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Can phytoplankton blooming be harmful to benthic organisms? The toxic influence of *Anabaena* sp. and *Chlorella* sp. on *Chironomus riparius* larvae

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HIGHLIGHTS

- Phytoplankton is available food for benthic macroinvertebrate in aquatic ecosystems
- We assessed the influence of phytoplankton on *Chironomus* larvae using toxicity tests
- *Anabaena* sp. caused oxidative stress and moderate DNA damage on *Chironomus* larvae
- Enzyme biomarkers revealed that *Chlorella* sp. caused oxidative stress to *Chironomus* larvae

GRAPHICAL ABSTRACT

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ABSTRACT

Cyanobacteria and microalgae are abundant biota groups in eutrophic freshwater ecosystems, serving as a food source for many aquatic organisms, including the larvae of non-biting midges (Chironomidae). Many species of cyanobacteria are toxin producers, which can act as stressors to other organisms. The present study aimed to analyze and compare the effects of dietary exposure to the common toxic cyanobacteria *Anabaena* sp. and non-toxic microalgae *Chlorella* sp. in *Chironomus riparius* larvae. Microcystin was detected and quantified in the methanolic extract of *Anabaena* sp. using the HPLC-DAD technique, and it was identified as microcystin-LR. Both *Anabaena* sp. and *Chlorella* sp. were suitable food sources to enable the survival of *C. riparius* larvae in laboratory conditions, causing negligible mortality and significant differences in the larval mass (ANOVA and Post hoc LSD test; $p < 0.05$) and hemoglobin concentration (Student's *t*-test; $p < 0.05$). Oxidative stress parameters such as advanced oxidation protein products (AOPP), thiobarbituric acid reactive substances (TBARS), catalase (CAT) and superoxide dismutase (SOD) activity, and DNA damage, were also investigated. One-way ANOVA, followed by the Post hoc LSD test, showed a significant increase in AOPP and CAT for the group of larvae fed with *Chlorella* sp. The same test showed moderate DNA damage in both groups of larvae, with greater damage in the group fed with *Anabaena* sp. Thus, *Chlorella* sp. and microcystin-LR producing *Anabaena* sp. are food sources that did not result in any drastic acute effect on the population level of *C. riparius* larvae. However, sub-individual-level

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endpoints revealed significant effects of the treatments, since they caused oxidative stress and DNA damage that may pose a danger to successive generations of test organisms.

1. Introduction

Water scarcity and multiple stressors, which include organic and inorganic pollution, geomorphological alterations, land-use changes, water abstraction, invasive species, and pathogens, have a strong negative influence on aquatic ecosystems, especially lentic ecosystems (Brondizio et al., 2019). The interaction between stressors causes a negative combined effect on aquatic biota, often by means of mechanisms unknown to the scientific audience (Jackson et al., 2016), since it is not clear how aquatic ecosystems respond to multiple stressors (Petrović et al., 2011).

One of the main stressors in freshwater ecosystems is eutrophication, the phenomenon caused by the inflow of nutrients from agricultural fertilizers, urban run-off, and sewage discharge into water bodies. Water enriched by nutrients, especially by nitrogen and phosphorus, is an effective medium for the abundant growth of phytoplankton, including cyanobacteria (Paerl, 2018). This problem is observed worldwide in many rivers, lakes, and reservoirs, especially during the summer months, when green scums, can be formed on the water surface. The scum is comprised of phytoplankton, often dominated by cyanobacteria which are usually producers of toxic substances (Falconer and Humpage, 2005). The increase in global temperatures associated with a resulting water scarcity and eutrophication contributes to cyanobacterial blooming (Huisman et al., 2018). Resulting blooms can have many different negative influences, on aquatic biota especially through the induced production of toxic secondary metabolites in a stressed environment (Falconer and Humpage, 2005; Huisman et al., 2018).

Cyanobacteria and microalgae are primary producers in aquatic systems, and a good source of nutrients and oxygen for the biota in them (Gangstad, 1959). *Anabaena* is filamentous nitrogen-fixing cyanobacteria whose strains can produce cyanotoxins such as microcystins, saxitoxins, anatoxins, and cylindrospermopsin (Ferrão-Filho and Kozlowsky-Suzuki, 2011). On the other hand, *Chlorella* is a genus of eukaryotic green microalgae from the class *Chlorophyceae*. *Chlorella* strains are not known as toxic algae, but some strains are completely indigestible for larvae (Marten, 2007). *Anabaena* and *Chlorella* are often the dominant species of phytoplankton found in freshwater, and during the summer season under favorable conditions they can cause blooming in lentic systems. Several previous studies have investigated whether phytoplankton, among them the *Chlorella* and *Anabaena* species, which are a source of proteins and nutrients, could be used as a food for chironomid larvae, thereby revealing the relative nutritional importance of particular phytoplankton species (Ali, 1990; Frouz et al., 2004a).

