Hepatic oxidative stress and neurotoxicity in *Pelophylax* kl. *esculentus* frogs: Influence of long-term exposure to a cyanobacterial bloom

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ABSTRACT

Although the long-term exposure of aquatic organisms to cyanobacterial blooms is a regular occurrence in the environment, the prooxidant and neurotoxic effects of such conditions are still insufficiently investigated *in situ*. We examined the temporal dynamics of the biochemical parameters in the liver of *Pelophylax* kl. *esculentus* frogs that inhabit the northern (N) side of Lake Ludaš (Serbia) with microcystins (MCs) produced in a cyanobacterial bloom over three summer months. The obtained data were compared with data on frogs that live on the southern (S), MC-free side of the same lake. Our results showed that the MC-producing bloom induced oxidative damage to proteins and lipids, observed as a decrease in the concentration of protein –SH groups and increased lipid peroxidation (LPO) in the liver of N frogs in comparison to S frogs. Glutathione (GSH) played a key role in the transient defense against the MC-induced development of LPO. The low glutathione peroxidase (GPx) activity detected in all groups of frogs from the N site was crucial for the observed prooxidant consequences. The bloom impaired cholinergic homeostasis as a result of a decrease in ChE activity. A delayed neurotoxic effect in relation to the prooxidant outcomes was observed. Our results also showed that even though the integrated biomarker response (IBR) of the antioxidant biomarkers increased during exposure, the individual biochemical parameters did not exhibit a well-defined time-dependent pattern because of specific adaptation dynamics and/or additional effects of the physicochemical parameters of the water. This comprehensive environmental ecotoxicological evaluation of the cyanobacterial bloom-induced biochemical alterations in the liver of frogs provides a new basis for further investigations of the prolonged, real-life ecotoxicity of the blooms.

Keywords: Oxidative damage; Antioxidant; Biotransformation; Cholinesterase; Temporal dynamic; Microcystin.

1. Introduction

The cause-effect relationship between anthropogenic-induced eutrophication and the development of cyanobacterial blooms in aquatic ecosystems represents an ecotoxicological problem of global concern (Merel et al., 2013). There is increasing evidence in scientific literature recognizing bloom occurrence as a powerful and increasing environmental stressor (Meriluoto et al., 2017). Cyanotoxins produced by a large number of cyanobacterial genera are known to exhibit negative effects at all levels of organization (from molecular to population) of all groups of aquatic and terrestrial organisms (Falfushynska et al., 2019; Gavrilović et al., 2014; Wiegand and Pflugmacher, 2005). These adverse changes in biological systems can cause a collapse of the integrity of the entire living network connected to the blooming ecosystem (Chen et al., 2009).

Microcystins (MCs) are the most common cyanotoxins found in aquatic environments during cyanobacterial bloom events. They are produced mainly by species of *Anabaena*, *Anabaenopsis, Nostoc, Oscillatoria, Microcystis* and *Planktothrix* genera. The mechanisms and levels of MC toxicity have been described in a number of scientific papers (Amado and Monserrat, 2010; Díez-Quijada et al., 2019; Rao et al., 2004; Wiegand and Pflugmacher, 2005). These hepatotoxins are potent inhibitors of serine/threonine protein phosphatases, but other molecular target sites in cells have also been established (such as the beta subunit of ATP-

synthase) (Chen and Xie, 2016; Mikhailov et., 2003). Recent data showed that MCs also induce neurotoxicity through different pathways (Hinojosa et al., 2019).

In studies in which prolonged exposure to environmentally relevant/low concentrations of MCs was examined, the importance of the oxidative stress pathway in the promotion of MC-induced toxicity was emphasized (Jiang et al., 2012; Wang et al., 2010). The prooxidant potential of MCs is due to their ability to disrupt the mitochondrial electron transport chain that consequently leads to increased generation of reactive oxygen species (ROS). The coordinated activity of antioxidant and biotransformation components of the cellular defense system plays a crucial role in the prevention of cyanotoxin-mediated redox imbalance (Amado and Monserrat, 2010). However, if adaptive protection is overwhelmed, the state of the oxidative stress can directly lead to oxidative damage of biomolecules (proteins, lipids and DNA), and even to cell death as the endpoint (Halliwell and Gutteridge, 2015).

Ecotoxicological investigations concerning the influence of cyanobacterial blooms on aquatic organisms in environmental conditions are of particular interest to the scientific community (Gavrilović et al., 2015; Ujvárosi et al., 2019). Although complex, only examinations *in situ* can provide real insight into the health of a bloom-impacted organism/ecosystem. A small number of studies dealing with the direct influence of MC-containing blooms on oxidative stress and neurotoxicity parameters in aquatic animals are found in scientific literature and have mainly focused on fish (Qiu et al., 2007). Even though frogs are among the most sentinel organisms used for environmental examinations of xenobiotics toxicity (D'Errico et al., 2018; Prokić et al., 2016), they are still one of the least studied groups in the context of cyanotoxin effects (Gavrilović et al., 2020; Ziková et al., 2013). Furthermore, the temporal dynamics of the responses of biochemical parameters to long-term exposure to the bloom are generally poorly

understood (Qiu et al., 2007). When considering that the blooming period can last for months and that MCs can persist for several days or even weeks in waterbodies, this type of exposure is almost the only present in nature (Jones and Orr, 1994; Pavagadhi et al., 2013). It is crucial to expand our knowledge of this type of exposure in order to explain the extent to which prolonged contact with cyanotoxins can compromise cellular homeostasis in the living world and how organisms cope with stress in the actual environment.

We monitored the cyanobacterial bloom-mediated changes in the biochemical parameters in the liver of *Pelophylax* kl. *esculentus* (Linnaeus, 1758) by comparing frogs that inhabited different (MC-free and MC-containing) sides of Lake Ludaš (Serbia). Our principal aim was to evaluate the actual environmental ecotoxicological impacts of the MC-producing cyanobacterial bloom over several months. The prooxidant and neurotoxic potential of the bloom, as well as the mode of action of the antioxidant and biotransformation defense components were investigated. Understanding the dynamics of the biochemical parameters during long-term exposure to the cyanobacterial bloom is expected to provide improved insight into its real-life ecotoxicity.

2. Materials and methods

2.1. Site selection and animal collection

Lake Ludaš was selected on the basis of previous studies on cyanobacterial bloom dynamics (Institute of Public Health, Subotica, 2018; Tokodi et al., 2018). It is located in the province of Vojvodina in northern Serbia (Fig. 1). The lake is 4.5 km long, with a total area of 3.28 km^2 and an average depth of 1 m (Tokodi et al., 2018). Although present on Ramsar List as

a wetland of international importance and protected as a Special Nature Reserve in Serbia, this lake has hypereutrophic character and long history of cyanobacterial blooming. Increased cyanobacterial abundance in the northern part of the lake, especially during summer months, has been documented since 1970 (Seleši, 2006). Periodic measurement of cyanotoxin concentrations in the lake water indicated different levels of MCs (0.02–603.61 μg L^{-1} for total MC) and negligible concentrations of saxitoxin (0.03–0.04 μg L^{-1}), while cylindrospermopsin has not been detected in Lake Ludaš (Tokodi et al., 2018, 2020). Regular monthly monitoring of phytoplankton community and physicochemical parameters of water is performed under the direction of Institute of Public Health, Subotica. Unlike the northern part of the lake that is characterized by cyanobacterial dominance, the primary production in the southern part was undertaken by a densely developed macrophyte cover. Both sides of the lake have similar physicochemical conditions, while high/low cyanobacterial abundance and presence/absence of cyanotoxins were marked as the dominant differences between the sides and the primary factor that affects biomarker responses in aquatic organisms (Gavrilović et al., 2020).

