Arch. Biol. Sci., Belgrade, 63 (4), 979-989, 2011 DOI:10.2298/ABS1104979P

BIOCHEMICAL AND ULTRASTRUCTURAL CHANGES IN THE LIVER OF EUROPEAN PERCH (*PERCA FLUVIATILIS* L.) IN RESPONSE TO CYANOBACTERIAL BLOOM IN THE GRUŽA RESERVOIR

BRANKA R. PERENDIJA[1], SVETLANA G. DESPOTOVIĆ[1], TIJANA B. RADOVANOVIĆ[1], JELENA P. GAVRIĆ[1], SLAVICA S. BORKOVIĆ MITIĆ[1], S. Z. PAVLOVIĆ[1], BRANKA I. OGNJANOVIĆ[2], SNEŽANA B. SIMIĆ[2], SNEŽANA B. PAJOVIĆ[3], and ZORICA S. SAIČIĆ[1]*

[1]*Department of Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade,* 11060 Belgrade, Serbia
[2] *Institute of Biology and Ecology, Faculty of Science, University of Kragujevac,* 34000 Kragujevac, Serbia
[3] *Laboratory of Molecular Biology and Endocrinology, Institute of Nuclear Sciences "Vinča",* 11001 Belgrade, Serbia

Abstract – We investigated the biochemical and ultrastructural changes in the liver of the freshwater fish, European perch (*Perca fluviatilis*), in response to *Aphanizomenon flos-aquae* bloom in the Gruža Reservoir, Serbia. The activities of total manganese- and copper zinc-containing superoxide dismutase (Tot SOD, Mn-SOD, Cu/Zn-SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and biotransformation phase II enzyme glutathione-S-transferase (GST), as well as concentrations of total glutathione (GSH) and sulfhydryl (-SH) groups were examined before and during the bloom period. Mn-SOD activity was significantly higher, while the activities of Cu/Zn-SOD, CAT and GSH-Px and the concentration of the -SH groups were significantly lower during the bloom. The ultrastructure of the liver revealed necrotic and apoptotic damage to the hepatocytes during the bloom period. Our work represents the first study to report the influences of an *Aphanizomenon flos-aquae* bloom in the Gruža Reservoir on antioxidant biomarkers and on histopathological alterations in the liver of the freshwater fish European perch (*Perca fluviatilis*).

Key words: *Perca fluviatilis*, liver, oxidative stress, antioxidant biomarkers, ultrastructural changes, cyanobacterial bloom

UDC 597.556.331.1:511.5

INTRODUCTION

The occurrence of cyanobacterial blooms in freshwater ecosystems has been frequently reported around the world. The proliferation of cyanobacteria and the formation of blooms in lakes and reservoirs is often a consequence of eutrophication in these freshwater bodies. Some cyanobacterial species have the potential to produce toxic secondary metabolites and the negative impacts of cyanotoxins on living organisms are of particular concern for scientists all over the world (Qiu et al., 2007; Clemente et al., 2010).

Recent evidence suggests that exposure to different cyanobacterial toxins causes oxidative stress in various organisms, due to the overproduction of reactive oxygen species (ROS) and to the changes in the level of antioxidant compounds in cells (Moreno et al., 2005; Smith et al., 2008; Amado and Monserrat, 2010). The highly reactive properties of ROS make them a potential threat to cellular macromolecules, and if the initiated oxidation processes are not inhibited by the enzymatic and nonenzymatic components of the antioxidant defense system, damage to the DNA, lipid peroxidation and dysfunction

of enzymes can result in necrotic or apoptotic cells (Smith et al., 2008).

Aquatic organisms can be directly exposed to cyanotoxins and therefore they are very good models to study the influences of cyanobacterial blooms on the cellular antioxidant defense system. Fish can be exposed to cyanobacterial toxins via direct feeding on phytoplankton, through epithelial absorption of dissolved toxins after lysis of blooms or from exposure through the food web (Amado and Monserrat, 2010). There is much evidence of the biochemical and ultrastructural alterations in different tissues of fish after exposure to cyanotoxins under laboratory conditions, but data on the effects of cyanobacterial blooms in natural conditions are very limited (Qiu et al., 2007).

Oxidative stress biomarkers and histopathology studies are valuable tools to monitor the effects of cyanotoxins on fish, especially in the liver, because it is the general detoxifying organ and the most important organ involved in the regulation of redox metabolism. The liver is also the main region of ROS generation and the target organ for different cyanotoxins (Moreno et al., 2005; Qiu et al., 2007).

Aphanizomenon flos-aquae (L.) Ralfs. is a freshwater filamentous cyanobacterial species from the *Nostocales* order. It is considered to be a threat to aquatic organisms due to the production of a variety of toxic and bioactive compounds, including some hepatotoxins, which can cause DNA strand breaking and the inhibition of protein synthesis (Preußel et al., 2006) and different neurotoxins (Ferreira et al., 2001). During the summer period, cyanobacterial blooms caused predominantly by *A. flos-aquae* are observed in the Gruža Reservoir (Ranković and Simić, 2005).