Chironomid larvae are common members of benthic communities in freshwaters, since they are the most dominant and diverse group of macroinvertebrates (Milošević et al., 2013). Exhibiting different lifestyles and feeding behavior, this group is responsible for the recycling of organic matter and plays a major role in aquatic food webs. In general, chironomid species (especially the subfamily *Chironominae*) prefer eutrophic aquatic systems, where they form biomass-rich populations (Takahashi et al., 2008). In addition, algal and cyanobacterial blooms frequently occur in eutrophic aquatic ecosystems, and they can be the source of toxic secondary metabolites, but also the main source of food for benthic organisms. When a phytoplankton bloom breaks down, the cells can reach the bottom, and most of the benthic organisms present in the water may come in contact with the cells and products which are released, including the toxic ones (Christoffersen, 1996). For example, cyanobacteria are the primary food component for larval *Chironomus crassicaudatus* (Ali, 1990), but on the other hand, some microalgae could be the primary food component for other chironomid species (Provost and Branch, 1959; Frouz et al., 2004b; Henriques-Oliveira et al., 2003). At the same time, some chironomids are very sensitive to environmental stress and as such are suitable organisms for biomonitoring and toxicity testing (Michailova et al., 2012). Thus, chironomid larvae are stressed by cyanotoxins (Mills and Wyatt, 1974; Laurén-Määttä et al., 1995; Chen et al., 2005; Martins et al., 2007; Michailova et al., 2012; Zanchett and Oliveira-Filho, 2013) and in some cases have showed the possibility of toxin accumulation and further trophic transfer to other biotic groups (Ferrão-Filho and Kozlowsky-Suzuki, 2011; Laurén-Määttä et al., 1995).

Having in mind the ecological relevance of chironomids in aquatic ecosystems, we used *Chironomus riparius* larvae as a model organism in the present study to test the effects of algal and cyanobacterial blooms, comparing the dietary exposure of developing *Chironomus riparius* larvae to a green algae species (*Chlorella* sp.) and a toxic cyanobacteria species (*Anabaena* sp.). To achieve this aim we (1) identified and quantified the type of toxin which *Anabaena* sp. produces; (2) tested the suitability of cyanobacteria and eukaryotic green algae

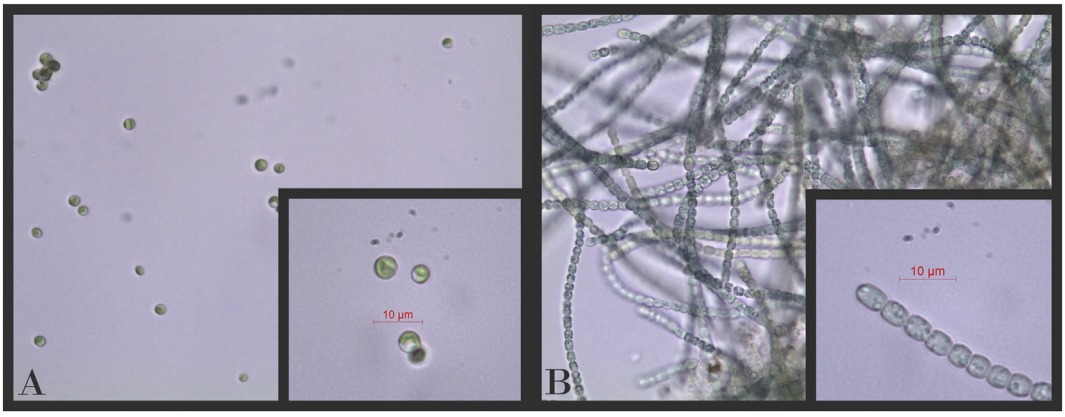

Fig. 1. A- *Chlorella* sp. (at 400× magnification) and detail of the cells with10 µm scale bar for size representation (at 1000× magnification); B- *Anabaena* sp. (at 400× magnification) and detail of the cells with10 µm scale bar for size representation (at 1000× magnification). Photograph taken by Leica MZ16A stereomicroscope and Leica DFC320 Digital Camera system.

strains as a food source for *C. riparius* larvae, and (3) determined the lethal and sublethal effects of cyanobacteria and green algae, as causative agents, on *C. riparius* larvae.

2. Materials and methods

2.1. Testing biomass

2.1.1. Algal cultures

Two strains of phytoplankton were tested: one strain of green algae *Chlorella* sp., and one toxic strain of cyanobacteria *Anabaena* sp. (Fig. 1).

Chlorella sp. was isolated from a freshwater pond in Southeastern Serbia (43°17′34″ N, 21°56′02″ E). Water samples were collected and initially examined under a light microscope to evaluate the content of the microalgal and cyanobacterial species. The strains were isolated and purified by repeated sub-culturing on solidified BG-11 media. The water samples were diluted and spread evenly over the surface of BG-11 agar plates using an "L" shaped glass rod. Seeded agar plates were incubated at 25 °C under constant cool LED lightning (26.81 μmol/s/m^2) until distinct colonies appeared. Individual colonies were picked up under a binocular microscope with a sterile loop and suspended in a fresh sterile liquid medium in order for them to gain biomass. They were identified using an identification key (Burchadt, 2014) to genus level.

The cyanobacterial strain of *Anabaena* genus was isolated from the soil on the bank of the Danube River in the Vojvodina region (Serbia) and cultivated in the laboratory as a part of the Culture Collection of Cyanobacteria (NSCCC) at the Department of Biology and Ecology in Novi Sad.