The study was carried out during the summer months of 2018 on two sides of the lake. Examinations at the northern (N) site (46° 06' 11" N, 19° 49' 16" E, 93 m.a.s.l.) included three sampling time points (N1 – June, N2 – July, N3 – August) that were chosen primarily because of the similar concentrations of MCs in the water, and because of the stability of the main physicochemical factors that are known to influence biochemical parameters (such as the temperature). Pre- and post-bloom periods were avoided due to reproductive (from March to mid-May) and/or seasonal effects on the oxidant/antioxidant biomarkers in frogs. The southern (S) site of the lake (46° 03' 59" N, 19° 49' 46" E, 95 m.a.s.l.) with one sampling time point in summer (August) was selected as the “reference site”.

Animal use was approved by the Serbian Ministry of Environmental Protection (Permission No. 353-01-1168/2018-04). Taxonomic determination of frogs was performed according to Arnold and Ovenden (1992) and Krizmanić (2008). Adult male frogs (n=10 for S, n=10 for N1, n=8 for N2 and n=10 for N3 frogs) were collected using hand nets and transported to the laboratory alive in cages with lake water. The total snout-vent length (SVL) and body mass (BM) of each frog were measured before dissection. The condition factor (CF) of frogs was calculated according to Bagenal and Tesch (1978) and served as an indicator of the overall health of the animals. The frogs were killed by decapitation. After dissection, the livers were weighed in order to determine the hepatosomatic index (HSI) as an additional indicator of the condition of the frog (Jelodar and Fazli, 2012). Liver samples were stored at −80°C until biochemical analysis.

2.2. Water, MC and phytoplankton analyses

Water samplings for the analyses of the physicochemical parameters, MC and phytoplankton were carried out at the same time/place as frog collection. Surface water near the shore of the lake was analyzed. Mobile water analytical equipment (YSI Multiparameter Water Professional Plus Water Quality Meter, USA) was used for *in situ* measurements of water temperature, pH, dissolved oxygen concentration and conductivity, while a Secchi disk served for determination of transparency. Water samples for the analyses of the other physicochemical parameters and MC concentrations were collected in 1-L glass bottles and transported in cool containers to the laboratory where they were processed within 12–24 h of collection. The

concentrations of ammonia, nitrite, nitrate, orthophosphates and total phosphorous were determined according to the Serbian National Standard for water quality using APHA (2017).

Sample preparation and quantification of the MCs was performed according to ISO 20179 (2005). The standards for the analyzed forms of MCs (MC-LR, MC-RR and MC-YR) were obtained from Cyano Biotech GmbH (Germany). The analysis was carried out on a HPLC system, Dionex Ultimate 3000, which consisted of an HPG-3200 Standard Binary Pump, WPS-3000 Autosampler, TCC-3000 Thermostatted Column Compartment, DAD-3000 and MSQ Plus Single Quadrupole Mass Spectrometer (Thermo Scientific, USA). A C18 Acclaim Polar Advantage II column with 3 μm particle size and 150 mm×3 mm I.D. (Thermo Scientific, USA) was used for chromatographic separations. Mass detection was performed by the single ion monitoring method at mass-to-charge ratios of 520, 996 and 1046 for MC-RR, MC-LR and MC-YR, respectively. The flow rate was 500 μL min^{-1}, the column temperature was 40°C and the injection volume was 20 μL. The limit of detection was 0.1 μg L^{-1} for each MC form.

Water samples for qualitative phytoplankton analysis were taken using a phytoplankton net with a mesh size of 25 μm (HYDRO-BIOS Apparatebau GmbH, Germany). Taxonomic identifications of phytoplankton species were made according to the widely used keys (John et al., 2011; Komárek, 2013; Komárek and Anagnostidis, 1998, 2005) and were performed under an upright microscope (DM750, Leica Microsystems, Germany) with an HI Plan 40×/0.65 (at 400× magnification) objective. The samples for phytoplankton quantification were taken in 0.5-L plastic bottles and preserved in Lugol's solution at a ratio of 1:250. Quantitative phytoplankton analysis was carried out using the Utermöhl (1958) method on an inverted microscope (INVE 500T, COLO Lab Experts, Slovenia) with an LWD Plan 40×/0.60 (at 400× magnification) objective. The density of phytoplankton taxa was presented as the number of cells mL^{-1}.

2.3. Tissue processing and biochemical analyses

After the liver was minced with scissors, one part of the tissue was homogenized for 45 s in 5 volumes of an ice-cold 25 mM sucrose buffer (pH 7.4) containing 10 mM Tris-HCl and 5 mM EDTA, using an Ultra Turrax homogenizer (T-18, IKA-Werk, Germany). The homogenates were sonicated at 10 kHz for 30 s on ice (Takada et al., 1982) with a Sonopuls ultrasonic homogenizer (HD 2070, Bandelin electronic, Germany). For determination of glutathione (GSH) concentration, the sonicates were centrifuged at 5,000 ×*g* for 10 min in 10% sulfosalicylic acid, and the protein precipitate was discarded. For measurement of the other biochemical parameters, the sonicates were centrifuged at 100,000 ×*g* for 90 min at 4°C to obtain the supernatant. Tissue processing for analysis of the level of lipid peroxidation (LPO) included homogenization and sonication of minced liver parts in 10 volumes of an ice-cold Tris-HCl buffer (pH 7.4), followed by centrifugation at 10,000 ×*g* for 10 min at 4°C in 40% TCA to obtain the supernatant.

The protein content was determined at 500 nm by the Lowry method (Lowry et al., 1951), with bovine serum albumin (BSA) as standard. Superoxide dismutase (SOD) activity was measured at 480 nm according to the assay described by Misra and Fridovich (1972), which is based on the ability of SOD to inhibit the autoxidation of epinephrine. The Claiborne (1984) method for catalase (CAT) analysis, which includes measurement of hydrogen peroxide degradation at 240 nm, was performed. Glutathione peroxidase (GPx) activity was assayed in accordance with the protocol outlined by Tamura et al. (1982), and glutathione reductase (GR) activity was estimated according to the assay described by Glatzle et al. (1974). Both methods for GPx and GR activities are based on the rate of NADPH oxidation. Glutathione-*S*-transferase

(GST) activity toward 1-chloro-2,4-dinitrobenzene (CDNB) was determined in accordance with Habig et al. (1974). The activities of all three GSH-related enzymes (GPx, GR and GST) were estimated at 340 nm. All antioxidant enzymatic activities were expressed in U mg^{-1} protein. GSH was determined according to the 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) enzymatic recycling method described by Griffith (1980). The concentration of GSH was expressed in nmol g^{-1} tissue. The concentrations of protein sulfhydryl (–SH) groups were estimated after incubation of tissue extracts with DTNB by the method of Ellman (1959) and expressed as nmol mg^{-1} protein. Both thiol determinations (GSH and protein –SH groups) were measured at 412 nm. The thiobarbituric acid-reactive substance (TBARS) assay described by Rehncrona et al. (1980) was used to measure the oxidative damage of lipids. Lipid peroxidation (LPO) level was evaluated at 532 nm and expressed in nmol mg^{-1} tissue. Liver cholinesterase (ChE) activity as a neurotoxicity parameter was detected at 412 nm with acetylcholine iodide as a substrate following the Ellman method (Ellman et al., 1961). ChE enzymatic activity was expressed as µmol min^{-1} g^{-1} tissue.

