

THE INFLUENCE OF MICROCYSTIN-LR AND
MICROCYSTIN-LR-PRODUCING *TRICHORMUS*
VARIABILIS (CYANOBACTERIA) ON GREEN
MICROALGAE IN LABORATORY CONDITIONS

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Abstract

The most studied cyanobacterial toxins are microcystins (MCs), of which microcystin-LR (MC-LR) is the most common, with the most experimentally documented toxicity to aquatic species. The aim of the present study is to examine the influence of an ecologically relevant MC-LR concentration (10 µg L⁻¹) on green microalgae (*Chlorella* sp., *Scenedesmus* sp., *Coelastrum* sp.) by determining the concentration of chlorophyll *a*. In addition, the influence of the *Trichormus variabilis* was monitored, characterized as MC-LR producer, on the growth of green microalgae, using the co-cultivation method. MC-LR was detected, quantified, and identified in the methanolic extract of *T. variabilis* using the high-performance liquid chromatography (HPLC). The study revealed that MC-LR significantly impacted green microalgae growth causing stimulation. In contrast, *T. variabilis* in co-culture exhibited only an inhibitory effect on the growth. The study revealed the allelopathic effect of environmentally relevant microcystin-LR concentration on green microalgae.

Key words: microcystins, chlorophyll *a*, growth, HPLC, co-culture

Introduction. Freshwater cyanobacteria produce the wide range of secondary metabolites [1]. These bioactive compounds, which can negatively or positively affect other organisms, are called allelochemicals [2] and can be toxic

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and non-toxic [1]. Allelopathic interactions between cyanobacteria and green microalgae in the aquatic environment, with favourable environmental conditions (high temperature and abundance of nutrients, especially phosphorus and nitrogen, increased light intensity, low turbulence), usually cause massive blooms, and as a result, many toxic secondary metabolites, including cyanotoxins, are produced, which can harm the aquatic environment [3]. The most commonly found cyanotoxins are microcystins (MCs), which have over 300 variants [4], including microcystin-LR (MC-LR). The prominent mechanism of their action is the inhibition of protein phosphatases (PPs), which can lead to cellular damage, oxidative stress, apoptosis, cytoskeletal dysfunction, cancer cell invasion, and autophagy [5]. Many genera of cyanobacteria produce MCs, where the most common are: *Microcystis*, *Anabaena* (*Dolichospermum*), *Planktothrix*, and *Aphanizomenon* [6]. One of the hypotheses is that MCs have allelopathic potential [7]. It is important to consider MCs as allelopathic compounds, particularly in the context of aquatic organisms that constantly interact with cyanobacteria, including green algae, because their interactions play a crucial role in ecosystem dynamics.

MCs can cause inhibition [8] or stimulation [9] of phytoplankton species. While previous studies have predominantly focused on higher concentrations of MC-LR, which may not accurately represent current conditions in freshwater ecosystems [10], examining the effects of environmentally relevant concentrations is crucial for gaining insights into the true ecological implications of this toxin. By exploring the impact of MC-LR at concentrations commonly found in freshwaters, a more precise and realistic perspective of its effects on organisms inhabiting these ecosystems, including algae, can be obtained.

This study was based on the hypothesis that environmentally relevant concentrations of MC-LR can modulate algal growth without necessarily leading to growth inhibition, expressing allelopathic potential. To explore these dynamics, the study used some of the most common algal species to investigate allelopathic interactions and the influence of MC-LR. To achieve this goal, this study (1) detected, identified, and quantified MC-LR produced by *Trichormus variabilis*; (2) examined the influence of an ecologically relevant concentration of MC-LR ($10 \mu\text{g L}^{-1}$) on the growth rate of green microalgae (*Chlorella* sp., *Scenedesmus* sp., *Coelastrum* sp.); (3) examined the influence of *T. variabilis* on the growth of green microalgae; (4) compared the results from these two separate experiments because the difference in the influence could suggest that *T. variabilis* produces other metabolites besides the toxin, which probably act synergistically with MC-LR.