Both cultures were prepared in 250 ml Erlenmeyer flasks at 24 °C, under cool LED lighting (26.81 μmol/s/m^2) for a 16-h photoperiod with constant aeration. The liquid medium used for cultivation was standard BG 11 (Rippka et al., 1979) for the *Chlorella* strain, while BG 11 with no nitrogen source was used for cultivating *Anabaena* sp. The cells were harvested by centrifugation (10 min at 5000 rpm) and used to feed the larvae. First and second instar larvae were fed with 5 mg while third- and fourth- instar larvae with 10 mg.

2.1.2. Test organism

The test organism used in this study was the non-biting midge *C. riparius* (Diptera, Chironomidae) at the larval stage (Fig. 2). The specimens of *C. riparius* used in the experiments were from the stock culture housed in the laboratory at the Faculty of Science and Mathematics, University of Niš, and they were reared according to OECD guidelines (OECD, 2004). The larvae were reared in glass tanks (with shredded cellulose paper as a sediment and a mixture of tap and deionized overlaying water) at a temperature of 23 °C ± 2 °C, for a 16-h photoperiod, with constant aeration, and they were fed with TetraMin® finely ground food. Freshly laid egg masses were collected and placed in Petri dishes filled with water from the stock culture aquaria (a mixture of tap and deionized water, pH 7.2 and total hardness 350 mg/L $CaCO_3$). The larvae hatched after 2 days. For the bioassay, 1st instar larvae from one egg mass were used for the experiment to maintain genetic uniformity. The larvae were placed in the test vessels at four days old.

Fig. 2. *Chironomus riparius* larvae and detail of the head (at 50× magnification) with 100 μm scale bar for size representation. Photograph taken by Leica MZ16A stereomicroscope and Leica DFC320 Digital Camera system.

2.1.3. Bioassay design

The study was carried out under laboratory conditions following OECD test number 218 guidelines (OECD, 2004). The experiment was performed in 700 mL glass jars, 8 cm in diameter for 12 days. Each jar had a 3 cm deep sediment layer made of quartz sand, and the jar was filled with 400 mL of mixed deionized and tap water (1:1). The test jars with the sediment were previously sterilized in a dry heat sterilizer (VimS elektrik SSW 120, Serbia) at 180 °C for 60 min, and placed in an isothermal room at 23 ± 1 °C with a 16:8 light-dark cycle. The treatments (*Chlorella* sp. and *Anaebena* sp.) and control consisted of 8 replicated test jars, each with 25 1st instar larvae of *C. riparius*. The larvae were fed every 48 h for 12 days with *Chlorella*/*Anaebena*. The control larvae were fed with TetraMin® fish food. Aeration was provided 24 h after the addition of the larvae to the testing jars, fixed approximately 2 cm above the sediment layer.

2.2. Fitness-related traits toxicity analysis

The mortality, growth (mass), and hemoglobin concentration were determined in order to test the suitability of the cyanobacteria and eukaryotic green algae strains as a food source for *C. riparius* larvae. The total number of 4th instar larvae was counted at the end of the experiment in order to determine the mortality rate. To measure the growth rates of the larvae, 10 individuals from each jar were removed and dried on filter paper for 5 min. They were then transferred to an analytical balance (Kern, Germany) and weighed. The results of the larval growth were given as the mean larval mass in milligrams, and they were used to define the suitability of the given phytoplankton feed to contribute to the larval mass gain/loss compared to the control food.

One of the traits of *Chironomus* larvae is that they possess hemoglobin (Myers et al., 1986). Hemoglobin is a respiratory pigment which is synthesized in the larval fat body and secreted into the hemolymph (Myers et al., 1986; Lee et al., 2006). This pigment has potential as a biomarker for environmental monitoring and has an important role in the oxidative process (Saffarini et al., 1991; Choi et al., 1999; Ha and Choi, 2008). The photometric method on a hematology analyzer (Medonic M16M/M20M, Sweden) was used to determine the concentration of hemoglobin in the hemolymph of the larvae (Yang et al., 2013). Testing was performed on fresh fourth instar larvae by decapitation of the larvae and extraction of the hemolymph from the larval body using a capillary tube. The concentration of hemoglobin was automatically expressed by the analyzer in g/L of hemolymph.

2.3. Toxin analysis

To identify and quantify the type of toxin produced by *Anabaena* sp., the following procedure was applied: 500 mg of dried *Anabaena* sp. biomass was extracted by the addition of 5 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 centrifuged at 4000 rpm for 10 min at 20 °C and the supernatant was collected and filtered through 0.22 μm filters (Agilent) (Minasyan et al., 2018).

The HPLC system (Agilent Technologies (USA), Series 1200, equipped with an Agilent photodiode array detector (DAD), an automatic injector, a binary pump, and Chem Station software) was used for detecting and identifying toxins. The extracts were separated by an analytical column Supelcosil ABZ Plus (Supelco, 150 × 4.6 mm, 5 μm) using water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% trifluoroacetic acid (TFA). The gradient applied was 20% B to 80% B for 30 min, the flow rate was 1 mL per min, 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 toxin were conducted by using the standards of microcystin-LR (LGC, Germany).

2.4. Oxidative stress

The oxidative stress on *C riparius* larvae was tested using four different biomarkers: lipid peroxidation (TBARS levels); advanced oxidation protein products AOPP; superoxide dismutase (SOD) and catalase (CAT).