All biochemical parameters were measured at 25°C using a UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan). All chemicals were obtained from Sigma (St. Louis, MO, USA).

2.4. Integrated biomarker response (IBR)

Considering that biomarkers usually exhibit different patterns of response, their complex changes are not always easy to interpret. We used integrated biomarker response (IBR) to merge all antioxidant parameters (SOD, CAT, GPx, GR and GST activities and GSH concentration) into one unifying value in order to estimate the differences between frog groups in response to cyanobacterial bloom-induced stress. IBR analysis was performed according to Devin et al.

(2014). The calculation was based on the original formula described by Beliaeff and Burgeot (2002). Our starting hypothesis for IBR calculation was that the antioxidant defense parameters increase during exposure to the bloom. For each biomarker, we computed the value $Y = (X - m)/s$, where X is the mean value for a group, m is the mean value for all groups and s is the standard deviation for all groups. The S value was calculated as $S = Y + |\text{Min}|$, where Min is the minimal value observed for all groups for each biomarker. All Si values were plotted on a radar diagram to obtain the total area $Ai = Si \times S_{i+1} \times \sin(2\pi/k)/2$, and the IBR = $\sum_{i=1}^{k} Ai$ was calculated. To limit the effect of biomarker arrangement, which is user-defined, a procedure that creates all possible circular permutations of k biomarkers was performed. The result was a matrix of 120 values for six biomarkers. We have presented the IBR value as the median value of all values (due to the non-normal distribution of the IBR values).

2.5. Statistical analysis

The Kolmogorov-Smirnov normality test preceded other statistical analyses. Data for all condition indices and biochemical parameters were normally distributed. For statistical comparison of the parameters among the four groups of frogs (S, N1, N2 and N3), one-way ANOVA (with Tukey's HSD for unequal N as post hoc analysis) was performed. The significant level was set at $p < 0.05$. In all figures and tables, the results are shown as the mean ± standard error (SE). Principal component analysis (PCA) was used to integrate and present the general patterns of the biochemical parameters in relation to the groups. All data were analyzed using statistical software STATISTICA 10.0, except the integrated biomarker response index that was calculated in R 3.4.1.

3. Results

3.1. Water, MC and phytoplankton analyses

The measured values of the physicochemical parameters and the concentrations of MCs are presented in Table 1. The water sample from the S site was MC-free. In contrast, we detected MC in all samples from the N site (about 1 µg L^{-1} of total MC). Slight differences in the concentrations of MC forms among N1, N2 and N3 samplings were observed. The counted values for total phytoplankton, total cyanobacteria and cyanobacterial species densities are shown in Table 2. The S site was characterized by the lowest phytoplankton and cyanobacterial abundance (with dominance of *Chroococcus* sp.). The water samples from the N site had about 30- to 60-times higher cyanobacterial densities compared to the S site. The highest phytoplankton abundance with a clear dominance of cyanobacterial species was detected in the N1 water sample, followed by the values counted in the N2 and then in the N3 sample. The main bloom-forming genera in this part of the lake were *Microcystis* and *Planktothrix*. We observed differences among the bloom water samples, with dominant presence of *M. flos-aquae*, *P. agardhii* and *M. wesenbergii* in the N1 sample, of *P. agardhii*, *M. wesenbergii* and *Jaaginema subtilissimum* in the N2 sample, and of *M. wesenbergii* and *P. agardhii* in the N3 sample.

3.2. Condition indices

The measured values for SVL, BM, CF and HSI are presented in Table 3. N1 frogs had a lower BM in comparison with S frogs ($p = 0.007$). Statistical analysis showed that there were no significant differences among the four groups of frogs for the SVL, CF and HSI condition indices.

3.3. Oxidative stress and neurotoxicity parameters

SOD activity (Fig. 2A) was higher in N3 frogs when compared to S frogs ($p = 0.0005$), while CAT activity (Fig. 2B) was higher in N3 frogs in comparison to all other frog groups ($p = 0.0012$ for S-N3, $p = 0.0123$ for N1-N3 and $p = 0.0081$ for N2-N3). All frogs from the N side of the lake showed lower GPx activities (Fig. 2C) and protein –SH group concentrations (Fig. 2G) than frogs from the S site (GPx: $p = 0.0002$ for S-N1, $p = 0.0029$ for S-N2 and $p = 0.0066$ for S-N3; –SH: $p = 0.0002$ for S-N1, $p = 0.0002$ for S-N2 and $p = 0.0002$ for S-N3). The concentration of the protein –SH groups was lower in N1 frogs when compared to N2 and N3 frogs ($p = 0.0017$ for N1-N2 and $p = 0.0002$ for N1-N3). Statistical analysis showed that no significant differences were determined among the four frog groups for GR activity (Fig. 2D). Frogs from N1 and N2 samplings had higher GST (Fig. 2E) activity in comparison with S and N3 individuals ($p = 0.0002$ for S-N1, $p = 0.0002$ for S-N2, $p = 0.0002$ for N1-N3 and $p = 0.0002$ for N2-N3). The concentration of GSH (Fig. 2F) in N2 frogs was significantly higher than in the other groups of frogs ($p = 0.0382$ for S-N2, $p = 0.0025$ for N1-N2 and $p = 0.0017$ for N2-N3). The level of LPO (Fig. 2H) was higher in N1 and N3 frogs as compared to the S group, and in N3 frogs as compared to the N2 group ($p = 0.0286$ for S-N1, $p = 0.0147$ for S-N3 and $p = 0.0381$ for N2-N3). ChE activity (Fig. 2I) was lower in N3 frogs in comparison to S frogs ($p = 0.0120$).

3.4. PCA analysis

Principal component analysis revealed clear differentiation among the four frog groups based on the biochemical parameters (Fig. 3). The highest percentage of dataset variation (51.90% of the total variability) was explained by the first two principal components (PCs); 30.88% for PC1 and 21.02% for PC2. Frogs from the S site had an opposite loading on PC1 to the other three groups, mainly due to the contribution of GPx activity and protein –SH group concentrations. PC2 distinguished N3 frogs from N1 and N2 frogs and the separation was mainly the result of differences in CAT and GST activities.

3.5. IBR analysis

The calculated values of the integrated biomarker response for each frog group (S, N1, N2 and N3) were used to create the star plot (Fig. 4). The IBR index provided a comparison between the groups for the analyzed set of antioxidant biomarkers. The values of the IBR index were as follows: 0.65 ± 0.02 for S frogs, 0.79 ± 0.03 for N1 frogs, 3.37 ± 0.04 for N2 frogs and 3.39 ± 0.09 for N3 frogs. The results showed that the S frogs had the lowest IBR, while an increasing trend in antioxidant response was observed in N1 to N3 frogs.

