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Original paper

Harmful effects of cyanobacterial blooms in Lake Ludaš (Serbia) on *Carassius gibelio* tissues

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Summary. Lake Ludaš is a protected nature reserve in Serbia that has been the focus of scientific interest, due to perpetual water quality deterioration that has led to consistent cyanobacterial blooms for almost five decades. To describe the possible effects of cyanotoxins on fish (*Carrasius gibelio*) inhabiting Lake Ludaš, several examinations were performed in November of 2018, including: assessment of water quality (physical and chemical parameters, as well as water microbiology), and assessment of several tissues of *C. gibelio* (micronucleus test, comet assay, and histopathological analyses). The water quality of Lake Ludaš has been categorized as class III, indicating moderate pollution. Physical and chemical parameters from November of 2018 seemed to be within acceptable levels. Examined fish tissues showed signs of DNA damage (highest in the blood, followed by the liver and gills), and histological alterations (in the liver and gills). Although other xenobiotics can induce similar changes, cyanotoxins could be the most likely cause of the observed alterations in *C. gibelio* tissues. The present study demonstrates the need to monitor this important wetland of international significance, since the living organisms in Lake Ludaš are showing signs of environmental stress suggesting that the preservation and natural balance of this ecosystem could be in danger.

Keywords: comet assay, cyanobacteria, cyanotoxins, fish, histopathology, micronucleus.

INTRODUCTION

Lake Ludaš, is an aquatic ecosystem that has been protected as a special nature reserve in Serbia for almost 70 years. However, anthropogenic activities have resulted in deterioration of this important aquatic ecosystem, which has led to consistent cyanobacterial blooms over the past 50 years (e.g. Seleši 1981; Dulić and Mrkić 1999; Simeunović 2009; Tokodi et al. 2018, 2020). One outcome of these blooms is production of cyanotoxins, secondary metabolites of cyanobacteria, which has been documented in several publications over the years (Simeunović 2009; Tokodi et al. 2018,

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2020). These toxic metabolites can exhibit their effects on all organisms in and around the aquatic ecosystem, including fish, via cyanotoxin accumulation in tissues, DNA damage, as well as alterations in histology (Drobac et al. 2016; Tokodi et al. 2018, 2020).

Detection of DNA damage is the most common method used to assess ecogenotoxicity: either by detection of DNA strand breaks using the comet assay, or detection of chromosome breakage by the micronucleus test. Blood is often used as the tissue of first choice, due to its high sensitivity, ease of manipulation, and the fact that cellular dissociation is not required. The liver is also often chosen because of its role in antioxidant defense and generally strong metabolic index. On the other hand, gills are of interest as they represent a soft tissue which is in direct contact with water, and which has an increased sensitivity to the mixtures of xenobiotics in it due to its unique role in respiration (Mallatt 1985).

Histopathology is a very important component of ecotoxicological research and environment monitoring (Van der Oost et al. 2003). The most important advantage of this method is its ability to clearly visualize changes within cells, tissues and organs, and thus indicate possible consequences on other levels of organization (Wester et al. 2002; Chiang and Au 2013). Since histological changes can also occur after exposure to very low concentrations of xenobiotics, these biomarkers are very important for early identification of damage to individual organs and potential health hazards (Chiang and Au 2013).

During our previous research on Lake Ludaš, the following dominant cyanobacterial species were detected: *Limnothrix redekei*, *Pseudanabaena limnetica*, *Microcystis aeruginosa*, *Planktothrix agardhii* and *Microcystis wesenbergii* (Tokodi et al. 2018, 2020). Because microcystin (*mcyE*) and saxitoxin (*sxtG* and *sxtS*) coding genes were detected in biomass samples (Tokodi et al. 2020), the presence of microcystins (MC-LR, dmMC-LR, MC-RR, dmMC-RR, MC-LF), and saxitoxins (STX) was not surprising (Tokodi et al. 2018, 2020). MC-RR was previously found in the reed rhizomes (*Phragmites communis*), cattails (*Typha latifolia*) and royal blue water lilies (*Nymphaea elegans*), as well as in muscle and gonad tissues of Prussian carp (*Carassius gibelio*) caught from the lake; whereas slightly higher concentrations of MC-LR were detected in their kidneys and intestines (Tokodi et al. 2018). Furthermore, histopathological alterations in fish liver, kidney, gills and intestines were observed (Tokodi et al. 2018, 2020), as well as DNA damage in the liver and gills of exposed fish (Tokodi et al. 2018). Thus, microcystin bioaccumulation and exposure can cause potential mutagenic effects in fish from Lake Ludaš.