A total of 10 larvae, previously weighed on an analytical balance, were collected after 12 days from each jar in all treatments. The larvae were homogenized for 30 s in 2.5 mL of a tris-ethylenediaminetetraacetic acid buffer (40 mM, pH 7.8; Sigma-Aldrich, St. Louis, MO, USA) using a homogenizer (IKA® Works de Brasil Ltd. Taquara, RJ 22713-00). The crude homogenate was centrifuged for 15 min at 500 *g* (4 °C), and the supernatant was pipetted into a new tube and centrifuged for 30 min at 12,000 g (4 °C). The resulting supernatant (postmitochondrial fraction) was used to measure the level of oxidative modification products and enzyme activity. The concentration of protein was determined according to the method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.4.1. Lipid peroxidation (TBARS levels) evaluation

Lipid peroxidation was estimated by the measurement of thiobarbituric acid reactive substances (TBARS) according to the method of Andreeva et al. (1988). The pink chromogen produced by the reaction of TBA with MDA was measured spectrophotometrically (Multiscan Ascent 96/384 plate reader, Thermo Labsystems) at 532 nm. The TBARS concentration was determined using an extinction coefficient of 1.56 × 105 M^{-1} cm^{-1} and the results were expressed in μmol/L and then converted to mg of proteins.

2.4.2. Evaluation of advanced oxidation protein products (AOPP)

The AOPP concentration was determined by the spectrophotometric method of Witko-Sarsat et al. (1996). Calibration was carried using chloramine-T solutions, which absorb the light at $\lambda = 340$ nm in the presence of potassium iodide. The data were calculated and expressed in μmol/L of chloramine T equivalents and finally related to the total protein levels (μmol/mg of proteins).

2.4.3. Catalase (CAT) activity evaluation

The CAT activity was determined by Goth's spectrophotometric method (1991), based on the ability of CAT to decompose the substrate (H_2O_2), whereby the enzymatic reaction was stopped by the addition of ammonium molybdate, and the resulting yellow complex of H_2O_2 and molybdate was measured at 405 nm against the reagent blank. The enzyme activity was expressed in U/L and then converted to mg of proteins.

2.4.4. Evaluation of superoxide dismutase activity (SOD)

The SOD activity was measured by the method of Minami and Yoshikawa (1979), based on formazan colored product formation. In the reaction with NBT (nitroblue tetrazolium), the superoxide anion, produced by pyrogallol autooxidation, forms a colored product. SOD, which is a superoxide anion scavenger, inhibits this reaction. The enzyme activity was expressed in U/L and then converted to mg of proteins. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of the NBT photoreduction rate.

2.5. DNA damage - comet assay

To determine whether cyanobacteria and green algae can damage DNA in somatic cells, fourth instar larvae of *C. riparius* were used.

Phosphate-buffered saline (PBS) without calcium or magnesium, as well as agarose for DNA electrophoresis and low-melting point agarose (LMA) were obtained from Alfatrade Enterprise D.O.O. (Serva Electrophoresis GmbH, Heidelberg, Germany). Forty 4th instar larvae of *C. riparius* from four groups i.e. negative, positive (H_2O_2, 20 mM, Bernabò et al. (2017)), *Chlorella* sp., and *Anabaena* sp., were transferred to Eppendorf tubes, homogenized, filtered and resuspended in 3 ml suspension buffer (1× PBS, 20 mM EDTA, 10% DMSO, pH 7.4). After centrifugation for 15 min at 1500 rpm at 4 °C (Eppendorf centrifuge 5415 R) the homogenate was resuspended in 150 μL of suspension buffer. From the cell suspension, 10 μL was mixed with 75 ml of 1% LMA, and 75 μL of this mixture was spread onto a microscope slide. To assess the genotoxic potential of green microalgae *Chlorella* sp. and cyanobacteria *Anabaena* sp., the extent of DNA damage in the larvae of *C. riparius* was measured both for untreated larvae, as a negative control, and larvae treated with 20 mM H_2O_2, as a positive control.

The comet assay was performed according to Singh et al. (1988) with minor modifications (Bernabò et al., 2017). Slides were stained with ethidium bromide (10 min in the dark) and analyzed under a Nikon (Ti-Eclipse) fluorescence microscope attached to a CCD (charge-coupled device) camera. DNA damage induced by *Chlorella* sp. and *Anabaena* sp. was evaluated visually (Collins, 2004), classifying the comets into five categories (0 to 4). The total comet score was calculated according to the following equation, modified from Miyaji et al. (2004): (% cells in class 0 × 0) + (%cells in class 1 × 1) + (%cells in class 2 × 2) + (%cells in class 3 × 3) + (%cells in class 4 × 4). The total score range was from 0 (all undamaged) to 400 (all maximally damaged).

2.6. Statistical analyses

All endpoints based on larval growth**,** oxidative stress and DNA damage parameters were analyzed for normality and homogeneity using the Kolmogorov-Smirnov (with Lilliefors significance Correction) statistic and tested for any significant difference by One-way ANOVA in the SPSS statistical software package (IBM Corporation, New York, United States), version 13.0 for Windows. ANOVA was followed by the LSD Post hoc test for pairwise comparison for growth, oxidative stress, and DNA damage parameters. The significance level was set at $p < 0.05$. Since the dataset on hemoglobin concentrations was composed of two groups, statistical differences were tested by Student's *t*-test. The Pearson correlation test was used to explore the relationship between AOPP concentration and CAT and SOD activities.