Materials and methods. Toxin analysis. To identify and quantify the MC-LR produced by *T. variabilis*, the following procedure was applied: about 0.05 g of dried biomass was extracted by the addition of 2 mL of extraction solvent containing 75% methanol and 25% water. It was subsequently sonicated in an ultrasound water bath for 30 min to achieve cell lysis. The extract was cen-

trifuged at $4000\times g$ for 10 min at 20°C and the supernatant was collected and filtered through $0.22\text{ }\mu\text{m}$ filters (Agilent) [11]. The HPLC system (Agilent Technologies (USA), Series 1200) equipped with an Agilent photodiode array detector (DAD) with an automatic injector, a binary pump was used for detecting and identifying toxins. Also, Chem Station software was used for analysing of obtained results. The extracts were separated by an analytical column Supelcosil ABZ Plus (Supelco, $150\times 4.6\text{ mm}$, $5\text{ }\mu\text{m}$) using water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% trifluoroacetic acid (TFA). The applied gradient was 20% B to 80% B for 30 min with the 1 mL per min flow rate. The injection volume was 10 μL , and the temperature of the column was 40°C . A photodiode array detector was used for scanning UV absorbance (190–300 nm) and evaluating the toxin spectrum. Identification and quantitation of the extracted toxin was done by comparing with MC-LR standard (Cyano Biotech GmbH, Germany; purity $> 95\%$). The concentration that was obtained using the HPLC software (ChemStation) was recalculated as the mass of the toxin in the total volume of the analyzed sample. Final results were obtained by recalculating this value of the mass in related to the dry biomass which was used for extraction.

Algal cultures and cultivation conditions. *Chlorella* sp., *Scenedesmus* sp., and *Coelastrum* sp. were isolated from a freshwater pond in southeastern Serbia and cultivated in the laboratory of the Department of Biology and Ecology, Faculty of Science and Mathematics in Niš. Microalgae were identified using an identification key to the genus level [12].

Trichormus variabilis 0441 was isolated from the Danube River in the Vojvodina region (Serbia) and cultivated in the laboratory at the Department of Biology and Ecology in Novi Sad (NSCCC).

All cultures were prepared in 250 mL Erlenmeyer flasks at 24°C under cool LED lighting ($26.81\text{ }\mu\text{mol/s/m}^2$) for a 16:8 light:dark photoperiod with constant aeration. Standard BG11 liquid medium [13] was used to cultivate all species.

Cyanobacterium identification. Species identification was performed using molecular methods and principles of polyphasic taxonomy. Molecular identification of the strain was performed in a previous study [14]. The morphological characterization of the strain was performed by observing the cellular morphology of the species during the stationary growth phase, using a Leica DM 100 light microscope (Leica Microsystem, Wetzlar, Germany) equipped with a Leica DFC290 digital camera. The estimation of protein, lipid and carbohydrate content was described in a previous study [15]. The phycobilin content was determined using the BENNETT and BOGORAD [16] method. Cyanobacterial biomass from the stationary growth phase was centrifuged at $4000\times g$ for 10 min, and the collected wet mass was dried to a constant mass and weighed. The dry biomass was then extracted in 0.01 M sodium phosphate buffer (pH 7.0) and stored in the darkness before disruption. Thereafter, cell walls were broken by continuous freezing and thawing to extract the phycobilins, and samples were sonicated for 5 min at

