Antioxidant Response in Gills of Eurasian Perch (*Perca fluviatilis*) to Cyanobacterial Bloom Exposure in the Gruža Reservoir

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Abstract

The aim of this study was to assess the impact of an *Aphanizomenon flos-aquae* bloom in the Gruža Reservoir on the antioxidant parameters measured in the gill cells of Eurasian perch (*Perca fluviatilis*). Effects of the bloom were evaluated through copper, zinc and manganese containing superoxide dismutase (CuZnSOD and MnSOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and biotransformation phase II enzyme glutathione-S-transferase (GST) activities and glutathione (GSH) and sulfydryl (–SH) groups concentrations. The activities of CuZn SOD and Mn SOD decreased during the cyanobacterial bloom compared to the period before the bloom. The opposite trend was observed for CAT and GSH-Px activities that increased. During the bloom GR and GST activities and GSH concentration decreased significantly. CuZn SOD, GR and GST activities are detected as the most important parameters for individual variations. Our results indicated that an *A. flos-aquae* bloom promotes oxidative stress in the gills of *P. fluviatilis*. The investigated parameters could be used in environmental risk assessment procedures to monitor the effects of *A. flos-aquae* blooms on fish. Observed changes in the antioxidant parameters in *P. fluviatilis* gills make them potential biomarkers in the research of ecological risk situations in freshwater ecosystems with frequent cyanobacterial blooms.

Keywords: Eurasian perch, *Perca fluviatilis*, gills, antioxidant parameters, cyanobacterial bloom.

Introduction

Human-induced eutrophication of freshwater ecosystems has many negative impacts on the environment. One of the consequences of eutrophication is characterized by the excessive abundance of cyanobacterial populations and the formation of cyanobacterial blooms (Ostojić et al., 2007). Cyanobacterial species can produce cyanotoxins, which adversely affect aquatic organisms (Zhang et al., 2016). *Aphanizomenon flos-aqaue* (Linnaeus) Ralfs ex Bornet & Flahault, 1888 blooms in freshwater ecosystems are increasing in many regions of the world under eutrophic conditions. *A. flos-aqaue* has been identified as the producer of a variety of different cyanotoxins, including anatoxin-a, cylindrospermopsin and saxitoxins (Ballot et al., 2010; Šulčius et al., 2015).

Previous studies showed that cyanotoxins cause toxicity to aquatic organisms by inducing oxidative stress (Guzmán-Guillén et al., 2015; Zhang et al., 2016). Evidences on the role of oxidative stress induced by cyanotoxins from *A. flos-aquae* include increased reactive oxygen species (ROS) production resulting in damage to DNA, proteins and lipids. Previous studies indicated that both ROS and cyanotoxins are removed by the activity of enzymatic and nonenzymatic components of the cellular antioxidant and biotransformation systems (Gavrilović et al., 2015; Zhang et al., 2015).

The prooxidant effects of cyanotoxins on fish has been extensively investigated in field and laboratory experiments, indicating that antioxidant biomarkers could be very useful in the biomonitoring of eutrophic

freshwaters where cyanobacterial blooms occur (Qiu et al., 2007; Gavrilović et al., 2014; Hauser-Davis et al., 2015; Zhang et al., 2015). However, most of the research has been conducted on herbivorous and omnivorous fish species that feed more frequently on toxic cyanobacteria. On the other hand, very few studies have examined the impact of cyanobacterial blooms and cyanotoxins on the antioxidant parameters in carnivorous fish species. Qiu et al. (2007) showed that the carnivorous fish are more vulnerable to cyanobacterial blooms due to their higher accumulation of cyanotoxins. They also indicated on lower correlations among antioxidant enzymes in tissues of carnivorous fish compared to phytoplanktivorous.

Perca fluviatilis Linnaeus, 1758 (Eurasian perch) is a carnivorous fish that has a wide distribution in fresh and brackish waters in the northern hemisphere. Negative effects of *A. flos-aquae* blooms on the antioxidant parameters in the liver and muscle of *P. fluviatilis* have been previously determined (Perendija et al., 2011; Gavrilović et al., 2015). However, how *A. flos-aquae* blooms influence these parameters in the gills of *P. fluviatilis* has not yet been specifically studied. It is important to investigate biochemical changes in the gills, because they are the first organs which come in contact with environmental factors and are the prime target of cyanotoxins. Gavrilović et al. (2014) showed that antioxidant enzyme activity in the gills was affected more than in the liver of cyprinids exposed to *A. flos-aquae* blooms. Furthermore, the high vascularity, large surface area and respiratory process of the gills facilitate greater toxicant interaction, while their detoxification system has lower activity than the liver (Chen et al., 2012).