3. Results

The most common types of cyanotoxins found in water are microcystins, among which microcystin-LR (MC-LR) has been regarded as the most common and most toxic variant. Using the HPLC-DAD technique, the microcystin-LR toxin was identified and quantified in a methanol extract of *Anabaena* sp. The UV spectrum for each separated fraction obtained in the HPLC chromatogram (Fig. 3) of the extract was checked and the MC-LR variant was identified by the characteristic UV spectrum (maximum absorption at 238 nm (Fig. 4)) and by comparing it with the characteristic retention time (6.893 min) from the chromatogram of MC-LR standard (Fig. 5). The microcystin-LR was quantified based on peak areas seen in the chromatograms of standard

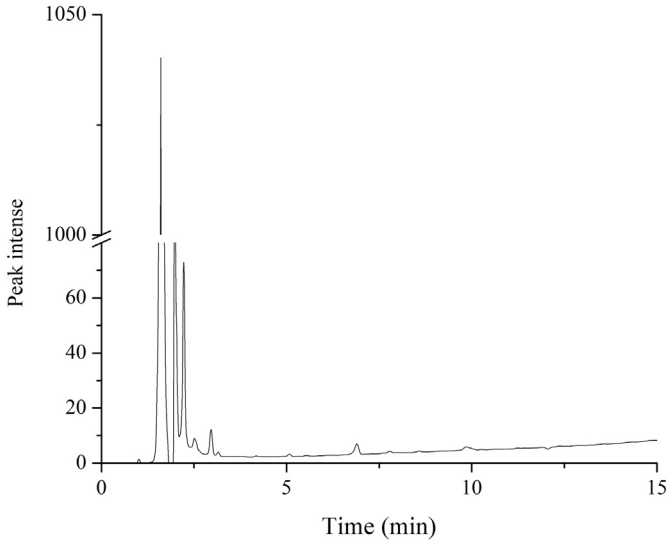

Fig. 3. HPLC chromatogram of methanol extract (*Anabaena* sp.)

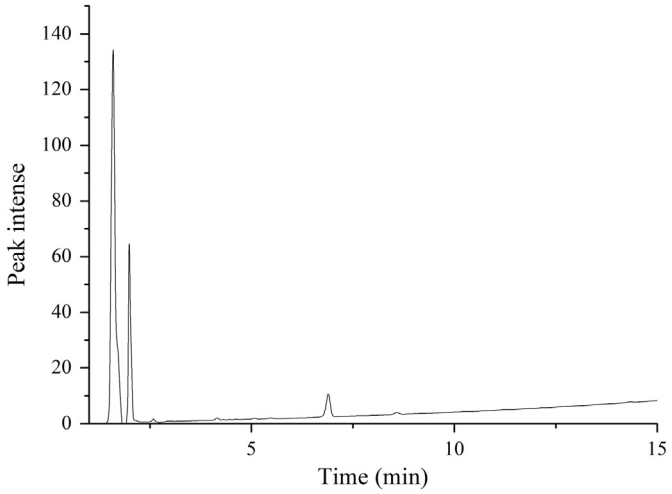

Fig. 5. HPLC chromatogram of microcystin-LR standard solution.

solutions and samples. The concentration of the microcystin-LR detected was approximately 1.9 μg/g of dry cyanobacterial mass.

None of the treatments in the experiment influenced the mortality rate (ANOVA, $p > 0.05$). More precisely, the mortality was always <10% in all treatments/control/replicas, which was within the acceptable limits for control according to OECD (OECD, 2004). Larvae exposed to different treatments showed significant variability in body mass (ANOVA, $F = 9.166$, $p = 0.007$), whereby chironomids fed with *Anabaena* sp. had a significantly higher weight than those in the *Chlorella* treatment (LSD test, $p < 0.05$; Table 1). However, larvae from both treatments had a lower mass compared to the larvae fed with the control food. Hemoglobin concentration followed the same pattern, and in the *Anabaena* treatment, the photometric method detected higher hemoglobin concentrations than for the *Chlorella* treatment (Table 1).

Multiple pairwise comparisons showed a significant difference in protein content between both treatments and the control group (LSD test, $p < 0.05$). The larvae fed with *Chlorella* sp. and *Anabaena* sp. contained a similar amount of proteins, but less than the control group of larvae.

The values of the oxidative stress parameters showed that the concentration of AOPP significantly varied between chironomid groups (ANOVA; $p < 0.05$, Table 1) and it was highest in the *Chlorella* treatment (mean = 0.286, SD = 0.104 μmol/mg prot), and significantly different to the control group (LSD test, $p < 0.05$; Table 2). There was no significant difference between the group of larvae treated by *Anabaena* sp. and the other groups.

Catalase (CAT) activity was lowest in the larvae treated with *Chlorella* sp. (mean = 0.029, SD = 0.011 μmol/mg prot.), making it significantly different from the control group of larvae and larvae treated with *Anabaena* sp. (LSD test, $p < 0.05$; Table 2).

Finally, the TBARS concentration and SOD activity showed no variation in pattern between the larvae treated with *Anabaena* sp., *Chlorella* sp. and those given TetraMin food.

When there was a rise in the concentration of AOPP in any of the groups of larvae fed with different kinds of food, there was a decrease in the CAT activity ($r = -0.77$, $p < 0.01$), which confirmed that chironomid larvae from both treatments experienced significant differences in oxidative stress (Table 3).