4. Discussion

The phenomenon of a cyanobacterial bloom in aquatic ecosystems has become a widespread and progressive environmental stressor in a eutrophication-laden world (Žegura et al., 2011). Previous laboratory and field studies showed that chronic exposure to environmentally relevant/low concentrations of cyanotoxins presents a serious risk to organism and ecosystem health (Calado et al., 2018; Chen et al., 2017; Pavagadhi et al., 2012; Zhang et al., 2009). Results obtained from this study provide the first insight into the complex dynamics of the biochemical parameters in the liver of frogs after exposure to an MC-producing cyanobacterial bloom over several months. Despite the relatively low total concentration of MCs (about 1 μg L^{-1}) during the monitored months, our data showed that the bloom induced hepatic oxidative stress and neurotoxicity in frogs from a natural habitat.

Adverse effects and bioaccumulation of MC-LR and MC-RR in plants and fish from Lake Ludaš has been reported (Tokodi et al., 2018). Even though we did not measure the bioaccumulation of MCs by frogs, there is evidence that adult frogs accumulate MCs in tissues at a higher level than fish (Papadimitriou et al., 2012). Recent studies showed that long-term exposure of aquatic organisms to cyanobacterial blooms results in the bioaccumulation of MCs, even in case of a low concentration of toxins in water (Zhang et al., 2009; Calado et al., 2018).

There is little information in scientific literature regarding the effects of MCs on the prooxidant/antioxidant balance in amphibians. Burýšková et al. (2006) studied the influence of complex bloom samples on *Xenopus laevis* embryos, but no significant changes in oxidative stress parameters were observed. Similar results were obtained on *X. laevis* tadpoles fed with cyanobacterial biomass containing MC-LR, which pointed to the involvement of special defense mechanisms against cyanobacterial toxicity (Ziková et al., 2013). Research on adult male frogs *Rana nigromaculata* showed that even low MC concentrations can cause oxidative damage and

ultrastructural changes in the liver (Zhang et al., 2013a). Results obtained on the testes of the same species showed a similar pattern of redox disbalance and revealed MC-induced reproductive toxicity (Jia et al., 2014; Jia et al., 2018; Zhang et al., 2013b, c).

In our study, the condition factor and hepatosomatic index showed that the bloom had no negative effect on the general health of *P.* kl. *esculentus* frogs. Similar results were obtained on *Coregonus lavaretus* after subchronic exposure to environmentally relevant densities of MC-producing *Planktothrix rubescens* (Ernst et al., 2007). Sun et al. (2013) also found that the MC-containing bloom did not affect the CF of juvenile *Hypophthalmichthys nobilis* fish, indicating that biochemical parameters provide more robust evidence of cyanotoxin effects.

Exposure to MCs induces excessive production of ROS at multiple levels in the cell, mainly as the outcome of mitochondrial dysfunction (Chen and Xie, 2016). Our study confirmed an important role of the oxidative stress/ROS pathway in the promotion of bloom toxicity. Numerous studies have shown that SOD and CAT activities increase when organisms are exposed to MCs (Li et al., 2005; Prieto et al., 2006). In our study, significant SOD/CAT cooperation was manifested as the last line of antioxidant defense since it was observed only in N3 frogs. The delayed activation of SOD and CAT was also reported in fish liver during prolonged exposure to MC-containing cyanobacteria (Jos et al., 2005). The established role of SOD is dismutation of the superoxide anion radical, and elevated activity of this enzyme causes increased formation of intracellular H_2O_2. This ROS is subsequently removed by the activity of CAT. However, Jiang et al. (2012) observed no changes in SOD and CAT activities in the liver of fish following sub-chronic exposure to environmentally relevant concentrations of MC-LR.

GPx has a crucial role in the protection of cellular membranes as it participates in the elimination of lipid peroxidation products (Halliwell and Gutteridge, 2015). It was shown that

cyanotoxin-mediated GPx deficiency is directly connected to high LPO in fish tissues (Silva et al., 2011). Zhang et al. (2013a) reported decreased GPx activity and an increased level of LPO in the liver of *R. nigromaculata* frogs after prolonged exposure to 1 μg L^{-1} MC-LR in laboratory-based conditions. This is basically in line with our findings that revealed the induction of LPO processes in N1 and N3 frogs in comparison to S frogs, while GPx activity in all groups from the N site remained suppressed during the entire monitoring period. Previous studies reported that LPO is one of the most important events responsible for MC-induced hepatotoxicity (Amado and Monserrat, 2010; Wiegand and Pflugmacher, 2005). However, the level of LPO that we detected in N2 frogs was not significantly different from the values in S frogs. Protection against LPO progression during prolonged exposure to low doses of MCs was shown to be the result of a temporary adaptive response that was conditioned by antioxidant and biotransformation activities (Zhao et al., 2009).

The main route for the detoxification of MCs is conjugation with GSH that is catalyzed by the biotransformation enzyme GST (Pflugmacher et al., 1998). Increased GST activity has been observed in different fish species that were exposed to low concentrations of MCs (similar to ours) under laboratory and field conditions (Jiang et al., 2012; Pavagadhi et al., 2012; Qiu et al., 2007). On the other hand, insufficient activity of GST can be a sign of its saturation with cyanotoxins or as the consequence of oxidative modifications of the enzyme protein (Pavagadhi et al., 2012). The activation of GSH was defined as the most important line of antioxidant and biotransformation defense that significantly participates in the protection of the cell membranes. An increase in the concentration of GSH was reported in the liver of fish exposed to an MC-producing cyanobacterial bloom, and was linked to the protection of cells from LPO (Bláha et al., 2004; Qiu et al., 2007). The data reported by these authors could explain the GST/GSH/LPO

pattern observed in the present study. Although higher GST activity in N1 and N2 frogs in comparison with S frogs suggested activation of the detoxification system, this was accompanied by a higher concentration of GSH only in N2 frogs, with unchanged LPO (in comparison with S frogs). Therefore, an initial increase in GST activity without a change in GSH compared to S frogs was not sufficient to prevent LPO in N1 frogs, whereas this defensive process in LPO-affected N3 frogs was at values that were similar to those observed in S frogs. A schematic representation of the relationship between GPx, GST, GSH and LPO in the bloom-impacted frogs is presented in Fig. 5. The observed defense response showed that GSH is one of the main factors responsible for the defense against the bloom-induced LPO. Increased GSH concentration can be the result of its enhanced *de novo* synthesis, which assumes a key role in the protection against oxidative stress caused by MCs (Chen and Xie, 2016; Chen et al., 2016).

In addition to LPO, the oxidative modification of proteins (such as carbonylation) is a common oxidative damage caused by MCs in tissues of many aquatic organisms (Amado and Monserrat, 2010; Gavrilović et al., 2020). Our findings suggest that exposure to the MC-producing bloom also induced the oxidation of protein –SH groups in frog liver. Changes in the redox state of thiol/disulfide couples could affect the structure and function of almost all proteins, including oxidation-reduction enzymes (Halliwell and Gutteridge, 2015). However, our study has shown that the activity of the redox-maintenance enzyme GR in the liver of *P.* kl. *esculentus* is not a sensitive biomarker of exposure to a MC-producing cyanobacterial bloom. Also, no changes in GR activity were detected in the liver, gills, muscle and brain of *Cyprinus carpio* fed on lyophilized *M. aeruginosa* extracts (Amado et al., 2011).