In this study we aimed to assess the impact of *A. flos-aquae* blooms in the Gruža Reservoir on antioxidant parameters (copper zinc and manganese containing superoxide dismutase (CuZn SOD and Mn SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and biotranformation phase II enzyme glutathione-S-transferase (GST) activities and glutathione (GSH) and sulfydryl (–SH) groups concentrations) in the gills of *P. fluviatilis*. The correlation structure of the parameters was also estimated and the parameters that mainly influence differentiation between samples were determined. We investigated the potential utility of the antioxidant parameters as biomarkers of cyanobacterial bloom exposure in the gills of *P. fluviatilis*.

Materials and Methods

The Gruža Reservoir (43° 55' 57'' N, 20° 40' 44'' E) is located in Central Serbia (Figure 1) and is mainly used to supply drinking water to the city of Kragujevac. It is a typical lowland reservoir with shallow and calm water and a considerably large surface area (Marinović et al., 2016). The reservoir has been under strong anthropogenic influences over the past several decades. Water quality from the reservoir is evaluated as β-mesosaprobic and has a hypereutrophic status with massive development of cyanobacteria during summer (Ostojić et al., 2007).

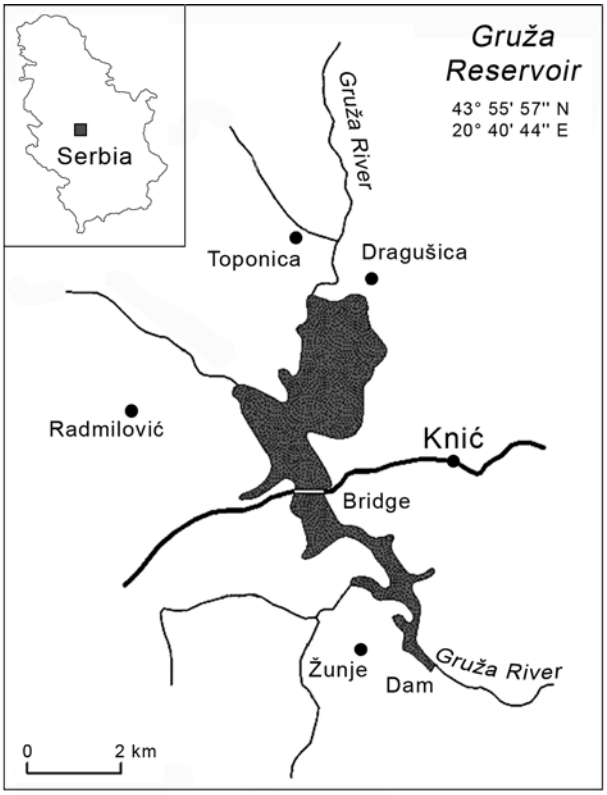

Figure 1: Map of the Gruža Reservoir.

The field work was conducted during two periods: in June before the cyanobacterial bloom and in August during the bloom. For analysis of the cyanobacterial community, samples were gathered by standard methods with a phytoplankton net (25 μm pore diameter) and Ruttner bottle (2 L). All samples were immediately preserved in 4 % formaldehyde. For qualitative analysis of the cyanobacteria, samples were microscopically examined. The quantitative analysis of the material was made using the Utermöhl method (1958) and it is expressed as the number of individuals per L (ind/L).

Fish individuals were caught by local anglers during the two aforementioned periods. The total length and weight of each fish was measured before dissection. Mean values for the total length and weight of the fish were 103 mm and 14 g before the bloom (n = 28) and 153 mm and 64 g during the bloom (n = 14). The fish were immediately killed by a blow to the head. The gills were excised and stored at –80°C for further biochemical analysis.