Given that excessive blooming still regularly occurs in Lake Ludaš, further investigation was performed in November of 2018, mostly focusing on the analysis of fish tissues in order to describe the harmful health effects of cyanobacterial blooms on these aquatic organisms. Therefore, the goal of the present investigation was to describe possible cyanotoxin effects on fish tissues via assessment of water quality: (1) physical and chemical parameters of the water; and (2) microbiology of the water; as well as assessment of several tissues of *Carrasius gibelio* through: (3) micronucleus assay; (4) comet assay; and (5) histopathological analyses.

MATERIALS AND METHODS

Fish sampling from natural populations conducted on Lake Ludaš during this study was approved by the Institute for nature conservation of Vojvodina province (Republic of Serbia) (permit number: 140-501-1316 / 2017-04).

Sampling

In November of 2018, water was sampled from the pier (46.103207°N, 19.821360°E) and center of Lake Ludaš (46.102159°N, 19.821149°E). Fish were sampled from the center of the lake using a standard electrofishing device and then sacrificed. Eight specimens of *Carrasius gibelio* (TL: 380 ± 26) were sampled and their liver, gills, spleen, intestine, gonads (ovaries and testes) and muscles were collected and fixed in 10% neutral-buffered formalin (NBF) for histopathological analyses. As a control, *Cyprinus carpio* specimens from the regular broodstock of the Department of Aquaculture, Hungarian University of Agriculture and Life Sciences, Hungary, were used. Fish were fed twice a day with commercial feed rich in proteins and were kept at 24 ± 1 °C, in a 12 h light/12 h dark cycle.

Analyses of water

Physical and chemical parameters

For *in situ* measurements in the field, multi-parameter WTW probes were used and the following physical and chemical parameters were determined: temperature, conductivity, O_2 concentration and O_2 saturation. TSS (total suspended solids), TOC (total organic carbon), NO_3, detergents, COD (chemical oxygen demand) and BOD (biological oxygen demand) were measured under laboratory conditions with a Pastel Ultraviolet (UV) Secomam.

Microbiology of water

Defined Substrate Technology was used to detect total coliforms (TC), *Escherichia coli* (EC) and enterococci (FE). This enables isolation and identification of certain groups of bacteria by enzymatic hydrolysis of specific substrates (Ed-

berg et al. 1990; Buckalew et al. 2006; Griffith et al. 2006). Quantification was performed with a Colilert Quanti-Tray 2000 system, which provides a Most Probable Number (MPN) result, based on color/fluorescence changes in 97 wells. For TC and EC, two dilutions were analysed (1:10 and 1:1000); while for FE only one dilution was analysed (1:10). Powdered reagents Colilert-18 and *E*-enterolert were used for cultivation of coliforms and enterococci respectively. A diluted water sample was mixed with powdered reagents and poured into a Quanty-Tray, a sterile plastic disposable 97-well tray. For TC and EC, trays were incubated at 37 °C for at least 18 h. FE were incubated for at least 24 h at 44 °C.

After incubation, the appearance of yellow color in the wells was indicative of the presence of TC. Under UV illumination at 365 nm, fluorescence in wells was used as an indicator of the presence of EC, in Colilert, and FE in Enterolert-E. The number of positive wells was scored and converted to MPN using tables provided by the manufacturer. Assessment of water quality was based on total coliforms, *E. coli* and enterococci classification scheme suggested by Kirschner et al. (2009) and was expressed as MPN/100 mL.

