The variant MC-RR is less frequently studied than MC-LR, and its specific interactions with phytoplankton remain underexplored.

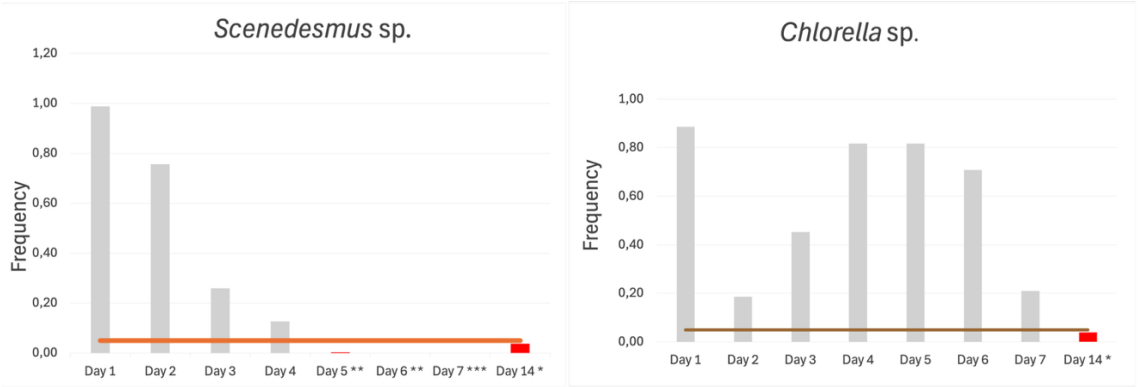

Figure 5. Bars represent the values for daily comparisons between the Control and MC-RR treatments, significant inhibition highlighted in red. The horizontal line denotes the significance

threshold at $p = 0.05$. A single asterisk (*) indicates $p < 0.05$, two asterisks (**) indicate $p < 0.01$, and three asterisks (***) indicate $p < 0.001$

The absence of any effect at 1 and 5 µg/L suggests that these concentrations are below the physiological detection or activation threshold of the microalgae under our culture conditions. The switch to significant stimulation at 10 µg/L is consistent with already mentioned hormetic type dose–response.

The difference in timing between the two species likely reflects intrinsic physiological or ecological differences: *Scenedesmus sp.* responded early (day 5) whereas *Chlorella sp.* only showed stimulation at day 14. This may reflect differences in growth rate, capacity to detect or metabolize MC-RR, or differential regulatory or adaptive mechanisms triggered by toxin exposure.

Our result is consistent with the notion that the effects of dissolved MCs may be species-specific, and that not all phytoplankton will respond adversely at low toxin levels. For instance, Babica et al. (2007) reported that microcystin effects vary greatly depending on both the phytoplankton species and the specific microcystin variant tested; in their study, some species showed resilience at low concentrations. Similarly, Sedmak et al. (2005) demonstrated morphological and physiological changes under microcystin exposure in representative phytoplankton, but typically at higher concentrations than those tested here.

Conclusion

These findings demonstrate the allelopathic potential of MC-RR, with species-specific effects on growth, reflecting differential sensitivity among phytoplankton taxa. The emergence of significant growth alteration at 10 µg/L suggests that this concentration may represent a biologically relevant threshold in ecosystems experiencing blooms. Monitoring concentrations approaching this level should be treated as potentially harmful to sensitive phytoplankton taxa.

Our experimental data demonstrate that while all tested cyanobacterial strains produce MC-RR, their tolerance to externally applied MC-RR varies considerably among strains. Whereas *Nostoc* sp. and *Microcystis* sp. tolerated up to 10 µg/L without inhibitory effects, *T. variabilis* suffered significant growth inhibition at the same concentration. Thus, the hypothesis that MC-RR-producing cyanobacteria will generally exhibit greater tolerance than green microalgae is only partially supported and appears to be highly strain-specific rather than a universal trait. Meanwhile, the green microalgae *Scenedesmus* sp. and *Chlorella* sp. displayed a stimulatory response to 10 µg/L MC-RR, suggesting that under certain conditions MC-RR may even enhance growth in non-cyanobacterial taxa. Results underscore the role of this toxin in influencing phytoplankton community structure and interactions in freshwater ecosystems.

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Conflict-of-Interest Statement

The authors declare no conflict of interest.

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