30-second intervals. The supernatant was separated by centrifugation (4000×g for 10 min), and absorbances were determined spectrophotometrically at 562 nm for phycoerythrin (PE), 615 nm for phycocyanin (PC), and 652 nm for allophycocyanin (APC) using phosphate buffer as a blank. The concentration of phycobilins was determined using the following formulae [15]:

$$PE \text{ [mg mL}^{-1}\text{]} = (A_{562} - (2.41 \times PC) - (0.849 \times APC))/9.62$$

$$PC \text{ [mg mL}^{-1}\text{]} = (A_{615} - 0.474 \times A_{652})/5.34$$

$$APC \text{ [mg mL}^{-1}\text{]} = (A_{652} - 0.208 \times A_{615})/5.09.$$

Experimental design. Two experiments were conducted to investigate the allelopathic potential of MC-LR. At first, the MC-LR standard was applied in an ecologically relevant concentration (10 µg L⁻¹), which treated green microalgae (*Chlorella* sp., *Scenedesmus* sp., and *Coelastrum* sp.). The second experiment included a co-cultivation method in which algal species (*Chlorella* sp., *Scenedesmus* sp., and *Coelastrum* sp.) were co-cultivated with the MC-LR-producing cyanobacterium *T. variabilis*. These experiments aimed to compare the effect of the pure toxin with the effect of the species that produces it.

Algal growth assay. The experimental strains (*Chlorella* sp., *Scenedesmus* sp., and *Coelastrum* sp.) were exposed to the same concentration (10 µg L⁻¹) of MC-LR in triplicate. Controls without MC-LR were also included in triplicate to ensure the comparison with treatments and validity of the test. Initial cell densities were determined spectrophotometrically based on the chlorophyll *a* (chl *a*) content (final chl *a* concentration in the experimental cultures was 0.1 µg chl *a* mL⁻¹).

Each sterile tube was supplemented with 10 mL of liquid BG11 medium and 1 mL of algal culture, and in the final concentration, 10 µg L⁻¹ of MC-LR (excluding controls) was added. The tubes were incubated at 24 °C under cool LED lighting (26.81 µmol/s/m²) for a 16:8 light:dark photoperiod for 14 days. Algal growth was monitored spectrophotometrically to determine the chl *a* concentration.

During the experimental period, 1 mL of the algal culture, which was pre-vortexed, was transferred to cuvettes, and the optical density at 678 nm, 720 nm, and 750 nm was measured using a spectrophotometer. The concentration of chl *a* was calculated using the following formula [16]:

$$\text{Chl } a \text{ (}\mu\text{g}\cdot\text{mL}^{-1}\text{)} = 14.96 \times (\text{OD}_{678} - \text{OD}_{750}) - 0.616 \times (\text{OD}_{720} - \text{OD}_{750}).$$

Co-cultivation experiment. Toxic *T. variabilis* and each of the green microalgae strains (*Chlorella* sp., *Scenedesmus* sp., and *Coelastrum* sp.) were added to sterile tubes in ratio 1:1, in three replicates for each strain. Due to the comparability and validity of the test, the growth of controls in three replicates (without *T. variabilis*) was monitored. All cultures were incubated at 24 °C under cool LED lighting (26.81 µmol/s/m²) for a 16:8 light:dark photoperiod for

14 days. The cell number was determined using a light microscope and a Thoma cell counting chamber.

Statistical analysis. A non-parametric *t*-test (Mann–Whitney U test, $p < 0.05$) was used to evaluate any significant difference in growth parameters between treated and control groups. The statistical analyses were performed using IBM SPSS Statistics.

Results and discussion. HPLC analysis of extracts. Using the HPLC-DAD technique, the MC-LR toxin was identified and quantified in a methanol extract of *T. variabilis*. Chromatogram (Fig. 1A, B) obtained by analyzing of extract was checked and the MC-LR variant was identified and quantified by the characteristic UV spectrum (maximum absorption at 238 nm) and by comparing it with the characteristic retention time obtained from the chromatogram of MC-LR standard. The concentration of the MC-LR detected in dried biomass of *T. variabilis* was approximately 13.795 µg/g, respectively.