The European perch (*Perca fluviatilis* L.) is a carnivorous freshwater fish found in Europe and Asia. It is a native fish widely distributed in Serbia and one of the dominant species present in the Gruža Reservoir. Juveniles feed on zooplankton, bottom invertebrate fauna and other perch fry, while adults feed on both invertebrates and fish.

In the present study *P. fluviatilis* was chosen as the test organism to investigate the influences of the *A. flos-aquae* bloom in the Gruža Reservoir on biochemical and ultrastructural parameters in liver. We determined the specific activity of antioxidant defense enzymes: total, manganese and copper zinc containing superoxide dismutase (Tot SOD, Mn-SOD, Cu/Zn-SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GSH-Px, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2) and biotransformation phase II enzyme glutathione-S-transferase (GST, EC 2.5.1.18), as well as concentrations of total glutathione (GSH) and sulfhydryl (-SH) groups. Histopathological examinations were studied using light and electron microscopy. All investigated parameters were measured before and during the cyanobacterial bloom.

Biomonitoring of the antioxidant biomarkers in fish could serve as an early warning signal of cellular damage resulting from exposure to cyanobacterial toxins in freshwater ecosystems. This study offers more information about the effects of cyanobacterial blooms on fish in connection with oxidative stress and ultrastructural changes in liver.

MATERIALS AND METHODS

Sampling site and fish collection

The Gruža Reservoir (43° 53’ 490” N, 20° 42’ 522” E) is one of the largest reservoirs in Serbia. It is characterized by a high primary production throughout the whole year, with a maximum of production during the summer period. A pronounced dominance of cyanobacteria and cyanobacterial blooms in the warmer period of the year indicates the eutrophic status of the reservoir (Ranković and Simić, 2005). In this study, measurements of environmental parameters as well as determination and quantification of the phytoplankton community were performed at the time of fish sampling.

The freshwater fish European perch (*Perca fluviatilis* L.) were caught by a local fisherman, before (28 specimens with the average length of 10.27 ± 0.27 cm and average mass of 14.13 ± 1.15 g) and during the bloom period (14 specimens with the average length of 15.32 ± 0.35 cm and average mass of 63.57 ± 3.57 g). The fish were brought alive to the laboratory where they were measured, weighed and immediately killed by a blow to the head. The liver was rapidly dissected out and frozen at -80°C for biochemical analysis. For histopathological study, the liver was immediately fixed in 10% neutral-buffered formalin until further processing.

Biochemical analysis

The liver was minced and homogenized in 5 vol. (Lionetto et al., 2003) of 25 mmol/L sucrose containing 10 mmol/L Tris-HCl, pH 7.5 at 4°C with an Ultra-Turrax homogenizer (Janke & Kunkel, IKA-Werk, Staufen, Germany), (Rossi et al., 1983). The homogenates were sonicated for 30s at 10 kHz on ice. One part of the sonicates was used for the determination of the concentration of GSH and centrifuged at 5,000 rpm for 10 min with 10% sulphosalicylic acid. The concentration of GSH was detected according to Griffith (1980) and expressed in nmol/g of tissue. Another part of the sonicates was centrifuged at 4°C at 100,000 *g* for 90 min (Takada et al., 1982) and the resulting supernatants were used for the measurement of enzyme activities and the concentration of -SH groups. The total protein concentration was determined according to the method of Lowry et al. (1951). Tot SOD activity was estimated by the epinephrine method (Misra and Fridovich, 1972), based on the capacity of SOD to inhibit the autooxidation of adrenaline to adrenochrome. The activity of Mn-SOD was obtained after the inhibition of Cu/Zn-SOD with KCN. Cu/Zn-SOD activity was calculated as the difference between Tot SOD and Mn-SOD. The activity of CAT was assayed by the method of Claiborne (1984), which is based on H_2O_2 degradation by the action of CAT contained in the examined samples. GSH-Px activity was evaluated following the oxidation of NADPH as a substrate with t-butyl hydroperoxide (Tamura et al., 1982). The activity of GR was estimated measuring NADPH oxidation as described by Glatzle et al. (1974). GST activity was detected by the procedure of Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. All enzyme activities were measured simultaneously in triplicate for each sample using a Nicolet Evolution 600 UV-Vis spectrophotometer and were expressed as specific in U/mg protein. The concentration of -SH groups was determined according to the method of Ellman (1959) and expressed in μmol/g of tissue.

Light and electron microscopy

All light and electron microscopy was performed at the Centre for Electron Microscopy, Faculty of Biology, University of Belgrade. For light microscopic analysis, the liver samples were fixed in 10% neutral buffered formaldehyde and routinely embedded in paraffin. Five-micron sections were deparaffinized, rehydrated and stained with propidium iodide for assessing hepatocyte apoptosis.

The liver samples were cut into small pieces, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), postfixed in 2% osmium tetroxide in the same buffer, dehydrated and embedded in Araldite. One-μm-thick sections were stained with basic fuchsine and methylene blue and observed under a Leica light microscope. Thin sections were cut by a Leica UC6 ultramicrotome, mounted on a copper grid, contrasted with uranyl acetate and lead citrate and observed with a Philips CM12 electron microscope (Eindhoven, The Netherlands).