Regarding the temporal dynamics of the biochemical parameters in the liver of the bloom-impacted frogs (from N1 to N3), slight (but mainly not significant) increases in SOD,

CAT, GPx and GR activities and a decrease in ChE activity were observed. The bell-shaped pattern for GSH concentration and the inverted bell-shaped pattern for the level of LPO revealed the correlation of these parameters. The dynamics of the concentrations of protein –SH groups and GST activity over time indicated a reduction in the level of protein oxidative damage and biotransformation, respectively. Taking all the data into account, the biochemical parameters did not exhibit a well-defined time-dependent pattern during the three months.

The integrated biomarker response is increasingly used in ecotoxicological studies concerning the effects of cyanotoxins (Calado et al., 2020). This multiple biomarker approach showed that acute exposure to low concentrations of MC-LR had toxic effects in *Geophagus brasiliensis* fish (Calado et al., 2019). The higher values of IBR in N2 and N3 frogs compared to N1 frogs demonstrated that the higher activation of the antioxidant defense over time was a response to bloom exposure. Cellular redox homeostasis is based on the combined activity of antioxidants, and successful defense is possible only if all components of the antioxidant system cooperate (Costantini et al., 2013). Although IBR values of the investigated antioxidant parameters from frog liver pointed to a potential adaptive response to the toxic cyanobacterial bloom, the single defense components were not coordinated, i.e. they were not activated at the same time. Considering all presented results, the activity of the antioxidant system in the liver of *P.* kl. *esculentus* was not sufficient to prevent bloom-mediated oxidative damage.

In environmental toxicological studies on cyanobacterial blooms, the possible presence of other xenobiotics in water that can cause prooxidant effects should not be overlooked. The elevated content of heavy metals and organic contaminants in the water are known to affect oxidative stress parameters (Falfushynska et al., 2010, 2012; Valon et al., 2013). Regular monthly monitoring showed that the concentrations of heavy metals (Fe, Zn, Cu, Cr, Cd, Pb, Ni,

Hg, Co, Mn) and different organic pollutants (pesticides, PAHs) in the water of Lake Ludaš did not exceed environmental quality standards for surface waters during our sampling in 2018 (Environmental Protection Agency – Ministry of Environmental Protection of the Republic of Serbia, 2019). Although previous *in situ* studies indicated that the presence of metal ions might promote the absorption of MCs in fish (Jiang et al., 2014), the co-toxic effects of the MCs with other pollutants are still not well known.

The modification of MC toxicity as a result of the presence of common nutrients in the water cannot be excluded in our research. It was demonstrated that NH_4^+, PO_4^{3-} and Cl^- intensified, while NO_3^- attenuated the toxic effect of MCs (Pavagadhi et al., 2013; Sun et al., 2011). The results of the present study are in agreement with this, and they indicate that the specific oxidant/antioxidant state (high GSH concentration and low LPO level) that was detected in N2 frogs was accompanied by the highest concentration of NO_3^- in water. Pavagadhi et al. (2013) reported that the interaction of MCs and NO_3^- could transform MCs over time or even change the bioavailability of MCs. The enhanced detoxification capacity of the GST-GSH system in N2 frogs could be the result of cooperative detoxification of NO_3^-.

Considering that only minor differences in other water parameters between samplings were detected, changes in the biochemical parameters appear to be mainly the result of the presence of cyanobacterial metabolites. However, modulation of prooxidant MC toxicity by coexposure to nonspecific cyanobacterial metabolites (such as lipopolysaccharides) produced by all cyanobacteria, as well as to unidentified cyanotoxins, has also been reported (Burýšková et al., 2006; Jaja-Chimedza et al., 2012). Based on the above, the effects of the cyanobacterial bloom observed in our study should not be simply linked to the detected concentrations of MCs. Slight differences in concentrations of different MC forms and different abundances of dominant

cyanobacterial species in different samples could have also had an additional effect. Most of the cyanobacterial genera and species detected in our study are known as potential cyanotoxin producers. The most numerous species found in the northern part of the lake (such as *M. flos-aquae*, *M. wesenbergii* and *P. agardhii*) are recognized as producers of MCs (Fastner et al., 1999; Luukkainen et al., 1994; Yasuno et al., 1998). However, cyanotoxin production is not species-specific but a strain-related feature, so that cyanobacterial abundance and concentrations of MCs were not correlated in our study.

The neurotoxic effect of the MC-containing cyanobacterial bloom on frog liver was also revealed in our study. The significantly lower ChE activity that was detected only in N3 frogs in comparison to S frogs, suggested that neurotoxicity was expressed with a delay in relation to prooxidant toxicity. Our data are consistent with research that reported the neurotoxic effects of MCs and a decrease in AChE activity in fish tissues (Calado et al., 2018; Wu et al., 2017).

5. Conclusions

This study revealed that prolonged exposure to an MC-containing cyanobacterial bloom altered the oxidative status of the liver of *P.* kl. *esculentus* frogs. Complex dynamics of the antioxidant and biotransformation parameters were observed, but the defense mechanisms were not capable of protecting cellular targets such as protein thiols and membrane lipids. However, an important role of GSH in the transient recovery from LPO is emphasized. A continuously low GPx activity and deficiency in cooperation of the antioxidant components were the main reasons for the failure of the defensive response in the bloom-impacted frogs. The neurotoxicity observed as the decrease in the ChE activity was manifested with a delay in relation to the prooxidant

effects. The response of the biochemical parameters did not exhibit a clear time-dependent pattern, suggesting the added influence of different factors, such as non-specific cyanobacterial metabolites and water-quality parameters. The possible presence of other prooxidant agents in the lake cannot be ignored. This work demonstrates the importance of *in situ* research as one of the most valuable approaches in efforts to elucidate the biochemical toxicity of blooms. Further comprehensive environment-based studies are urgently needed to expand the comparatively scarce knowledge of the effects of long-term exposure to cyanobacterial blooms.

Declaration of Competing Interest

The authors have no competing interest to declare.

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Figure captions

Fig. 1. Map of Lake Ludaš (Serbia) with the southern (S) and northern (N) sampling sites marked.

Fig. 2. Biochemical parameters in the liver of *P.* kl. *esculentus* frogs from the southern (S) and northern (N) sites at Lake Ludaš, obtained at three time points (N1, N2 and N3). A – superoxide dismutase (SOD), B – catalase (CAT), C – glutathione peroxidase (GPx), D – glutathione reductase (GR), E – glutathione-*S*-transferase (GST), F – glutathione (GSH), G – protein sulfhydryl (–SH) groups, H – lipid peroxidation (LPO) and I – cholinesterase (ChE). The data are expressed as the mean ± SE. Different letters indicate significant differences between the groups (Tukey's HSD for unequal N, $p < 0.05$).