The total comet score in the comet assay for the fourth instar larvae of *C. riparius* significantly differed (ANOVA, $F = 9.894$, $p = 0.005$) between the control groups and those treated with *Chlorella* sp. and *Anabaena sp.* (Table 4).

The DNA damage was significantly higher in the *Anabaena* sp. treated group of larvae than in the negative control group. On the other hand, the total comet score in the *Chlorella* sp. treated group of larvae was not significantly higher than the negative control group.

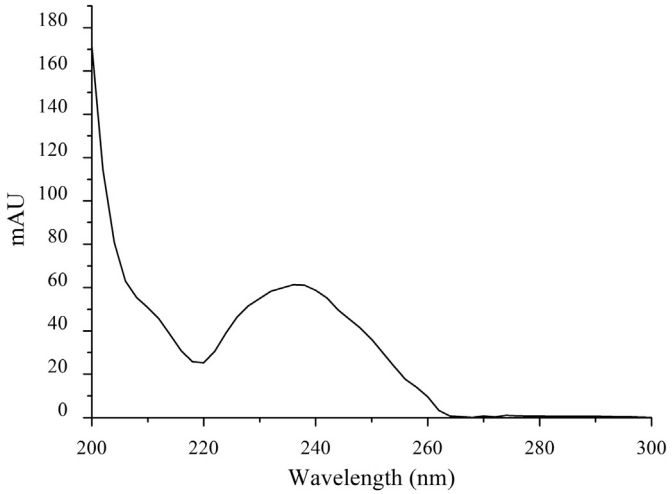

Fig. 4. Characteristic UV spectrum of microcystin-LR.

4. Discussion

Since phytoplankton is an available food source for *Chironomus* larvae, many toxic species, such as *Anabaena* sp., and non-toxic *Chlorella* sp. can be available to them. This is the first study to reveal the effects of dietary exposure to toxic and non-toxic phytoplankton on benthic

Table 1
The effect of the phytoplankton diet on growth and hemoglobin concentration from the hemolymph of chironomid larvae, expressed as the mean ± SD of larval mass. Values within the same column not sharing a common superscript letter are significantly different: [a] $p < 0.05$ (ANOVA and Post hoc LSD test for larval mass and Student's *t*-test for hemoglobin concentration). If superscript letters are missing, all the values are significantly different among the groups.

Treatment	Mean larval mass (mg)	Mean hemoglobin concentration (g/L)
Chlorella sp.	0.51 ± 0.08	Under detection level
Anabaena sp.	3.61 ± 0.15[a]	10.5 ± 3.05
Control	5.14 ± 2.33[a]	27.3 ± 8.20

Table 2
The mean ± SD concentrations of oxidative stress parameters in *C. riparius* larvae. Values within the same column not sharing a common superscript letter are significantly different: $p < 0.05$.

	Proteins mg%	AOPP μmol/mg prot.	TBARS μmol/mg prot	SOD U/mg prot	CAT U/mg prot
Chlorella sp.	63.4 ± 24.006[a]	0.286 ± 0.104	0.025 ± 0.043[a]	2.91 ± 3.092[a]	0.029 ± 0.011
Anabaena sp.	66.63 ± 11.47[a]	0.184 ± 0.032[a]	0.026 ± 0.004[a]	6.47 ± 2.004[a]	0.089 ± 0.013
Control (Tetramin)	106.23 ± 8.034	0.122 ± 0.01[a]	0.024 ± 0.003[a]	2.25 ± 0.434[a]	0.059 ± 0.004

C. riparius larvae, by scaling the endpoints of the biological parameters for the tested larvae.

The consumption and composition of the food depend on the properties of both the larvae (size, motility, the capability of digestion) and the cyanobacteria or algae (size, ease of harvest, density, nutritional value), considering that some filamentous cyanobacteria and microalgae are relatively indigestible (Tarkowska-Kukuryk, 2013; Marten, 2007). Tarkowska-Kukuryk (2013) showed that chironomid larvae are important consumers of algae in a shallow phytoplankton dominated lake, and also that consuming algae is taxon-dependent, without any significant changes during the development of the larvae. Contrary to their research, the present study showed that the mass and hemoglobin concentration of larvae fed with *Chlorella* sp. were significantly lower than both the other treatment and the control. The reason for this occurrence could be that *Chlorella* sp. is not digestible to the same extent as the cyanobacteria *Anabaena* sp. Due to the presence of a rigid cell wall, which is composed mainly of cellulose, *Chlorella* sp. cannot be fully utilized by many organisms (Yamada and Sakaguchi, 1982). The capacity to digest cellulosic compounds in *Chironomus* larvae is low or even nonexistent, but some larvae can complete their development on a diet restricted to bacteria (Koroiva et al., 2013).