Cyanobacterium identification. Microscopy showed that the observed strain is a heterocystic trichal, unbranched, unsheathed cyanobacteria with long, wavy filaments (Fig. 1C). The trichomes had a transverse and intercellular division

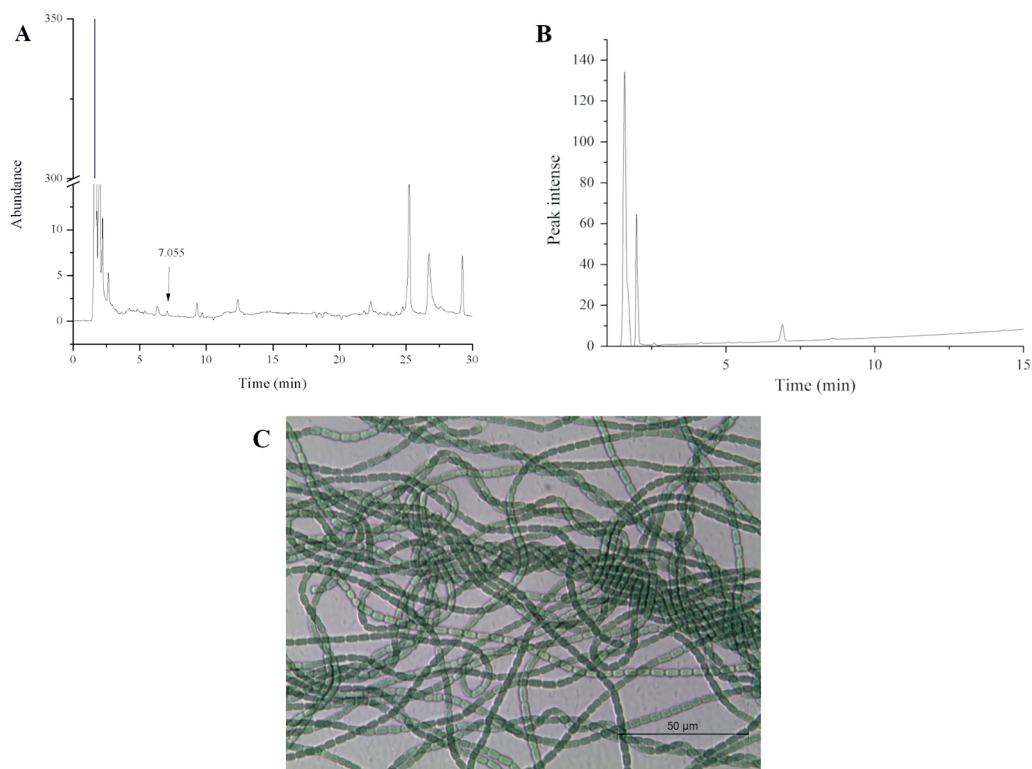


Fig. 1. A, B – HPLC chromatogram of methanol extract of *T. variabilis*;
C – Micrography of *T. variabilis*

and were formed of cylindrical cells. Further molecular analysis has characterized this cyanobacterium as *T. variabilis* [14]. The analysis of the phycobiliprotein distribution in *T. variabilis* shows the presence of all three phycobilins (PC, APC, PE) in different proportions. In 0.1 g of dry biomass, the PC, APC, and PE ratios were 269.8, 215.6, and 27.5 mg mL⁻¹, respectively. The content (% of dry biomass) of proteins, carbohydrates, and lipids of *T. variabilis* was 42%, 25%, and 4.7%, respectively [16].

Effects of MC-LR on algal species. The environmentally relevant concentration of MC-LR (10 µg L⁻¹) significantly affected ($p < 0.05$) the production of chl *a* of the examined green microalgae. Initially, inhibition was observed in all species on the second day of the experiment. Subsequently, the MC-LR significantly stimulated the production of chl *a* (Fig. 2) in *Chlorella* sp. (from the 4th