Fig. 3. Principal component analyses (PCA) of the biochemical parameters (SOD, CAT, GPx, GR, GST, GSH, protein –SH groups, LPO and ChE) in the liver of *P.* kl. *esculentus* frogs from the southern (S) and northern (N) sites at Lake Ludaš, obtained at three time points (N1, N2 and N3). A – projection of the cases on the PC-plane, B – projection of the variables on the PC-plane.

Fig. 4. Integrated biomarker response (IBR) in the liver of *P.* kl. *esculentus* frogs from the southern (S) and northern (N) sites at Lake Ludaš, obtained at three time points (N1, N2 and N3). The antioxidant parameters (SOD, CAT, GPx, GR, GST and GSH) were selected to calculate IBR.

Fig. 5. Schematic representation of the relationship between GSH-based antioxidant machinery (GPx, GST and GSH) and lipid peroxidation (LPO) in the liver of *P.* kl. *esculentus* frogs from the northern (N) site at Lake Ludaš, obtained at three time points (N1, N2 and N3). ↑ indicates significantly higher value with respect to the southern (S) site; ↓ indicates significantly lower value with respect to the S site; − indicates no significant difference with respect to the S site.

Table 1

Water-quality parameters and concentration of microcystin forms (MC-LR, MC-RR and MC-YR) in water samples from the southern (S) and northern (N) sites at Lake Ludaš, obtained at three time points (N1, N2 and N3) (nd – not detected).

	S	N1	N2	N3
Temperature (°C)	27.40 ± 0.76	26.03 ± 0.41	26.23 ± 0.15	26.47 ± 0.42
pH	9.30 ± 0.05	9.42 ± 0.07	9.00 ± 0.04	9.67 ± 0.04
O_2 (mg L^{-1})	8.86 ± 0.83	15.48 ± 0.68	14.39 ± 0.49	13.35 ± 0.50
Conductivity (µS cm^{-1})	1795 ± 7.09	898 ± 6.35	921 ± 1.76	936 ± 7.77
Transparency (cm)	64.00 ± 2.52	16.00 ± 0.29	16.50 ± 0.29	16.33 ± 0.33
Ammonia, NH_4-N (mg L^{-1})	0.56 ± 0.03	0.41 ± 0.02	0.66 ± 0.01	0.27 ± 0.01
Nitrite, NO_2-N (mg L^{-1})	< 0.002	0.012 ± 0.001	0.019 ± 0.001	< 0.002
Nitrate, NO_3-N (mg L^{-1})	< 0.1	0.45 ± 0.03	7.00 ± 0.49	< 0.1
Orthophosphates, PO_4-P (mg L^{-1})	0.013 ± 0.001	0.03 ± 0.003	0.02 ± 0.002	0.04 ± 0.004
Total phosphorous, P (mg L^{-1})	0.19 ± 0.01	0.57 ± 0.02	0.07 ± 0.01	0.70 ± 0.03
MC-LR (µg L^{-1})	nd	0.50 ± 0.11	0.40 ± 0.08	0.60 ± 0.13
MC-LL (µg L^{-1})	nd	0.25 ± 0.05	0.40 ± 0.08	0.41 ± 0.09
MC-YR (µg L^{-1})	nd	nd	0.20 ± 0.04	nd
MC-Total (µg L^{-1})	nd	0.75 ± 0.16	1.00 ± 0.20	1.01 ± 0.22

Table 2

Densities (cell mL^{-1}) of total phytoplankton, total cyanobacteria and cyanobacterial species in water samples from the southern (S) and northern (N) sites at Lake Ludaš, obtained at three time points (N1, N2 and N3); the most numerous and blooming cyanobacterial species are in bold (the counting error for dominant taxa was <1%).

	S	N1	N2	N3
Phytoplankton	42,742	2,242,076	1,758,639	1,179,073
Cyanobacteria	37,324	2,182,173	1,707,413	1,160,141
Anabaena sp.	420	0	20,012	11,086
Aphanizomenon sp.	0	3,675	0	0
Dolichospermum flos-aquae	0	0	885	0
Anabaenopsis circularis	0	885	0	531
Anabaenopsis cunningtonii	136	3,276	2,347	496
Anabaenopsis elenkinii	0	9,209	8,147	549
Aphanocapsa sp.	0	0	5,224	72,274
Chroococcus sp.	27,840	89	13,415	0
Raphidiopsis raciborskii	60	8,678	33,870	19,658
Cuspidothrix sp.	0	1,860	1,240	6,075
Gloeocapsa sp.	0	0	3,188	0
Komvophoron sp.	0	1,771	2,037	638
Merismopedia sp. 1	0	0	2,125	4,959
Merismopedia sp. 2	0	0	0	5,419

Microcystis wesenbergii	1,878	**112,547**	**180,021**	**665,610**
Microcystis flos-aquae	0	**1,560,200**	4,516	62,126
Oscillatoria sp. 1	1,350	0	0	0
Oscillatoria sp. 2	840	0	0	0
Oscillatoria sp. 3	1,518	0	0	0
Phormidium sp.	1,000	0	0	0
Planktolyngbia limnetica	48	34,402	13,814	10,201
Jaaginema subtilissimum	1,998	93,685	**167,359**	37,775
Limnothrix sp.	152	39,316	47,108	6,730
Geitlerinema amphibium	0	20,898	50,650	0
Planktothrix agardhii	84	**279,064**	**1,117,585**	**253,641**
Pseudanabaena limnetica	0	12,618	33,870	2,373

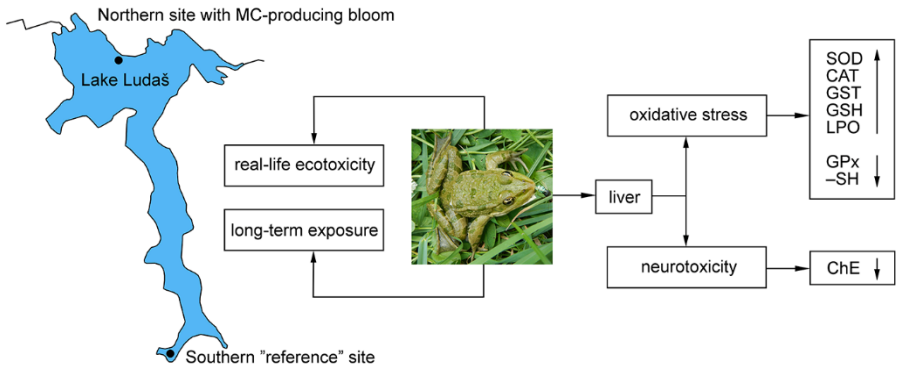
Table 3

The total snout-vent length (SVL), body mass (BM), condition factor (CF) and hepatosomatic index (HSI) of *P.* kl. *esculentus* frogs from the southern (S) and northern (N) sites at Lake Ludaš, obtained at three time points (N1, N2 and N3). The data are expressed as the mean ± SE. Different letters indicate significant differences between the groups (Tukey's HSD for unequal N, $p < 0.05$).