Statistical analyses

The data are expressed as mean ± S.E. (standard error). The non-parametric Mann-Whitney U-test was used to search for significant differences between means. Significance of the results was ascertained at $p<0.05$. The analytical protocols described by Dinneen and Blakesley (1973) were followed.

Table 1. Physico-chemical parameters of water and quantification of the cyanobacterial community measured before and during the bloom period in the Gruža Reservoir.

	Before bloom	**During bloom**
Temperature (°C)	24.8	27.0
pH	8.86	8.76
O_2 (mg/L)	8.96	6.70
O_2 (%)	117.2	91.2
Nitrate (mg/L)	16.8	1.5
Phosphate (mg/L)	4.90	0.13
Ammonia (mg/L)	0.08	0.00
Total hardness	144	138
Conductivity (μS/cm)	288	278
Cyanobacteria (ind/L)	-	420 000

RESULTS

Physico-chemical parameters of the water and quantification of the cyanobacterial community in the Gruža Reservoir are shown in Table 1. Before the bloom period, no cyanobacterial individuals were detected in the fixed water samples, but during the bloom period, the analysis of water samples revealed a clear predominance of the cyanobacterial genera *Aphanizomenon*. The species *A. flos-aquae* was the predominant photoautotrophic constituent in the examined water samples.

The specific activities of Tot SOD, Mn-SOD and Cu/Zn-SOD are shown in Fig. 1. The results of our investigations revealed that during the bloom period, Mn-SOD activity was considerably higher, while the activity of Cu/Zn-SOD was considerably lower than in the period before the bloom. Tot SOD activity showed no discernible differences between the investigated periods. The examined specific activities of CAT and GSH-Px in the liver of *P. fluviatilis* were significantly decreased during the bloom period, while no differences were observed in the GR and GST activities (Fig. 2). No statistically significant difference was noticed for the concentration of total GSH, while the concentration of the -SH groups diminished significantly in the liver of *P. fluviatilis* during the cyanobacterial bloom (Fig. 3).

Propidium iodide staining showed that the bloom period induced apoptotic changes in the liver, resulting in a higher number of the hepatocytes' containg apoptotic nuclei (Fig. 4). Ultrastructural examination of the hepatocytes revealed a profound alteration of almost all membrane-bound organelles

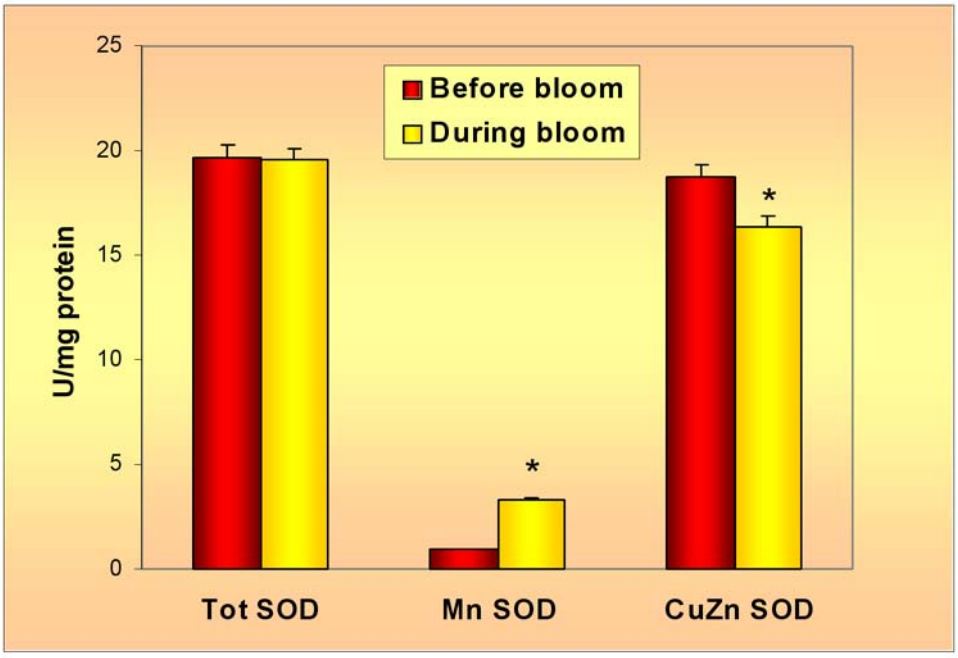

Fig. 1. Specific activities (U/mg protein) of total, manganese and copper zinc containing superoxide dismutase (Tot SOD, Mn-SOD and Cu/Zn-SOD) in the liver of European perch (*Perca fluviatilis*) before and during cyanobacterial bloom in the Gruža Reservoir. The data are expressed as mean ± S.E. The non-parametric Mann-Whitney *U*-test was used to seek significant differences between means. A minimum significance level of *p<0.05 was accepted.