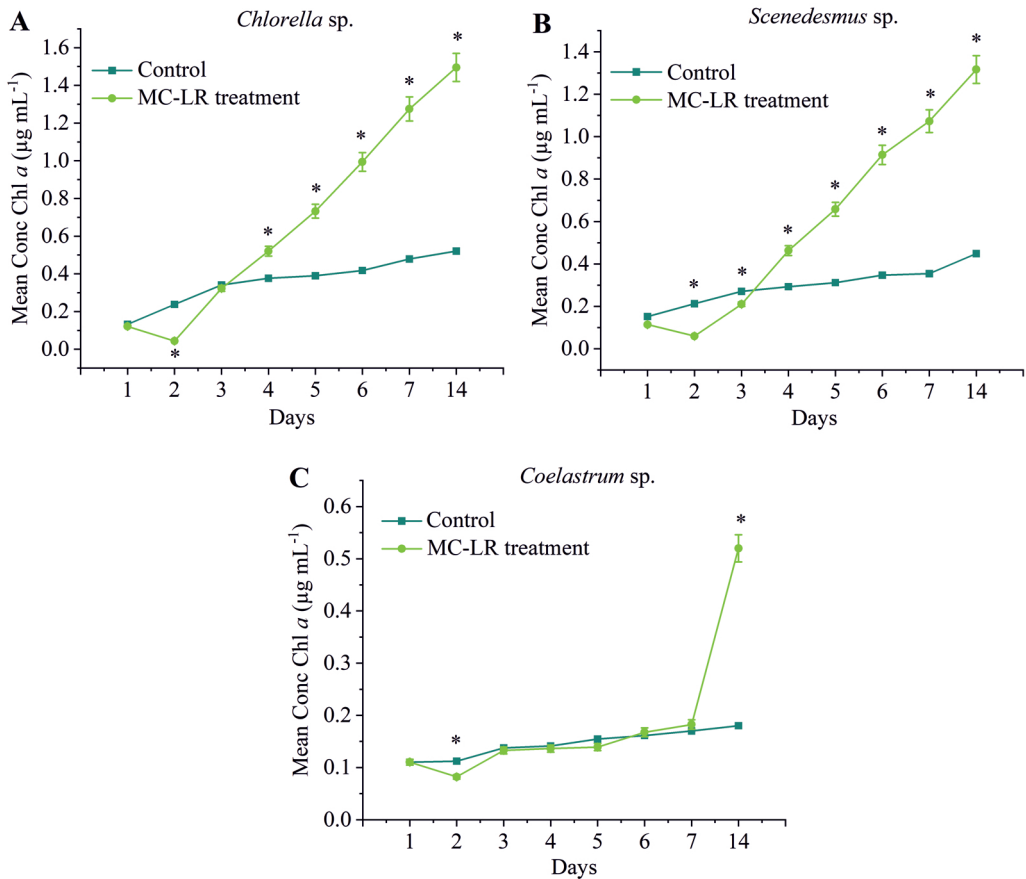


Fig. 2. Effects of MC-LR on green microalgae. Spectrophotometric comparison of the growth rate based on chl *a* (µg mL⁻¹) concentration in control (without MC-LR) and treatment conditions (10 µg L⁻¹ MC-LR) in exponential growth phases (mean, $n = 3$) for 14 days. Growth rate of **A** – *Chlorella* sp.; **B** – *Scenedesmus*; **C** – *Coelastrum* sp. *signifies a statistically significant difference between MC-LR treatment and control (t -test, Mann–Whitney U test: $*p < 0.05$)

day), *Scenedesmus* sp. (from the 3rd day) and *Coelastrum* sp. (on the 14th day) of exposure.

The action of MC-LR is complex because its influence can vary due to various biotic and abiotic factors, but also depending on the concentration at which the tested strains are treated [17]. Although some authors [17,18] suggest that environmental concentration, commonly below $10 \mu\text{g L}^{-1}$ [18], has no significant effect on phytoplankton growth, the results of the present study showed the opposite.

Green microalgae can recover from oxidative stress caused by MCs due to the activation of protective mechanisms such as the production of intra- and extracellular polysaccharides [19]. Here, the initial inhibition in tested green algae during the first two days of exposure to MC-LR was observed and followed by growth stimulation in response, consistent with previous evidence that some green algae can rapidly recover from MCs toxicity.