Tissues were homogenized in a 1:5 ratio in an ice-cold 25 mM sucrose solution (pH 7.5) containing 10 mM Tris-HCl and 5 mM EDTA (Lionetto et al.,

2003). The homogenates were sonicated for 30 s at 10 kHz and centrifuged at 100 000 × *g* for 90 min at 4°C. The resulting supernatants were used for biochemical measurements.

The antioxidant parameters were measured simultaneously in triplicate for each sample (the means were used for further calculations) using a UV-Vis spectrophotometer Nicolet Evolution 600. The activities of CuZn SOD and Mn SOD were measured by the method of Misra and Fridovich (1972), which is based on the autoxidation of adrenaline to adrenochrome. CAT activity was determined according to Claiborne (1984), where a decrease in H_2O_2 consumption was measured. GSH-Px activity was quantified by the technique of Tamura et al. (1982) in which oxidized glutathione (GSSG) produced by peroxidase activity is coupled to the reaction catalyzed by GR. The activity of GR was assayed according to the method described by Glatzle et al. (1974). GST activity was determined by monitoring the conjugation rate of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH according to Habig et al. (1974). All enzymatic activities were calculated in terms of the sample protein content (Lowry et al., 1951) and expressed in U/mg protein. The GSH concentration was determined by the procedure of Griffith (1980) which is based on the oxidation of GSH by 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) and the reduction of GSSG in the presence of GR. The concentration of –SH groups was estimated by the method of Ellman (1959), using DTNB as substrate. GSH and –SH groups concentrations were expressed as nmol/g tissue. All chemicals were the products of Sigma (St. Louis, MO, USA).

The obtained results for the antioxidant parameters were reported as means ± standard error. For statistical comparisons of data before and during the bloom, the non-parametric Mann-Whitney *U*-test was performed and *p* values below 0.05 were regarded as significant. In addition, principal component analysis (PCA) was used to define the most important parameters, which could be used as key factors for individual variations. All statistical analyses were performed using STATISTICA 6.0 software.

Results and Discussion

Cyanobacterial blooms are recognized as a growing problem in freshwater ecosystems, because 70% of them are toxic. *A. flos-aquae* is one of the most common toxin-producing cyanobacteria in temperate climate regions (Ballot et al., 2010; Zhang et al., 2015). In this study, prior to the cyanobacterial bloom in the Gruža Reservoir, no cyanobacteria were detected. During the bloom 420000 ind/L were detected and *A. flos-aquae* was the predominant cyanobacterial species present in the water samples.

Previous studies showed that *A. flos-aquae* blooms could alter the antioxidant system and induce ROS production, resulting in oxidative stress in different fish tissues (Perendija et al., 2011; Gavrilović et al., 2015; Zhang et al., 2015). Modulations of antioxidant and biotransformation parameters in fish have often been discussed as early warning signals of potential cyanobacterial toxicity to individuals or whole populations (Paskerová et al., 2012). Fish are among the vertebrates most likely to be affected by cyanotoxins. However, carnivorous fish are particularly important experimental models for investigating the effects of cyanobacterial blooms in terms of their position on the top of the aquatic food chain. Kopp et al. (2013) detected the highest concentrations of cyanotoxins in tissues of carnivorous *P. fluviatilis* compared to the concentrations in the other 15 investigated fish species with different feeding types.

CuZn SOD and Mn SOD activities in the gills (Figure 2) of *P. fluviatilis* decreased during the cyanobacterial bloom, which indicated on disturbed functionality of the primary cell defence against ROS. CuZn SOD catalyses the dismutation of anion radicals ($O_2^{\bullet-}$) to molecular oxygen and hydrogen peroxide (H_2O_2) in cytosol, while Mn SOD is active in the mitochondrial compartments of cells. Many studies showed that cyanotoxins have enzyme-inhibiting activity due to their capacity to inhibit protein synthesis (Runnegar et al., 2002). Our previous results also revealed that *A. flos-aquae* bloom inhibit CuZn SOD and Mn SOD activities in the gills of cyprinid fish species (Gavrilović et al., 2014). A similar decrease of SOD activity was demonstrated by Chen et al. (2012) in the gills of zebrafish after exposure to microcystins.