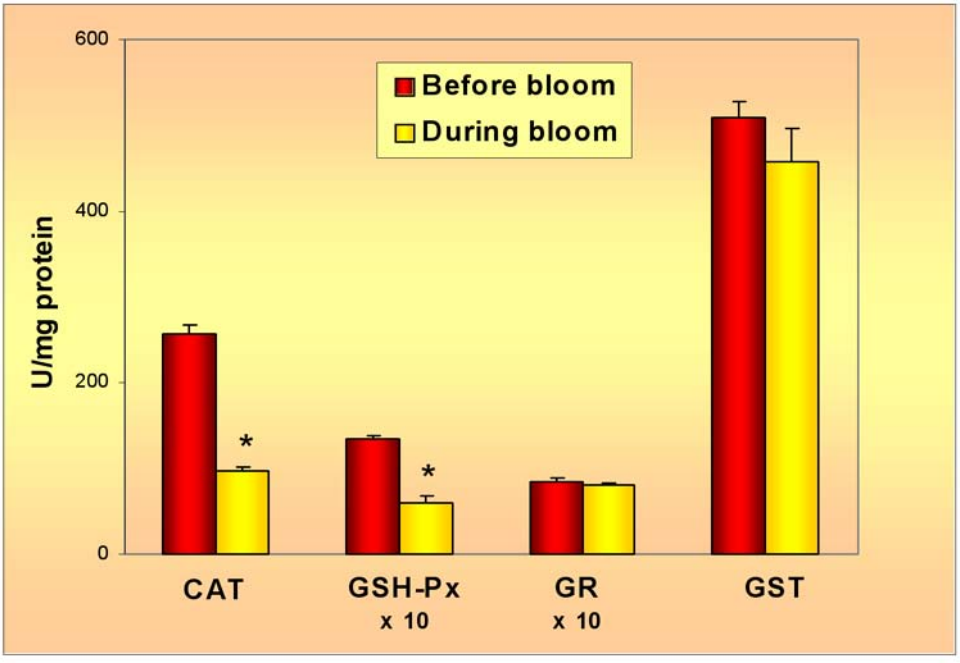

Fig. 2. Specific activities of catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and biotransformation phase II enzyme glutathione-S-transferase (GST) in the liver of European perch (*Perca fluviatilis*) before and during cyanobacterial bloom in the Gruža Reservoir. The data are expressed as the mean ± S.E. The non-parametric Mann-Whitney *U*-test was used to seek significant differences between means. A minimum significance level of *p<0.05 was accepted.

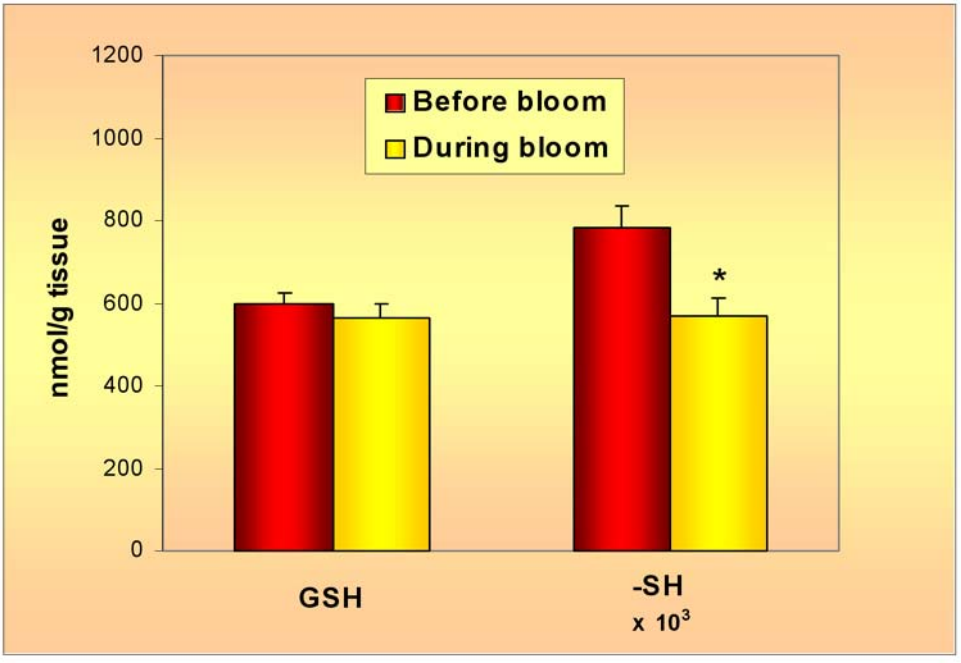

Fig. 3. Concentrations of total glutathione (GSH) and sulfhydryl (-SH) groups in the liver of European perch (*Perca fluviatilis*) before and during cyanobacterial bloom in the Gruža Reservoir. The data are expressed as mean ± S.E. The non-parametric Mann-Whitney *U*-test was used to seek significant differences between means. A minimum significance level of *p<0.05 was accepted.