One of the reasons why the examined phytoplankton species can have a higher growth rate in the presence of MC-LR compared to standard conditions may be

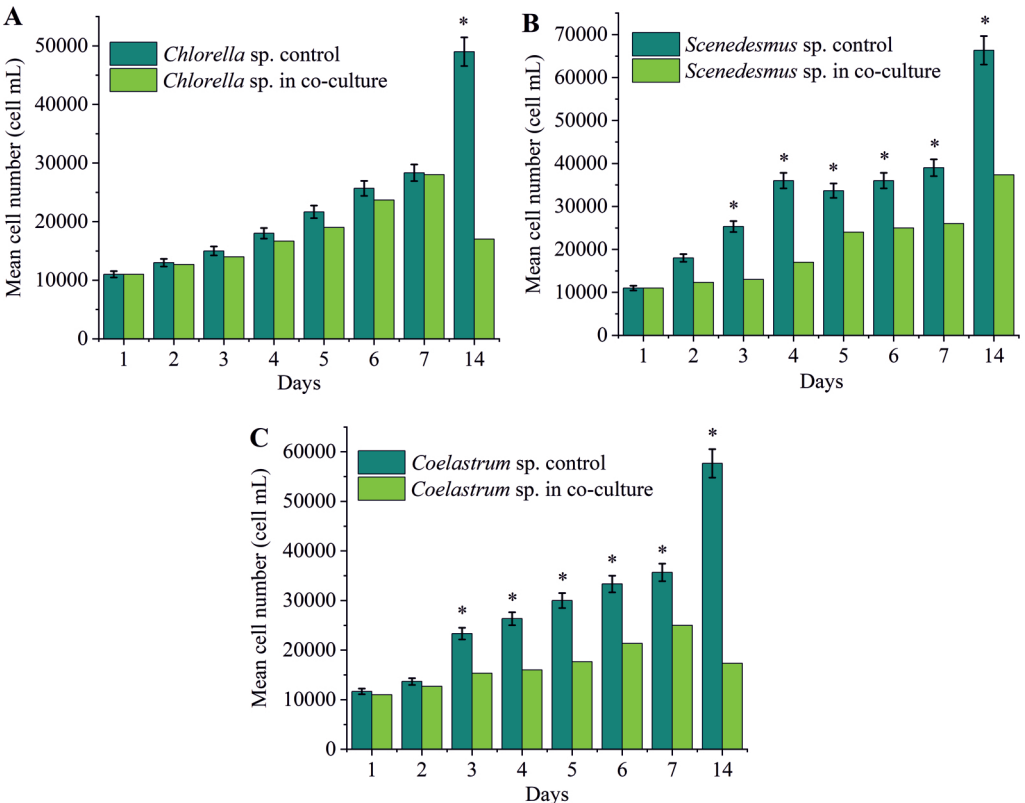


Fig. 3. Effect of *T. variabilis* on green microalgae in co-culture. Comparison of the cell number in control (monoculture) and co-cultivation (co-culture with *T. variabilis*) conditions (mean, $n = 3$) for **A** – *Chlorella* sp.; **B** – *Scenedesmus* sp; **C** – *Coelastrum* sp. *signifies a statistically significant difference between co-culture and control (t -test, Mann–Whitney U test: $*p < 0.05$)

in the decomposition of toxins. Studies indicated that bacteria could degrade MCs [20], and during the decomposition of MC-LR, a process of mineralization occurs, i.e., the conversion of carbon from MC-LR into CO₂. It is possible that besides the tested strains of phytoplankton, there were also dynamic communities of heterotrophic bacteria in the medium, so the effect of accompanying bacteria cannot be excluded entirely. There is a possibility, if the hypothesis is true, that the microalgae in which the stimulating effect was observed used CO₂ in their metabolism, which accelerated their growth.