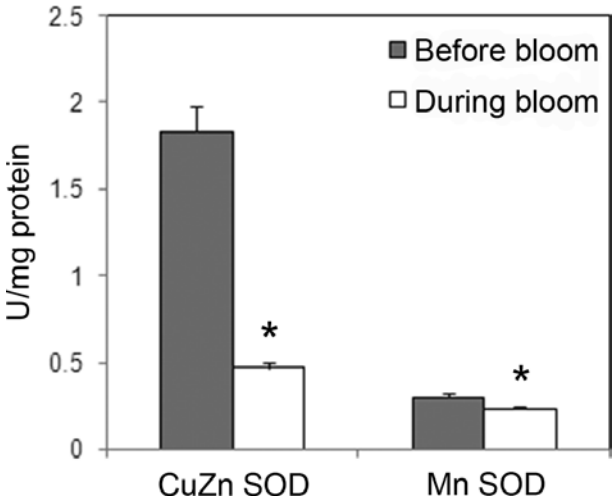

Figure 2: CuZn SOD and Mn SOD activities (U/mg protein) in the gills of *P. fluviatilis* collected before and during the *A. flos-aquae* bloom in the Gruža Reservoir. Asterisk indicates statistically significant differences between samples from different investigated periods (p<0.05).

The activities of CAT and GSH-Px in the gills of *P. fluviatilis* are shown in Figure 3. Both H_2O_2 dismutazing enzymes increased during the cyanobacterial bloom. Although H_2O_2 is less harmful compared to $O_2^{\bullet-}$ and hydroxyl radicals ($^{\bullet}OH$), it can react with redox active metals through the Fenton reaction. The result of this reaction is the homolysis of H_2O_2 to 2 $^{\bullet}OH$, which is associated with H_2O_2 toxicity (Flores-Rojas et al., 2015). The results suggest that CAT and GSH-Px play an important role as antioxidant enzymes in the gills of *P. fluviatilis* exposed to the cyanobacterial bloom. The elevated activities of CAT and GSH-Px give evidence that these defence mechanisms remain active against H_2O_2 induced oxidative stress. A similar increase of CAT activity was demonstrated in the gills of *Rutilus rutilus* exposed to the *A. flos-aquae* bloom (Gavrilović et al., 2014). Previous studies showed that promotion of oxidative stress by cyanotoxins can increase the activity of some antioxidant enzymes as a mechanism for scavenging ROS. That increase can arise from activation of existing enzymes or from *de novo* enzyme synthesis (Hou et al., 2015). However, our results are not consistent with a previous study that showed decrease in CAT and GSH-Px activities in the gills of zebrafish that were treated with microcystins (Chen et al., 2012).

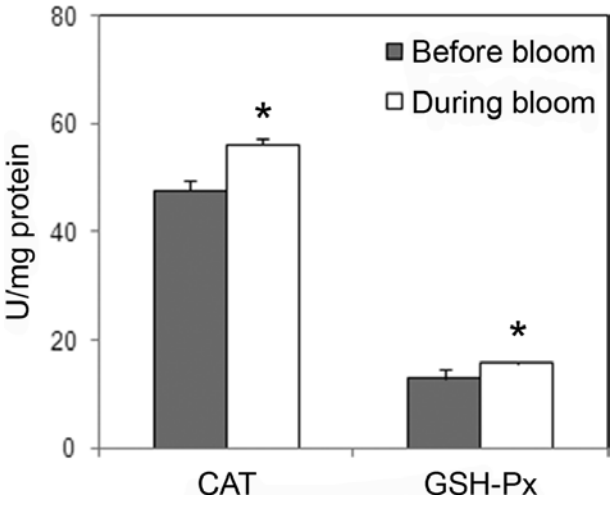

Figure 3: CAT and GSH-Px activities (U/mg protein) in the gills of *P. fluviatilis* collected before and during the *A. flos-aquae* bloom in the Gruža Reservoir. Asterisk indicates statistically significant differences between samples from different investigated periods ($p<0.05$).