	S	N1	N2	N3
SVL (cm)	6.12 ± 0.15 [a]	5.83 ± 0.13 [a]	5.95 ± 0.16 [a]	6.11 ± 0.22 [a]
BM (g)	32.12 ± 1.73[a]	23.28 ± 1.77[b]	25.51 ± 1.49[ab]	29.45 ± 2.09[ab]
CF	13.99 ± 0.51 [a]	11.51 ± 0.24 [a]	12.11 ± 0.53 [a]	13.26 ± 1.22 [a]
HSI	2.01 ± 0.11 [a]	1.86 ± 0.17 [a]	1.97 ± 0.10 [a]	1.74 ± 0.12 [a]

Highlights

- Long-term *in situ* exposure to a cyanobacterial bloom affects biomarkers in frogs.
- An MC-producing bloom induces oxidative damage of hepatic proteins and lipids.
- The bloom impairs cholinergic homeostasis through a decrease in ChE activity.
- Complex environmental factors can modify the toxicity of blooms.
- Further studies of prolonged, real-life ecotoxicity of blooms are warranted.



Graphical Abstract

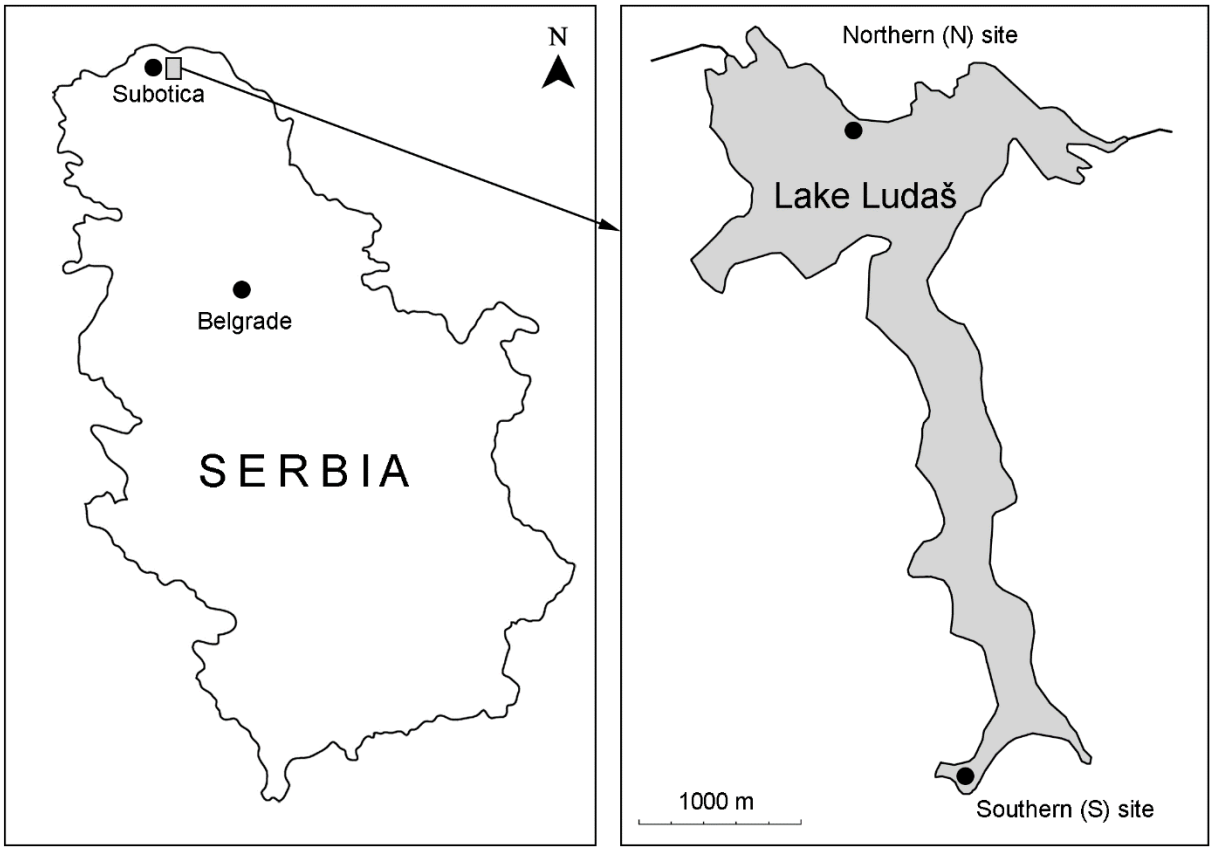

N
Subotica
Belgrade
SERBIA
Northern (N) site
Lake Ludaš