Effects of *T. variabilis* on microalgae in co-culture. The toxic *T. variabilis* caused significant growth inhibition ($p < 0.05$) of all tested strains in co-culture (Fig. 3). It was proposed that cell extracts containing the toxin may exhibit more significant activity than pure toxins alone, indicating a potential synergistic interaction between allelopathic substances and the toxin [3]. The differences between the effect of the pure toxin and *T. variabilis* in co-culture can

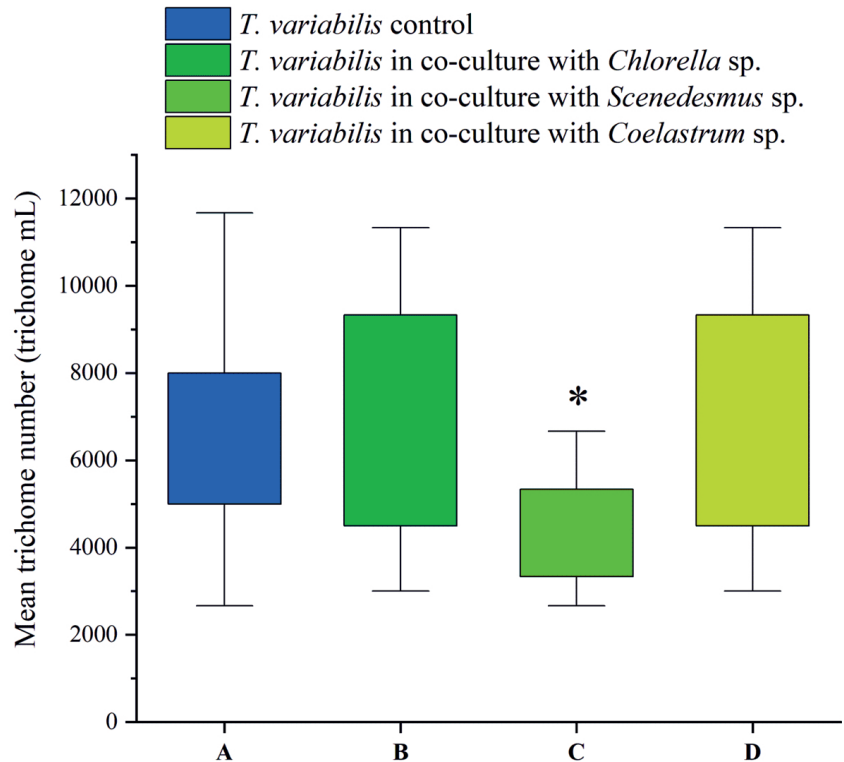


Fig. 4. The influence of green microalgae on the growth of *T. variabilis* in co-culture. Box plots with minimum and maximum values (mean, $n = 3$) of trichome number (trichome mL) of *T. variabilis* in **A** – control conditions (monoculture); and co-culture with **B** – *Chlorella* sp.; **C** – *Scenedesmus* sp.; **D** – *Coelastrum* sp. *signifies a statistically significant difference between control and co-culture (t -test, Mann–Whitney U test: $*p < 0.05$)

be explained by the fact that this toxin-producing species probably produces, in addition to MC-LR, some other compounds with allelopathic potential that alone or combined with the toxin have a negative effect on microalgal species.

Green microalgae responses to the presence of *T. variabilis* in co-culture differ (Fig. 4). *Chlorella* sp. and *Coelastrum* sp. did not affect the growth of *T. variabilis*. However, *Scenedesmus* sp. caused inhibition of *T. variabilis* growth after the 4th day of co-cultivation ($p < 0.05$). It is possible that *Scenedesmus* sp. can defend against the toxic effect of cyanobacteria by producing secondary metabolites with allelopathic activity, which can inhibit the growth of cyanobacteria.

Conclusions. The study revealed the allelopathic effect of environmentally relevant MC-LR concentration on phytoplankton species, and a clear difference between the producer (*T. variabilis*) and product (MC-LR) potential was established.

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