Cyanotoxins were shown to be conjugated with GSH and it is a common biotransformation step catalysed by GST. The existence of a cyanotoxin-GSH complex formed through GST catalysis in aquatic organisms, suggests that GST plays an important role in cyanotoxin detoxification (Li et al., 2014). This reaction could reduce the intracellular pool of GSH (in addition to consumption of GSH by GSH-Px) and thus lower the protection against oxidative stress (Hauser-Davis et al., 2015). During the cyanobacterial bloom in the Gruža Reservoir, GR and GST activities (Figure 4) and GSH concentration (Figure 5) decreased significantly in in the gills of *P. fluviatilis*. Decreased activity of GST can be a sign of saturation due to substrate inhibition caused by the presence of high cyanotoxin concentrations. GST activity decreases *in vivo* were detected in *Cyprinus carpio* exposed to *Aphanizomenon* and *Planktothrix* biomasses (Palíková et al., 2007). Our previous results revealed that *A. flos-aquae* bloom inhibit GR and GST activities in the gills of cyprinids (Gavrilović et al., 2014). Decreased GSH concentration in *P. fluviatilis* may be related to GSH-Px activity increase and GR activity depletion in response to the cyanobacterial bloom toxicity. GR is responsible for preserving the reductive environment of the cell through the maintenance of the GSH intracellular pool in a reduced state (Chen et al., 2012; Paskerová et al., 2012). Furthermore, cyanotoxins from *A. flos-aquae,* such as cylindrospermopsin, are recognized as potent inhibitors of GSH synthesis (Runnegar et al., 2002). Hou et al. (2015) indicated that GSH depletion in zebrafish that were treated with microcystins reveals the crucial role of GSH in cellular antioxidant protection and microcystin detoxification. Chen et al. (2012) also observed a decrease in GST activity and GSH concentration in the gills of zebrafish after administration of microcystins.

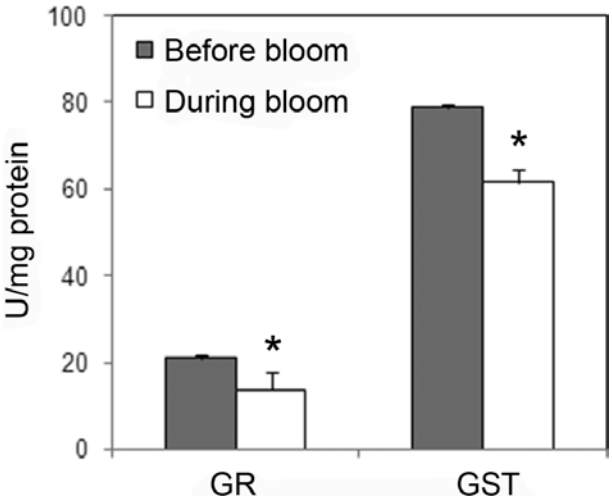

Figure 4: GR and GST activities (U/mg protein) in the gills of *P. fluviatilis* collected before and during the *A. flos-aquae* bloom in the Gruža Reservoir. Asterisk indicates statistically significant differences between samples from different investigated periods ($p<0.05$).

Presence of xenobiotics in water can cause oxidative modification of proteins in aquatic organisms (Borković-Mitić et al., 2016; Prokić et al., 2016). The concentration of –SH groups is a protein oxidation biomarker. Change in the concentration of –SH groups in the gills of *P. fluviatilis* (Figure

5) has not been observed in this study. Previous works revealed the prooxidant effect of *A. flos-aquae* bloom on the liver and muscle proteins in *P. fluviatilis* (Perendija et al., 2011; Gavrilović et al., 2015). No change in concentration of –SH groups in *P. fluviatilis* gills could be a consequence of this tissue's ability to better withstand protein oxidation caused by *A. flos-aquae* blooms compared to the liver and muscle. However, the complete antioxidant system that was investigated in this tissue was still significantly affected.

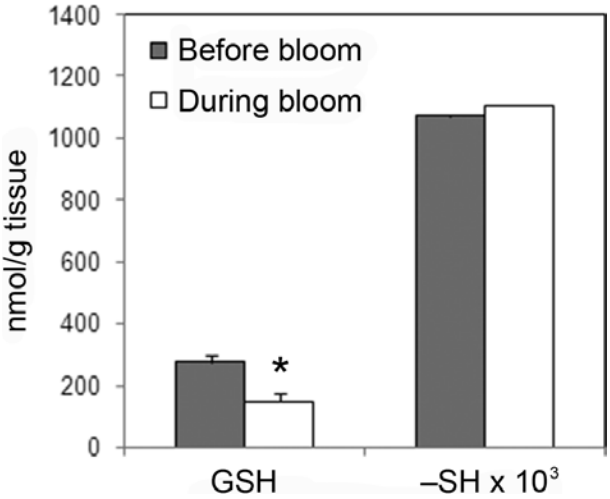

Figure 5: GSH and –SH groups concentrations (nmol/g tissue) in the gills of *P. fluviatilis* collected before and during the *A. flos-aquae* bloom in the Gruža Reservoir. Asterisk indicates statistically significant differences between samples from different investigated periods ($p<0.05$).