1000 m
Southern (S) site

Fig. 1

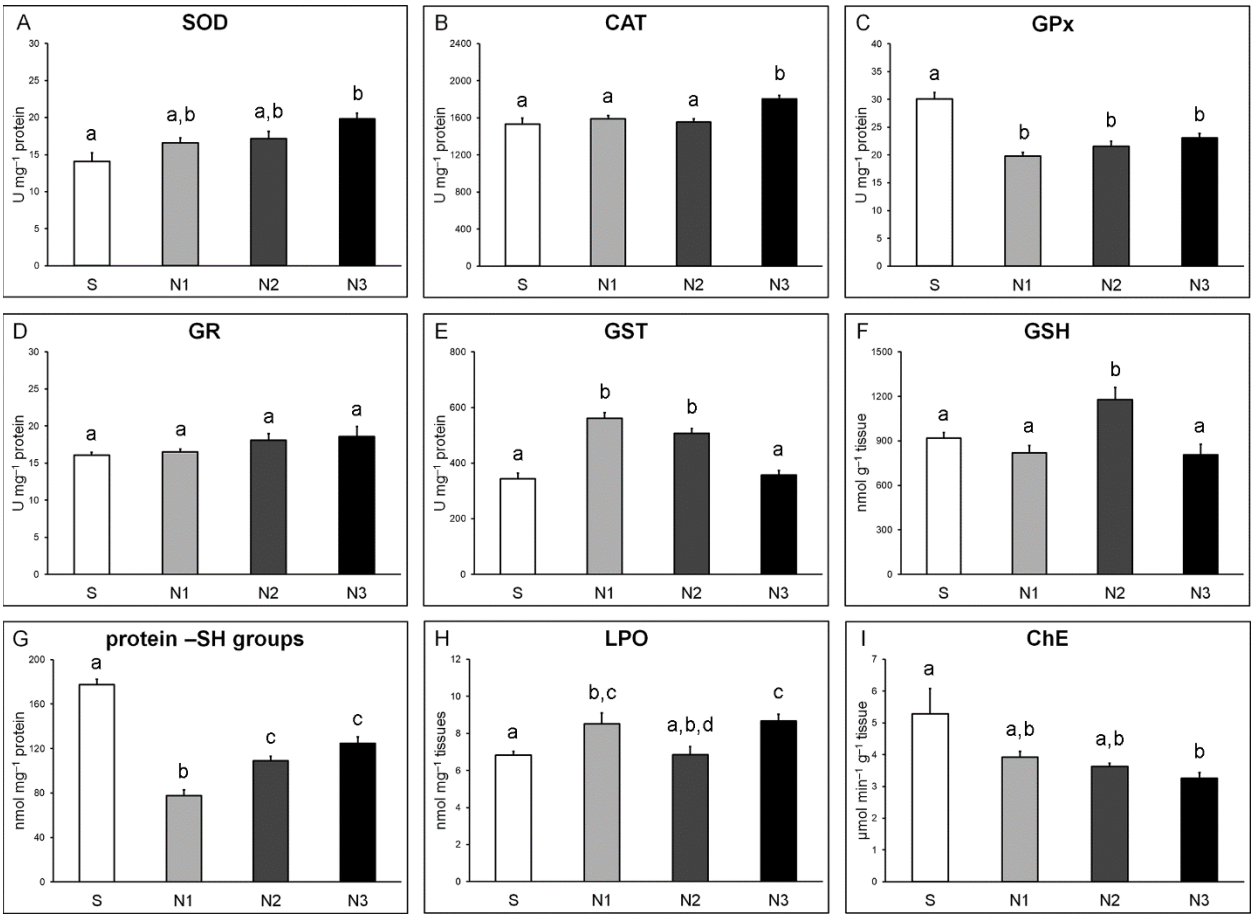

A
SOD
U mg⁻¹ protein
a
a,b
a,b
b
S
N1
N2
N3
B
CAT
U mg⁻¹ protein
a
a
a
b
C
GPx
U mg⁻¹ protein
a
b
b
b
D
GR
U mg⁻¹ protein
a
a
a
a
E
GST
U mg⁻¹ protein
a
b
b
a
F
GSH
nmol g⁻¹ tissue
a
a
b
a
G
protein –SH groups
nmol mg⁻¹ protein
a
b
c
c
H
LPO
nmol mg⁻¹ tissues
a
b,c
a,b,d
c
I
ChE
µmol min⁻¹ g⁻¹ tissue
a
a,b
a,b
b

Fig. 2

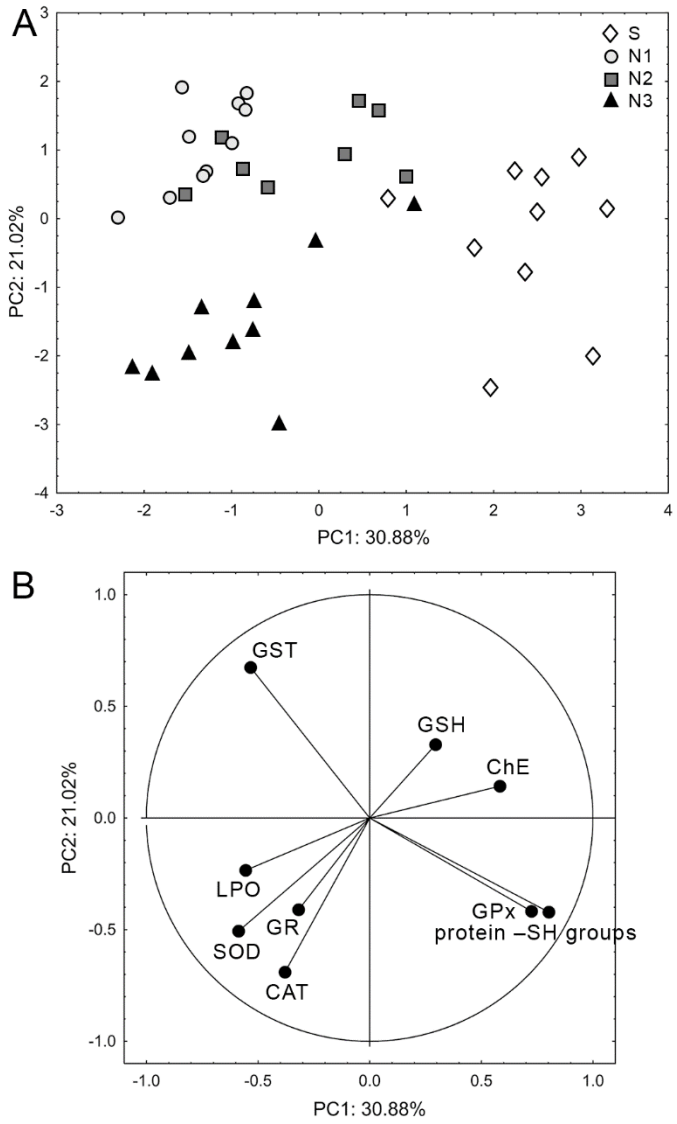

A
S
N1
N2
N3
PC2: 21.02%
PC1: 30.88%
B
GST
GSH
ChE
LPO
GR
SOD
CAT
GPx
protein –SH groups
PC2: 21.02%
PC1: 30.88%

Fig. 3

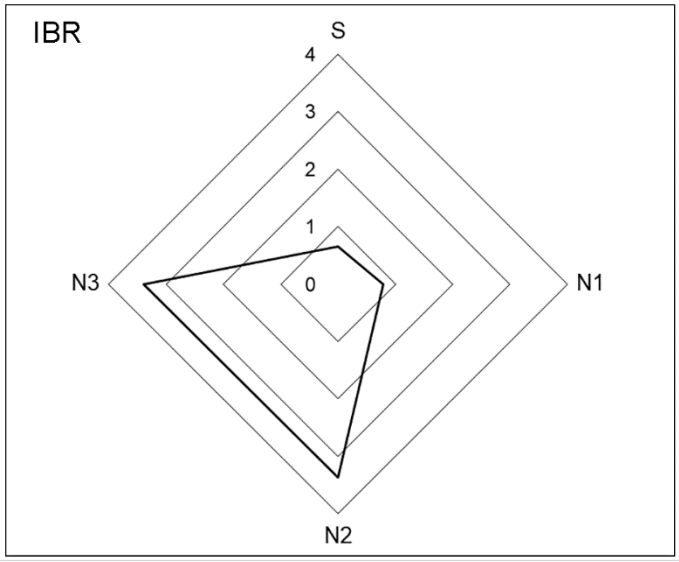


Fig. 4

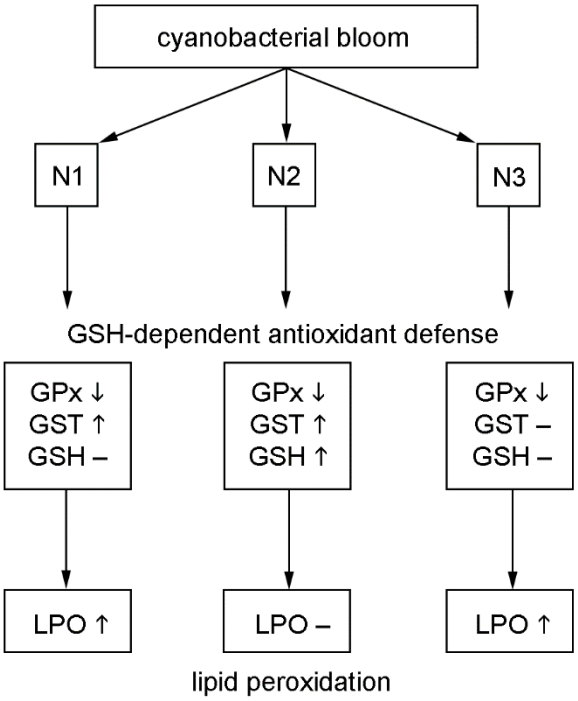

cyanobacterial bloom
N1
N2
N3
GSH-dependent antioxidant defense
GPx ↓
GST ↑
GSH –
GPx ↓
GST ↑
GSH ↑
GPx ↓
GST –
GSH –
LPO ↑
LPO –
LPO ↑
lipid peroxidation

Fig. 5