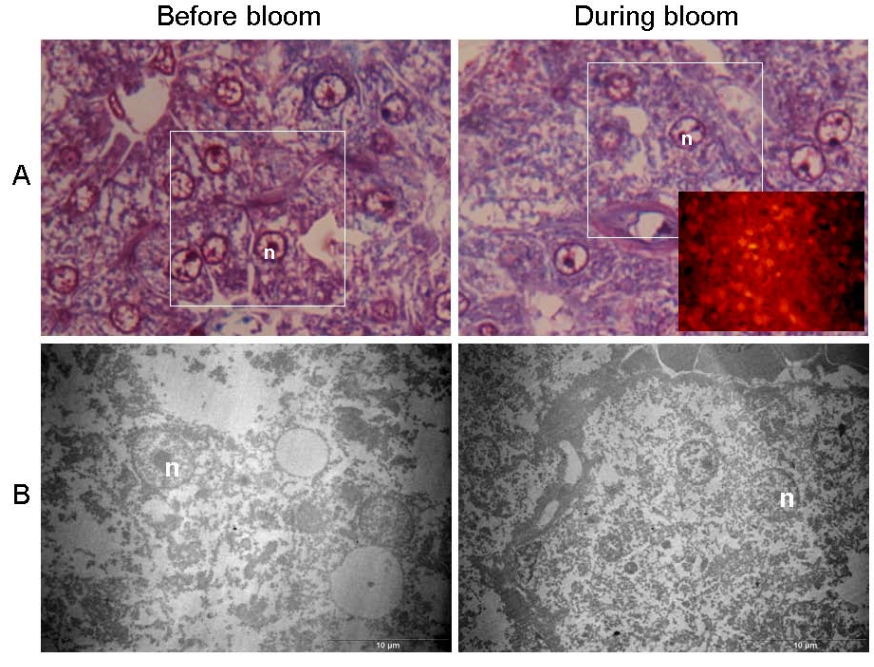

Fig. 4. Light (A) and electron (B) microscopy of the liver of European perch (*Perca fluviatilis*) before and during cyanobacterial bloom in the Gruža Reservoir. Propidium-iodide staining (inset) shows apoptotic changes during bloom period. Mag. x 100, orig. n -nucleus.

usually observed prior to cell death. More prominent changes were observed in the hepatocyte mitochondria. In addition, some hepatocytes showed signs of necrosis.

DISCUSSION

Cyanobacteria are known for mass development and blooms in eutrophic freshwater ecosystems worldwide. Some of these photosynthetic prokaryotes are capable of producing a variety of toxins, so that cyanobacterial blooms can have a negative impact on the health of animals living in aquatic ecosystems. It was previously shown that oxidative stress and alterations in the antioxidant biomarkers could be induced by various cyanotoxins in many animals, including fish (Smith et al., 2008; Amado and Monserrat, 2010; Silva et al., 2011). In spite of extensive studies of cyanotoxin-mediated deleterious effects, the reports on fish species have mainly been focused on examinations under laboratory conditions while, *in situ* studies have been limited (Qiu et al., 2007; Clemente et al., 2010).

The bloom-forming cyanobacteria *A. flos-aquae* have been identified as producing some secondary metabolites which display hepatotoxicity and neurotoxicity. Such compounds may significantly affect the antioxidant defense system and induce liver damage (Liu et al., 2006). In our study, *P. fluviatilis* was collected before and during an *A. flos-aquae* bloom in the Gruža Reservoir in order to investigate changes in the antioxidant biomarkers (specific activities of Tot SOD, Mn-SOD, Cu/Zn-SOD, CAT, GSH-Px, GR and GST, as well as concentrations of total GSH and -SH groups) and ultrastructural alterations in liver.

Antioxidant defense enzymes such as CAT and SOD act synergistically in the protection of cells by maintaining the cellular redox status (Kono and Fridovich 1982). The endogenous scavenger SOD catalyses the dismutation of the highly reactive superoxide anion ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2), while CAT is responsible for the elimination of H_2O_2. The activity of Tot SOD showed no significant changes in this study. We also determined the activities of two constitutive SOD isoforms, Cu/Zn-SOD and Mn-SOD. Although these enzymes catalyze the same reaction, they are structurally different and have different cellular location: Cu/Zn-SOD is found in the cytosol, while Mn-SOD is a mitochondrial enzyme (Pérez-Jiménez et al., 2009). However, there was a significant decrease in Cu/Zn-SOD activity, while the activity of Mn-SOD increased during the bloom period. The observed reduction in the activity of Cu/Zn-SOD in the liver of *P. fluviatilis* could lead to the accumulation of $O_2^{\bullet-}$ in the cell, contributing to cyanotoxin-induced liver toxicity. The increase in Mn-SOD activity in this study could be interpreted as an early adaptive response to oxidative stress in the mitochondrial compartment. Previous studies showed that oxidative substances in cells might cause an elevation of some antioxidant enzymes as a defense mechanism (Li et al., 2010; Pavlović et al., 2010).

The activity of CAT diminished in the liver of *P. fluviatilis* during the bloom period in the Gruža Reservoir. Our result agrees with reports by Atencio et al. (2008), whose investigations showed that microcystins generated oxidative stress in liver of Tenca fish (*Tinca tinca*) by decreasing the activity of antioxidant defense enzymes SOD and CAT. Kono and Fridovich (1982) revealed that the high production of $O_2^{\bullet-}$ inhibited CAT activity, so the reduction in the activity of SOD might be responsible for the depletion in the activity of CAT. Liu et al. (2006) also reported that the activities of SOD and CAT in the liver of mice injected with extracts of *A. flos-aquae* were lower than in the control animals. Decreased activities of SOD and CAT were also demonstrated in the livers of rats exposed to microcystin-LR (Moreno et al., 2005). However, the toxic effects of microcystins on the oxidative stress biomarkers in fish cell lines showed that CAT activity in cells treated with the toxin decreased significantly compared to the control, while the activity of SOD increased (Puerto et al., 2009). Some other authors obtained different results in the activity of CAT. Clemente et al. (2010) did not observe differences in CAT activity in the liver of freshwater fish *Geophagus brasiliensis* collected from the Alagados

Reservoir in Brazil, which was investigated in periods of low and high concentrations of the cyanobacteria *Cylindrospermopsis raciborskii*. In contrast, CAT activity in the liver of the silver carp (*Hypophthalmichthys molitrix*) was significantly higher during *Microcystis* blooms in Lake Taihu in China than in the periods before and after blooms (Li et al., 2007).