The genotoxic effect of *Chlorella* sp. on the larvae was not significant in this study, which has also been reported in previous studies demonstrating the genotoxic and antigenotoxic effects of this phytoplankton, mostly on *Vertebrata*. For example, water extracts of *Chlorella* sp. have been reported to show a significant reduction of DNA damage in cultured fibroblasts from both young and old human individuals (Makpol et al., 2009). The treatment of fibroblasts alone did not show any significant change in DNA damage. Also, Makawy et al. (2016) reported that the cotreatment of monosodium glutamate and aqueous extracts of *Chlorella vulgaris* reduced DNA damage in mice. A study by Zamri et al. (2018) showed that an increase in DNA damage in cigarette smoke-exposed rats was reduced after treatment with *Chlorella vulgaris*. In contrast, Zhang et al. (2017) showed the genotoxic effect of the *Chlorella vulgaris* growth factor (CGF) and water extract in human lung carcinoma cells (A549 and NCI- H460). According to Yusof et al. (2010), DNA damage was increased in the hepatoma cell line (HepG2) at all concentrations (0–4 mg/mL) of *Chlorella vulgaris* water extract tested. Finally, these studies show conflicting results regarding the effect of *Chlorella*, mostly extracts, on DNA damage in different organisms, thus stressing the need for further studies on its genotoxic effects on biota in aquatic ecosystems.

Since the mass and growth of larvae fed by *Chlorella* sp. were low, it was expected that other metabolic parameters would also be substantially altered. The level of AOPP in *C. riparius* larvae showed that the concentration was significantly higher in the treatment with *Chlorella* sp. than in the other two groups. An increased concentration of AOPP in this group of larvae indicates that *Chlorella* sp. is an agent that can cause the oxidative modification of proteins. In addition, the negative correlation between AOPP concentration and CAT activity suggests that increased oxidative stress and the oxidative modification of CAT could be the reason for decreased CAT activity. Such a result was concordant with a previous study in which *Chlorella vulgaris* was also able to reduce catalase activity (CAT) in liver cancer induced rats (Sulaiman et al., 2006; Martínez-Francés and Escudero-Oñate, 2018). Finally, the concentration of TBARS was no different from the control group in the present study in larvae treated with *Chlorella* sp. This result indicates that *Chlorella* sp. does not cause the modification of lipid components in the somatic cells of *C. riparius* larvae.

Table 3
Pearson correlation test in a group of larvae treated with *Chlorella* sp.
**Correlation is significant at the 0.01 level (2-tailed).

Correlations		SOD U/ml	SOD U/mg prot	CAT U/L	CAT U/mg prot
AOPPumol/L	Pearson Correlation	−0.215	−0.200	−0.771**	−0.774**
	Sig. (2-tailed)	0.503	0.533	0.003	0.003
	N	12	12	12	12

The hemoglobin concentration in larvae treated with *Chlorella* sp. was significantly lower than the control group and larvae fed with *Anabaena* sp., which was followed with a significant increase in the AOPP level. The increase of stress enzymes in the case of a decreased hemoglobin concentration can be explained by their compensatory role relative to the decrease in hemoglobin, with the purpose of maintaining radical detoxification (Choi et al., 1999). The potential of hemoglobin as a biomarker for environmental stress has been shown in a study by Ha and Choi (2008) in which the chemical environmental contaminants, bisphenol A and chlorpyriphos, altered the concentration of hemoglobin. The expression of hemoglobin genes shows a chemical-specific response, whereby exposure to fenitrothion caused a decrease in body weight concomitantly with a decrease in the Hb gene expression of *Chironomus tentans* (Lee et al., 2006). The present study, along with previous research, confirms hemoglobin as a promising endpoint in further toxicity testing or bioassays.

There are a few studies on the effects of the *Chlorella* species on the survival and development of invertebrates (Ahmad et al., 2001; Raja and Kumar, 2016). The percentage mortality of *Aedes aegipty* larvae fed with *Chlorella vulgaris* after six days was 100% (Ahmad et al., 2001). In contrast, growth in the larvae of the silkworm, *Bombyx mori* L., diet-supplemented with *Chlorella pyrenoidosa* extracts even improved (Raja and Kumar, 2016). The present study has shown the opposite outcome to previous research, since the *Chlorella* sp. treatment did not affect the mortality rate, but it had a negative influence on growth of the larvae.

Since the present study is the first study to analyze the influence of live untreated, physically and chemically unchanged cells of *Chlorella* sp. along the scale of the biological and ecological levels of organization, it can offer missing pieces of information about the unresolved harmful effects of the *Chlorella* species on aquatic invertebrates. Concordant to the effects caused by *Chlorella* sp. as an agent, the CAT activity was significantly affected and increased in larvae fed with *Anabaena* sp. Other enzymes examined were not significantly altered in this treatment, albeit the Comet assay showed that DNA damage was significantly more severe than in the group treated with *Chlorella* sp. Considering that the strain of *Anabaena* sp. tested is a producer of microcystin-LR, and its cells were well digested by the larvae, increased CAT and DNA damage could be a response to the stress associated with exposure to the toxin. It has already been shown that microcystins have the potential to induce the excessive formation of reactive oxygen and nitrogen species, which can lead to DNA damage (Zanchett and Oliveira-Filho, 2013; Zegura, 2016). The results of the Comet assay show that the consumption of *Anabaena* sp. can cause DNA damage in the somatic cells of *C. riparius* larvae. Comet class 2, observed in groups exposed to

Table 4
Genotoxic effect of dietary exposure of *C. riparius* larvae to *Chlorella* sp. and *Anabaena* sp. [a]Values represent the mean ± SD from three independent experiments. Values within the same column not sharing a common superscript letter are significantly different: [a] $p < 0.05$.