Using PCA, all the antioxidant parameters measured in the present study were distinguished on the ordination plots corresponding to the first (PC1) and second (PC2) principal components (Figure 6). PCA is used to estimate the correlation structure of the variables and to obtain more information on the variables that mainly influence the sample similarities and differences. PCA showed that PC1 and PC2 explain 76.13% of the total variance of data, with 61.10% for the first principal component and 15.03% for the second. PC1 which is more valuable for the explanation of variability is characterized by negative loading of CuZn SOD, Mn SOD, GR and GST activities and GSH concentration and positive loading of CAT and GSH-Px activities and –SH groups concentration. The obtained correlation structure of the variables is in accordance with the results presented in Figs. 2-5, since CuZn SOD, Mn SOD, GR, GST activities and GSH concentration have a similar decreasing pattern during the cyanobacterial bloom, while CAT and GSH-Px activities increased during the bloom. Furthermore, CuZn SOD, GR and GST activities were detected as the most important parameters for individual variations. However, despite the individual variations in the gill antioxidant parameters of *P. fluviatilis*, cyanobacterial blooms induced similar antioxidant responses in the species.

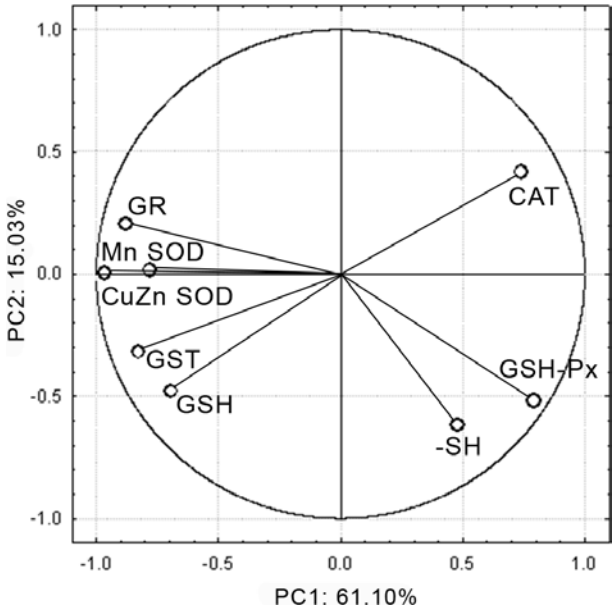

Figure 6: Ordination diagram of principal component analysis (PCA) of the antioxidant parameters (CuZn SOD, Mn SOD, CAT, GSH-Px, GR and GST activities and GSH and –SH groups concentrations) in the gills of *P. fluviatilis* collected before and during the *A. flos-aquae* bloom in the Gruža Reservoir.

Conclusions

Our findings demonstrate that the cyanobacterial bloom in the Gruža Reservoir disrupts oxidant/antioxidant balance in the gills of *P. fluviatilis*, leading to the induction of potential oxidative stress. *A. flos-aquae* bloom provoked an increase in CAT and GSH-Px activities, accompanied by depletion of CuZn SOD, Mn SOD, GR and GST activities and GSH concentration. CuZn SOD, GR and GST activities that were detected as the most important parameters for individual variations showed similar response to the bloom exposure. Characterization of the activities and concentrations of the antioxidant and biotransformation parameters in the gills of *P. fluviatilis* before and during the *A. flos-aquae* bloom in the Gruža Reservoir improved our understanding of the early biochemical and physiological responses of carnivorous fish to cyanobacterial blooms. Changes in the investigated parameters in *Perca fluviatilis* gills make them potential biomarkers for monitoring *Aphanizomenon flos-aquae* bloom exposure and useful tools in the research of ecological risk situations in freshwater ecosystems with frequent cyanobacterial blooms.

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