The depletion of GSH-Px activity in the liver of *P. fluviatilis* during the bloom period also was also observed. The antioxidant enzymes CAT and GSH-Px act cooperatively as scavengers of H_2O_2 and the concomitant decrease in the activity of both enzymes can cause the accumulation of H_2O_2 in the cells, thus creating oxidative damage. The diminished GSH-Px activity observed in the liver of *P. fluviatilis* corresponds with the results of Moreno et al. (2005), whose investigations showed a depletion of GSH-Px activity in the liver of rats exposed to microcystin-LR. However, Puerto et al. (2009) reported the enhancement of GSH-Px activity in fish cells treated with microcystins. Since GSH-Px is capable of reducing H_2O_2 to water and organic hydroperoxides to their corresponding alcohols utilizing GSH as a reducing equivalent, it is important to maintain the cytosolic concentration of reduced GSH. The biological function of GR is to preserve an intracellular reducing environment, which is critical to the cell against oxidative stress. This enzyme catalyzes the regeneration of the reduced form GSH from the oxidized form GSSG (Zhao et al., 2009; Li et al., 2010). The activity of GR observed in our study did not show significant changes in liver of *P. fluviatilis* as a response to cyanobacterial bloom. Our findings are in contrast with the results of some other studies, including the reduction of GR activity in fish cells treated with microcystins (Puerto et al., 2009), as well as GR depletion in the liver of rats exposed to microcystin-LR (Moreno et al., 2005).

The hepatic GSH concentration is a critical factor for maintaining a normal cellular redox balance and protecting the hepatocytes against oxidative stress (Zhao et al., 2009; Li et al., 2010). GSH is also a substrate in conjugation of a wide variety of electrophilic substrates, catalyzed by biotransformation phase II enzyme GST (Li et al., 2010). For the cyanobacterial toxin microcystin, biotransformation via GST by conjugation to GSH was reported for different aquatic organisms, including fish, crustaceans and molluscs (Pflugmacher et al., 1998). In our study, no significant changes were observed in the activity of GST or in the concentration of total GSH in the liver of *P. fluviatilis* between the two investigated periods in the Gruža Reservoir. Qiu et al. (2007) obtained similar results for GSH concentration in the liver of four fishes from Lake Taihu in China, while Atencio et al. (2008) also showed no discernible changes in the GSH level in the *T. tinca* liver after exposure to cyanobacterial cells containing microcystins. In contrast to our results, a decreased GSH concentration and an increased GST activity were found in the liver of mice injected with extracts of *A. flos-aquae*, compared to the control animals (Liu et al., 2006), as well as in the liver of the freshwater fish *H. molitrix* after injection with extracted hepatotoxic microcystins (Li et al., 2007). The results of Qiu et al. (2007) were also at variance with our results as an increase in GST activity was observed in the livers of four fish species during *Microcystis* blooms in Lake Taihu in China.

In the present study, the *A. flos-aquae* bloom caused the depletion of hepatic -SH concentration in *P. fluviatilis*, suggesting an environmental impact on -SH group metabolism. The homeostasis of -SH groups is a very important factor for the maintenance of the cellular redox status. Earlier studies have shown that alterations in the concentration of -SH groups can affect the structure and function of proteins and induce changes in antioxidant defense enzyme activities (Kovačević et al., 2006).

Our findings suggest that the *A. flos-aquae* bloom induced oxidative stress in terms of decreased Cu/Zn-SOD, CAT and GSH-Px activities, as well as concentration of -SH groups, which could result in an increased vulnerability of the cell to ROS. The insufficiency of antioxidant defense mechanisms could, in sensitive fish species, potentially result in effects

on the health status, especially if other stressors are involved at the same time, which was the case in the eutrophic freshwater ecosystems. Although the cyanobacterial bloom caused biochemical disturbances in fish liver, the GR and GST activities and concentration of total GSH remained unaltered, showing that these biomarkers were not for providing evidence of hepatocellular injury in *P. fluviatilis* as a response to the *A. flos-aquae* bloom.

The ultrastructural examinations in this study showed that the hepatocytes of *P. fluviatilis* revealed profound changes of almost all membrane-bound organelles during the cyanobacterial bloom. The alterations to the endomembrane system were quite pronounced and the hepatocytes displayed degenerated organelles and morphologic changes in the nuclei, which resulted in the death of cells. Some hepatocytes showed signs of necrosis, while propidium iodide staining revealed that the bloom period induced apoptotic changes in the liver, resulting in a higher number of apoptotic nuclei in the hepatocytes of *P. fluviatilis*.