Analyses of fish

Genotoxicity – micronucleus and comet assay

Eight specimens of *Carrasius gibelio* were sampled for genotoxocity analysis. Blood was used for detection of chromosome aberrations with a micronucleus test, while blood, liver and gills were used for detection of DNA damage using a comet assay.

Micronucleus test

For micronucleus analysis, about 100 µL of blood was smeared on a glass slide directly on site. After drying (1 h), microscopic slides were submerged in cold methanol for 30 min to enable fixation. Smears were stained with 20 µL of acridine orange (final concentration 2 µg/mL) and observed under a fluorescence microscope (Leica, DMLS, Germany). Micronucleus was determined as a structure which is 1/16 to 1/3 of the main nucleus (Fenech et al. 2003). Micronuclei were analysed in 2000 randomly selected cells.

Comet assay

Cell suspensions of the liver and gills were prepared based on a protocol described in Sunjog et al. (2014). Briefly, tissue samples were cut into small pieces in Hank's Balanced Saline Solution (HBSS) and exposed to trypsin (final concentration 0.05%, 10 min, room temperature). Trypsin digestion was stopped by addition of HBSS medium. Suspensions of blood, liver, and gills cells were centrifuged (455 ×*g* for 10 min) and diluted in HBSS to obtain approximately 50,000 cells/mL. Cell viability was assessed by differential acridine orange/ethidium bromide (AO/EB) staining (Squier and Cohen 2001). Samples showing more than 70% cell viability were further processed for the comet assay.

The alkaline comet assay was performed as described in a previous study by Sunjog et al. (2014). Briefly, slides precoated with two layers of the 1% NMP (normal melting point) agarose were covered with 70 µL of 1% LMP (low melting point) agarose mixed with 30 µL of cell suspensions. For each cell type, one slide was prepared per specimen. Slides were immersed in freshly made, ice-cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO- for blood cells, pH 10) and held at 4 °C for 16-18 h. After lysis, slides were covered with cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) to enable denaturation (20 min, 4 °C) and electrophoresis was performed (0.75 V/cm, 300 mA, 20 min, 4 °C). The slides were neutralised (0.4 M Tris, pH 7.5, 15 min, 4 °C) and fixed in cold methanol (15 min, 4 °C). Slides were stained using acridine orange (final concentration 2 µg/mL) and examined under a fluorescence microscope (Leica, DMLS, Germany). A total of 50 comets for each sample were randomly scored and analysed by the Comet IV computer software (Perceptive Instruments, UK). Tail intensity (% of DNA in the comet tail; TI) was used to express the level of DNA damage.

Histopathology

For histological analyses, tissue samples from several organs (liver, gills, intestine, spleen, ovaries, testes and muscles) were collected and fixed in 10% NBF. Samples were processed by a standard histological procedure as described in our previous studies (Tokodi et al. 2020; Drobac Backović et al. 2021; Marinović et al. 2021). Gill and muscle samples were decalcified with 75% rapid decalcifier solution (Apex Engineering Products Corporation) before histological processing, which included dehydration of samples in a graded ethanol series, clearing in xylol and subsequently embedding in paraffin wax blocks. Three 5 µm thin sections per tissue per specimen were cut and placed onto glass slides and stained with a standard haematoxylin and eosin (H&E) staining procedure. The sections were examined under a microscope (Nikon Eclipse 600) and photographed (QImaging Micro Publisher 3.0 digital camera).

RESULTS

Assessment of water quality

Electrical conductivity was high, while remaining parameters were rather low, with slightly higher values measured in the center of the lake, compared to pier samples (Table 1).

Table 1. Physical and chemical parameters of water from Lake Ludoš in November 2018.