Treatment	Comet class					Total comet score[a]
	0	1	2	3	4	
Negative control	86.2 ± 0.62	13.8 ± 0.74	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	13.8 ± 2.60[a]
Positive control	59.1 ± 0.62	24.1 ± 0.41	10.1 ± 0.52	4.2 ± 0.94	2.5 ± 0.35	66.9 ± 5.83
Chlorella sp.	73 ± 0.20	21.9 ± 0.31	5.1 ± 0.24	0.00 ± 0.00	0.00 ± 0.00	32.1 ± 19.94[ab]
Anabaena sp.	69.7 ± 0.94	17.6 ± 0.27	12.7 ± 0.60	0.00 ± 0.00	0.00 ± 0.00	43.05 ± 12.55[b]

Anabaena sp. agents, describes the DNA damage as moderate. The increased damage can be due to the production of microcystin-LR, since the exposure of DNA to different biotoxins is believed to lead to DNA damage and a variety of genetic disorders (Singh and Sharma, 2018). The mechanism by which microcystins function, including the degree of potential DNA damage, has been poorly investigated in invertebrates. However, microcystin-LR can induce mutations in mammalian cells and interfere with DNA damage repair processes, which contributes to genetic instability (Zegura, 2016). In previous studies, the effects of microcystin-containing cyanobacteria extract and pure microcystin-LR caused DNA damage in rice seedlings and the spermatogenic cells of rats (Singh and Sharma, 2018; Lone et al., 2015). It has been suggested that microcystins cause chromosome damage by increasing the frequency of the micronucleus (MN) in mouse erythrocytes and in the human TK6 cell line (Menezes et al., 2013).

A study by Palus et al. (2007) reported a slight increase in DNA damage and frequency of micronuclei in human lymphocytes induced by selected cyanobacterial extracts (*Microcystis aeruginosa*, *Aphanizomenon flos-aquae*, *Pseudoanabaena* sp., and *Anabaena* sp.). In previous research on microalgae and micrograzer interaction (Berry et al., 2008; Kaczorowska and Kornijów, 2012; Toporowska et al., 2014), it was shown that the ingestion of different toxin-producing cyanobacteria can lead to the accumulation of toxins in *Chironomus* spp. larvae, as well as mortality and a decreased growth rate in Daphnia. Based on this, the presumption was that direct ingestion of a pure strain of microcystin-LR producing *Anabaena* sp. could have a similar effect on micro grazers, such as chironomid larvae.

Microcystin-LR inhibits protein phosphatases, leading to the hyperphosphorylation of many cellular proteins (Zegura, 2016). However, the present results indicate that the proteins in the somatic cells of *C. riparius* larvae were not modified by the toxin present in the *Anabaena* sp. strain that was used. The reason for such a scenario could possibly be the low concentration of microcystin-LR used in this bioassay, insufficient to cause protein modification in the chironomid larvae.

The *Anabaena* sp. treatment in this study did not affect the mortality or development of *C. riparius* larvae. This contrasts with research carried out by Laurén-Määttä et al. (1995) in which *Chaoborus* larvae were stressed by microcystin-LR, with a statistically significant increase in mortality through trophic transfer, by feeding on contaminated *Daphnia*. The absence of mortality may either be due to the low quantity of toxin produced by *Anabaena* sp., or due to the larvae acquiring resistance, which can be enabled by the significant presence of the strong antioxidant glutathione (GSH) (Toporowska et al., 2014). Nevertheless, the microcystin-LR detected is a dangerous agent for other aquatic organisms in the food web, because cyanotoxins are described as resistant to chemical and biological degradation and can bioaccumulate in the food chain (Takser et al., 2016; Ferrão-Filho and Kozlowsky-Suzuki, 2011).

5. Conclusion

Dietary treatment with different phytoplankton strains did not affect the survival rate of *C. riparius* larvae. Nevertheless, the parameters of sublethal effects revealed the adverse influence of *Anabaena* sp. and *Chlorella* sp. on the larvae, by making significant alterations in oxidative stress enzyme activity and moderate DNA damage. Despite toxic cyanobacteria *Anabaena* sp. causing significant DNA damage, it also provided more nutrients than *Chlorella* sp., and enabled better growth. Although non-toxic and rich in nutrients, *Chlorella* sp. may also be an unsuitable agent for *C. riparius* larvae in the case of algal bloom. Assuming that *Chlorella* sp., has low digestibility, as the main source of food, this microalga can cause oxidative stress and the slow growth and underdevelopment of chironomid larvae. The present study reveals the importance of the potential toxic influence of phytoplankton to benthic macroinvertebrates. It opens a new avenue for including this type of effect in aquatic ecosystem bioassessment through the prism of multistressor conditions. In addition, further research should be focused on the possible cumulative effects of toxin produced by phytoplankton blooming and damage of cell structures on the offspring of *C. riparius*.

CRediT authorship contribution statement

Nikola Stanković: Conceptualization, Investigation, Writing - original draft.**Ivana Kostić:**Formal analysis.**Boris Jovanović:**Writing - review & editing.**Dimitrija Savić-Zdravković:**Methodology, Investigation. **Sanja Matić:**Formal analysis.**Jelena Bašić:**Formal analysis.**Tatjana Cvetković:**Formal analysis.**Jelica Simeunović:**Resources.**Djuradj Milošević:**Project administration, Formal analysis, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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