Underdal et al. (1999) showed that the liver of mice treated with saline extracts of *A. flos-aquae* exhibited necrotizing hepatocytic damage, with swollen and degenerated mitochondria and cytoplasmic and nuclear vacuolation. In contrast, Liu et al. (2006) showed that the livers of mice injected with extracts of *A. flos-aquae* were not significantly different from those of the controls. Qiu et al. (2007) examined ultrastructural changes in the livers of four fishes with different trophic levels exposed to toxic cyanobacterial blooms in a large Chinese lake. During the cyanobacterial bloom, carnivorous fish exhibited the most serious injury to the hepatocytes, e.g. swollen endomembrane system and morphologically altered nuclei. It should be pointed out that the freshwater fish *P. fluviatilis* in our study is also a carnivorous species, which might be responsible for its low resistance to cyanobacterial bloom.

In conclusion, the results of present investigation show that a *A. flos-aquae* bloom is capable of inducing an oxidative stress response in fish, linked with an inhibition of the majority of the investigated antioxidant biomarkers. This study indicates that the antioxidant defense system of fish could provide useful information for the biomonitoring study of cyanobacterial influences on organisms that live in aquatic environments. Different antioxidant parameters are included in this study, but the activities of Cu/Zn-SOD, Mn-SOD, CAT and GSH-Px, as well as the concentration of -SH groups, seemed to be the most potent biomarkers in the liver of the freshwater fish *P. fluviatilis* exposed to a cyanobacterial bloom. The present results also establish the capacity of an *A. flos-aquae* bloom to induce ultrastructural alterations in the liver and therefore its toxicity potential to fish. To our knowledge, this is the first study to report the influences of an *Aphanizomenon flos-aquae* bloom in the Gruža Reservoir on antioxidant biomarkers and on the histopathological changes in the liver of the freshwater fish European perch (*Perca fluviatilis*).

Acknowledgements - This study was supported by the Ministry of Education and Science of the Republic of Serbia, Grants Nos. 173041 and III 43002. The authors are thankful to Radmila Paunović-Štajn, MSc., for proofreading the manuscript.

REFERENCES

Amado, L. L., and *J. M. Monserrat* (2010). Oxidative stress generation by microcystins in aquatic animals: Why and how. *Environ. Inter.* **36,** 226-235.

Atencio, L., Moreno, I., Jos, A., Pichardo, S., Moyano, R., Blanco, A., and *A. M. Camean* (2008). Dose-dependent antioxidant responses and pathological changes in tenca (*Tinca tinca*) after acute oral exposure to *Microcystis* under laboratory conditions. *Toxicon* **52**, 1-12.

Claiborne, A. (1984). Catalase activity. In: Handbook of Methods for Oxygen Radical Research, 9 (Ed. R. A. Greenwald), 283-284. CRC Press Inc., Boca Raton.

Clemente, Z., Busato, R. H., Ribeiro, C. A. O., Cestari, M. M., Ramsdorf, W. A., Magalhaes, V. F., Wosiack, A. C., and *H. C. S. Assis* (2010). Analyses of paralytic shellfish toxins and biomarkers in a southern Brazilian reservoir. *Toxicon* **55**, 369-406.

Dinneen, L. C., and *B. C. Blackesley* (1973). A generator for the sampling distribution of the Mann Whitney U statistic. *Appl. Stat.* **22,** 269-273.

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70-77.

Ferreira, F. M. B., Soler, J. M. F., Fidalgo, M. L., and *P. Fernández-Vila* (2001). PSP toxins from *Aphanizomenon flos-aquae* (cyanobacteria) collected in the Crestuma-Lever reservoir (Douro river, northern Portugal). *Toxicon* **39**, 757-761.

Glatzle, D., Vulliemuier, J. P., Weber, F., and *K. Decker* (1974). Glutathione reductase test with whole blood a convenient procedure for the assesment of the riboflavin status in humans. *Experientia* **30**, 665-667.

Griffith, O. W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* **106**, 207-212.

Habig, W. H., Pubst, M. J., and *W. B. Jakoby* (1974). Glutathione S-transferase. *J. Biol. Chem.* **249**, 7130-7139.

Kono, Y., and *I. Fridovich* (1982). Superoxide radical inhibits catalase. *J. Biol. Chem.* **257**, 5751-5754.

Kovačević, T. B., Borković Mitić, S. S., Pavlović, S. Z., Radojičić, R. M., and *Z. S. Saičić* (2006). The concentrations of antioxidant compounds in the hepatopancreas, the gills and muscle of some freshwater crayfish species. *Acta Biol. Hung.* **57**, 449-458.

Li, L., Xie, P., Li, S., Qiu, T., and *L. Guo* (2007). Sequential ultrastructural and biochemical changes induced *in vivo* by the hepatotoxic microcystins in liver of the phytoplanktivorous silver carp *Hypophthalmichthys molitrix*. *Comp. Biochem. Physiol.* C **146**, 357-367.

Li, Z., Zlabek, V., Grabic, R., Li, P., and *T. Randak* (2010). Modulation of glutathione-related antioxidant defense system of fish chronically treated by the fungicide propiconazole. *Comp. Biochem. Physiol.* C **152**, 392-398.