Physical and chemical parameters	pier	center
temperature (˚C), *in situ*	10.8	10.8
concentration O_2, *in situ* (mg/l)	9.83	11.9
saturation O_2, *in situ* (%)	94.7	104.6
conductivity, *in situ* (μS/cm)	1066	1060
TSS (mg/dm^3)	17.2	26.8
TOC (mg/dm^3)	11.5	11.9
NO_3 (mg/dm^3)	≤0.5	≤0.5
detergents (mg/dm^3)	2.9	3.1
COD (mgO_2/dm^3)	32.5	34.0
BOD (mgO_2/dm^3)	16.6	17.2

TSS = total suspended solids; TOC = total organic carbon; COD = chemical oxygen demand; BOD = biological oxygen demand.

Water microbiology

The numbers of TC, FE, and EC were assessed, as these groups are included in the national regulations of Serbia in assessing the ecological status of surface waters (Rulebook, Official Gazzete 74/2011). TC was estimated at 48800/100 mL and FE at 2014/100 mL. EC was not detected. According to these numbers, Lake Ludaš belongs to III class ecological status.

Assessment of fish tissue damage

Micronucleus

The overall frequency of MN was low, ranging from 0.5‰ to 1‰. Moreover, there was no significant correlation between the frequency of MN in the blood and values obtained from alkaline comet assay of the same tissue.

Alkaline comet assay

Data obtained by alkaline comet assay indicated variation of DNA damage within the investigated tissues (Fig. 1). Statistically significant highest damage was obtained in blood (TI% was 37.24 ± 15.54, mean ± SD), followed by the liver (TI% was 23.93 ± 4.79, mean ± SD), and gills (TI% was 16.99 ± 6.71, mean ± SD).

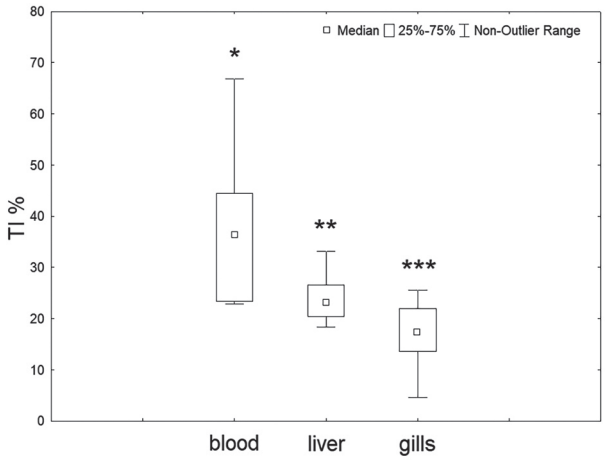

Fig. 1. Box plots representing DNA damage (TI%) of blood, liver and gills in *Carrasius gibelio*; *, **, *** show the statistical difference in DNA damage between the tissues.

Histopathology of fish tissues

Control specimens raised at the Department of Aquaculture, Hungarian University of Agriculture and Life Sciences displayed normal histological structure in all organs (Fig. 2A, D). Livers and gills of fish caught from Lake Ludaš displayed several histopathological alterations. Livers predominantly displayed a normal histological structure having a cord-like parenchymal structure and polygonal hepatocyte. However, in several cases the cord-like structure was disrupted and cells lost their polygonal shape (Fig. 2B). Hepatocytes were much larger than control hepatocytes and displayed intensive hypertrophy and vacuolization (Fig. 2B). Nuclei were frequently condensed, the nucleoli were not visible, and in some cases, pyknosis was observed (Fig. 2C).

Gills displayed more intense alterations. Proliferations of the interlamellar cell mass and lifting of the primary and secondary epithelium were frequently observed (Fig. 2E). In some cases, we also observed circulatory disturbances in the form of telangiectasia (Fig. 2F) and hemorrhage. The intestines, spleen, gonads (ovaries and testes) and muscles of fish caught from Lake Ludaš displayed no histopathological alterations.