Lionetto, M. G., Caricato R., Giordano, M. E., Pascariello M. F., Marinosci, L., and *T. Schettino* (2003). Integrated use of biomarkers (acetylcholinesterase and antioxidant enzyme activities) in *Mytilus galloprovincialis* and *Mullus barbatus* in an Italian coastal marine area. *Mar. Poll. Bull.* **46**, 324-330.

Liu, Y., Chen, W., Li, D., Shen, Y., Li, G., and *Y. Liu* (2006). First report of aphantoxins in China-waterblooms of toxigenic *Aphanizomenon flos-aquae* in Lake Dianchi. *Ecotoxicol. Environ. Saf.* **65**, 84-92.

Lowry, O. H., Rosebrough, N. L., Farr, A. L., and *R. I. Randall* (1951). Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Misra, H. P., and *I. Fridovich* (1972). The role of superoxide anion in the autoxidation of epinephrine and simple assay for superoxide dismutase. *J. Biol. Chem.* **247**, 3170-3175.

Moreno, I., Pichardo, S., Jos, A., Gómez-Amores, L., Mate, A., Vazquez, C. M., and *A. M. Cameán* (2005). Antioxidant enzyme activity and lipid peroxidation in liver and kidney of rats exposed to microcystin-LR administered intraperitoneally. *Toxicon*, **45**, 395-402.

Pavlović, S. Z., Borković Mitić, S. S., Radovanović, T. B., Perendija, B. R., Despotović, S. G., Gavrić, J. P., and *Z. S. Saičić* (2010). Seasonal variations of the activity of antioxidant defense enzymes in the red mullet (*Mullus barbatus* L.) from the Adriatic Sea. *Mar. Drugs* **8**, 413-428.

Pérez-Jiménez, A., Hidalgo, M. C., Morales, A. E., Arizcun, M., Abellán, E., and *G. Cardente* (2009). Antioxidant enzymatic defenses and oxidative damage in *Dentex dentex* fed on different dietary macronutrient levels. *Comp. Biochem. Physiol.* C **150**, 537-545.

Pflugmacher, S., Wiegand, C., Obermm, A., Beattie, K. A., Krause, E., Codd, G. A., and *C. E. V. Steinberg* (1998). Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. *Biochim. Biophys. Acta* **1425**, 527-533.

Preußel, K., Stüken, A., Wiedner, C., Chorus, I., and *J. Fastner* (2006). First report on cylindrospermopsin producing *Aphanizomenon flos-aquae* (Cyanobacteria) isolated from two German lakes. *Toxicon* **47**, 156-162.

Puerto, M., Pichardo, S., Jos, A., and *A. M. Camean* (2009). Oxidative stress induced by microcystin-LR on PLHC-1 fish cell line. *Toxicol. Vitro* **23**, 1445-1449.

Qiu, T., Xie, P., Ke, Z., Li, L., and *L. Guo* (2007). In situ studies on physiological and biochemical responses of four fishes with different trophic levels to toxic cyanobacterial blooms in a large Chinese lake. *Toxicon* **50**, 365-376.

Ranković, B., and S. Simić (2005). Fitoplankton akumulacionog jezera Gruža. In: Akumulaciono jezero Gruža (Eds. Lj. Čomić and A. Ostojić), 65-78. Faculty of Science, Kragujevac.

Rossi, M. A., Cecchini, G., and *M. M. Dianzani* (1983). Glutathione peroxidase, glutathione reductase and glutathione transferase in two different hepatomas and in normal liver. IRCS *Med. Sci. Biochem.* **11**, 805.

Silva, C. A., Oba, E. T., Ramsdorf, W. A., Magalhães, V. F., Cestari, M. M., Ribeiro, C. A. O., and *H. C. S. Assis* (2011). First report about saxitoxins in freshwater fish *Hoplias malabaricus* through trophic exposure. *Toxicon* **57**, 141-147.

Smith, J. L., Boyer, G. L., and *P. V. Zimba* (2008). A review of cyanobacterial odours and bioactive metabolites: Impacts and management alternatives in aquaculture. *Aquaculture* **280**, 5-20.

Takada, Y., Noguchit, T., and *M. Kayiyama* (1982). Superoxide dismutase in various tissues from rabbits bearing the Vx-2 carcinoma in the maxillary sinus. *Cancer Res.* **42**, 4233-4235.

Tamura, M., Oschino, N., and *B. Chance* (1982). Some characteristics of hydrogen and alkyl hydroperoxides metabolizing systems in cardiac tissue. *J. Biochem.* **92**, 1019-1031.

Underdal, B., Nordstoga, K. and *O. M. Skulberg* (1999). Protracted toxic effects caused by saline extracts of *Aphanizomenon flos-aquae* (Cyanophyceae/Cyanobacteria). *Aquat. Toxicol.* **46**, 269-278.

Zhao, Y., Seefeldt, T., Chen, W., Wang, X., Matthees, D., Hu, Y., and *X. Guan* (2009). Effects of glutathione reductase inhibition on cellular thiol redox state and related systems. *Arch. Biochem. Biophysic.* **485**, 56-62.