DISCUSSION

Although the physical and chemical parameters in samples collected in November 2018 do not seem alarming, measurements from our previous research (Tokodi et al. 2020) indicate that during 2018 the pH has been mostly high (almost 9), O_2 saturation showed high values with hypersaturation in July, and as was noted in November, electrical conductivity remained high throughout the entire year. Similar findings were reported in recent years by the Institute

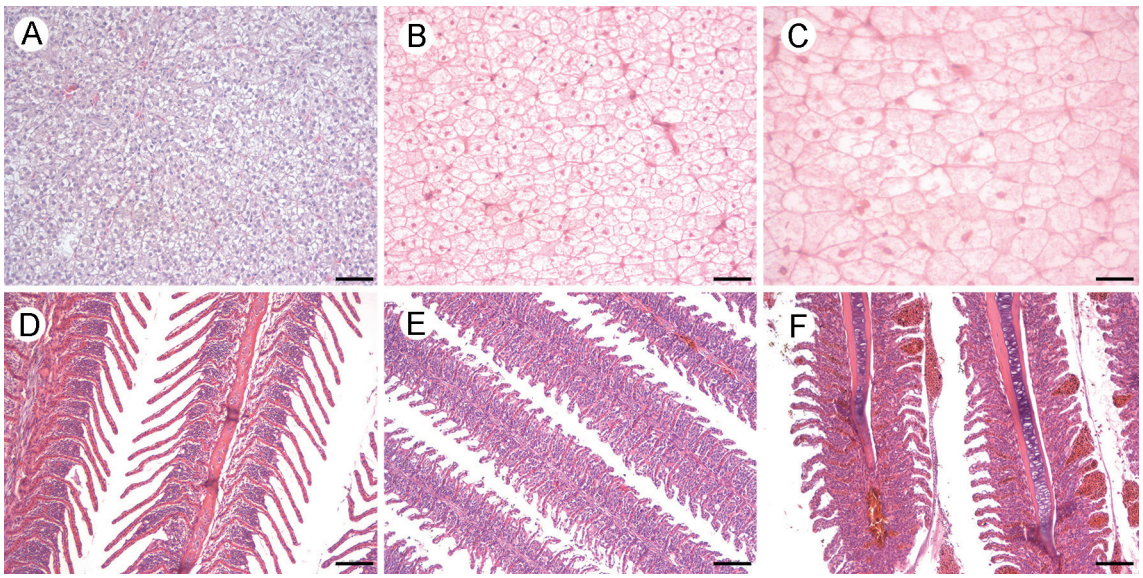

Fig. 2. Histological observation of liver (**A**-**C**) and gills (**D**-**F**) of control fish (**A**, **D**) and fish caught from Lake Ludaš in November 2018 (**B**, **C**, **E**, **F**). (**A**) Control specimens displayed normal hepatic structure. Fish caught from Lake Ludaš displayed a slight loss of the cord-like parenchymal structure and hepatocyte hypertrophy (**B**) as well as condensation of nuclei (**C**). (**D**) Control specimens displayed a normal gill structure. Fish from Lake Ludaš showed epithelial hyperplasia and lifting (**E**) as well as telangiectasia (**F**). H&E staining. (Scale bars: D-F = 100 μm; A, B = 50 μm; C = 25 μm.)

for Public Health, Subotica that performs regular monitoring of Lake Ludaš (Institute for public health, Subotica, 2019). According to the investigated microbiological parameters, the ecological status of Lake Ludaš can be categorized as class III, which is an indication of moderate pollution. With the absence of EC in the water, it can be assumed that there was no recent influx of fecal contamination since EC represents the main indicator of recent fecal pollution. Furthermore, both annual reports on water quality, as well as our previous research (Tokodi et al. 2018, 2020), demonstrate continuous cyanobacterial blooming and the presence of several potentially toxic as well as invasive cyanobacterial species. Additionally, in previous years and earlier in 2018, several microcystin (MC) variants and low concentrations of saxitoxins (STX) were also reported in the water, and MC and STX coding genes in biomass samples (Tokodi et al. 2018, 2020). Even though the specific values of cyanotoxins in the Lake Ludaš were not analyzed at the moment of fish sampling during the present study, their presence was noted in previous studies (e.g. Svirčev et al. 2014, 2017; Tokodi et al. 2018, 2020). Earlier generated data clearly shows that cyanotoxins were constantly present, including the year when fish were sampled and analyzed, and therefore could have had an impact on fish tissues, especially since harmful effects in the biota are developed over time. Hence, potential effects found in fish do not represent only the current situation, but are a consequence of long-term exposure, as evidenced by earlier literature.

The absence of correlation between results from our comet assay and micronucleus tests is not surprising, given that such discrepancies are common in environmental conditions during monitoring of water pollution. Strand breakage (measured in the comet assay), and chromosome breakage (measured in the micronucleus test) are quite distinct types of DNA lesions in terms of the nature of DNA damage and severity. Micronuclei (MN) derive from whole-chromosome damage such as aneugenesis (chromosomes that fail to be integrated within the nucleus of a daughter cell) or clastogenesis (chromosomal fragmentation) and usually relate to errors during cell division, and for this reason are unlikely to be repaired (Martins and Costa 2015). On the other hand, our study confirms that the comet assay is more sensitive and can also identify differences in fish tissue responses. Results show that blood is the most sensitive tissue, while the gills presented with the lowest DNA damage. Previous studies on species from the Cyprinid family reported the same results in some cases (Kostić et al. 2016, 2017), and opposite results in other cases (Sunjog et al. 2013, 2014, 2016; Tokodi et al. 2018). Research performed in Brazilian reservoirs with cyanobacterial blooming and cyanotoxins in the water documented the accumulation of microcystin (up to 37.09 ng/g in muscle and 804.0 μg/g in liver) in all of the sampled fish, and significant differences between the micronucleus count in experimental and control fish. This indicated a potential mutagenetic effect in the exposed fish (*Oreochromis niloticus*) (Vasconcelos et al. 2013). Generally, fish tissues used in

ecogenotoxicology proved to be a valuable parameter, and a realistic indicator of water status described in Lake Ludaš.

Although investigation was performed in late autumn, observed histopathological alterations were similar to previous seasons in 2018 (Tokodi et al. 2020). These mostly include loss of the cord-like parenchymal structure and hepatocyte hypertrophy, as well as condensation of nuclei in the liver, and epithelial hyperplasia and lifting, as well as telangiectasia in the gills. Previous research performed in Lake Ludaš indicated deleterious effects in the liver and gills, but also the kidneys and intestines (Tokodi et al. 2018). Similarly, histopathological damage of the same tissues, including muscles, was also detected and described in fishponds from Serbia with observed cyanobacterial blooming, thus indicating potential health problems that could arise from consuming the affected fish (Drobac et al. 2016). Widespread effects of MCs on fish tissues has been thoroughly described in a review by Svirčev et al. (2015), and include the obaserved histopathological changes described in this research. Although some of the observed histopathological changes can be reversible if the irritant is no longer present, considering that blooms are constantly noted, it is more probable that these changes will persist. The described negative effects of cyanotoxins suggest that cyanobacteria and their toxins are a relevant problem that may affect the health of both fish and other organisms in the vicinity of the affected aquatic ecosystem.

CONCLUSIONS

In the present study, water quality of Lake Ludaš has been categorized as class III, indicating moderate pollution. Although the physical and chemical parameters of water collected in November of 2018 do not seem alarming, measurements in earlier seasons of the same and previous years showed high pH levels and electrical conductivity (Tokodi et al. 2020). Furthermore, previous research (including earlier results from the same year of the present study) also documented the continuous presence of blooming cyanobacteria and cyanotoxins in the lake that could potentially affect aquatic organisms (Tokodi et al. 2018, 2020). Several methods were employed to assess ecogenotoxicity, focusing mostly on fish tissues. DNA damage of blood, liver and gills, together with histological alterations in the liver and gills of fish sampled in Lake Ludaš were found. Albeit some other xenobiotics can induce similar changes, cyanotoxins could be the most likely cause of the observed and described changes in *Carrasius gibelio*. The study demonstrates the need to monitor this important wetland of international significance, since the living organisms present in Lake Ludaš display signs of environmental stress suggesting that preservation and natural balance of the ecosystem could be in danger.

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