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Cyanobacterial effects in Lake Ludoš, Serbia - Is preservation of a degraded aquatic ecosystem justified?

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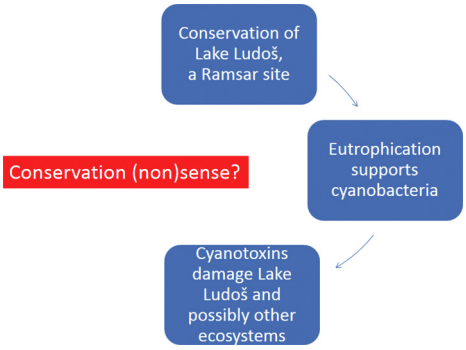
HIGHLIGHTS

- Deterioration of Lake Ludoš water quality and cyanobacterial blooming
- Microcystins detected in water, plants and animals in the lake.
- DNA damage and deleterious histopathological effects observed in fish.
- Hydrogen peroxide treatment not effective in laboratory tests
- Urgent measures for wetland restoration required to prevent possible dissemination.

GRAPHICAL ABSTRACT



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ABSTRACT

Cyanobacteria are present in many aquatic ecosystems in Serbia. Lake Ludoš, a wetland area of international significance and an important habitat for waterbirds, has become the subject of intense research interest because of practically continuous blooming of cyanobacteria. Analyses of water samples indicated a deterioration of ecological condition and water quality, and the presence of toxin-producing cyanobacteria (the most abundant *Limnothrix redekei*, *Pseudanabaena limnetica*, *Planktothrix agardhii* and *Microcystis* spp.). Furthermore, microcystins were detected in plants and animals from the lake: in macrophyte rhizomes (*Phragmites communis*, *Typha latifolia* and *Nymphaea elegans*), and in the muscle, intestines, kidneys, gonads and gills of fish (*Carassius gibelio*). Moreover, histopathological deleterious effects (liver, kidney, gills and intestines) and DNA damage (liver and gills) were observed in fish. A potential treatment for the reduction of cyanobacterial populations employing hydrogen peroxide was tested during this study. The treatment was not effective in laboratory tests although further in-lake trials are needed to make final conclusions about the applicability of the method. Based on our observations of the cyanobacterial populations and cyanotoxins in the water, as well as other

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aquatic organisms and, a survey of historical data on Lake Ludoš, it can be concluded that the lake is continuously in a poor ecological state. Conservation of the lake in order to protect the waterbirds (without urgent control of eutrophication) actually endangers them and the rest of the biota in this wetland habitat, and possibly other ecosystems. Thus, urgent measures for restoration are required, so that the preservation of this Ramsar site would be meaningful.

1. Introduction

Water is a fragile but crucial resource for humanity, and an essential part of natural ecosystems and climate regulation. Due to economic activities, human population growth and urbanization there has been a general decline in water quality which has led the European Union to develop and adopt the EU Water Framework Directive (WFD, 2000). The goal of this directive is to achieve 'good status' (both ecological and chemical) for all ground and surface waters in order to protect human health, water supplies, natural ecosystems and biodiversity. The quality indicators consist of biological (phytoplankton, aquatic flora, benthic invertebrates, fish), hydromorphological and physicochemical factors. Further, good chemical status is understood as compliance with all the relevant chemical quality standards.

Lake Ludoš is one of the aquatic ecosystems protected as a nature reserve in Serbia since 1955, and since 1977 it has been on the list of wetlands of international importance, the so-called Ramsar List (Reis et al., 2017). Therefore the stability and well-being of this ecosystem is of great concern. The protected area is a habitat for endemic and relict plant species including *Orchis mascula* (L.), *Plantago schwarzenbergiana* (Schur.) and *Tripolium pannonicum* (Jacq.). There are also indigenous fauna, especially birds, with some on the International Union of Conservation of Nature (IUCN) Red list, namely white-headed duck *Oxyura leucocephala* (Scopoli), ferruginous duck, *Aythya nyroca* (Güldenstädt), and moustached warbler *Acrocephalus melanopogon* (Temminck) (http://www.serbia.com/srpski/posetite-srbiju/prirodne-lepote/reke-i-jezera/ludasko-jezero-specijalni-rezervat-prirode-star-milion-godina/).

Intense anthropogenic activities have been contributing to the deterioration of Lake Ludoš water quality, increasing biological production and the quantity of sludge in the lake (Institute of Public Health, Subotica, 2011, 2012). This development has resulted in a decline of species diversity. Changes in water quality have also contributed to the increase in the occurrence and abundance of cyanobacterial species in Lake Ludoš (Institute of Public Health, Subotica, 2011, 2012, 2013, 2014, 2015). Anthropogenic nutrient loading and climate change (warming, altered rainfall) synergistically enhance cyanobacterial blooms in aquatic ecosystems (Pearl and Paul, 2012), which could further worsen this problem in the future.

The first scientific environmental investigation of Lake Ludoš dates back to the 1950s, however, more systematic research started in the 1970s (Table 1). Records show a continuous occurrence of cyanobacteria since the 1970s, especially during the summer months (Gajin et al., 1983; Branković and Budakov, 1994; Branković et al., 1998; Nemeš and Matavulj, 2006; Svirčev et al., 2007; Filipović and Obradović, 2008).

One of the first reports from Seleši (1981) recorded a bloom of *Microcystis aeruginosa* Kützing in the 1970s. Similar observations were made in the 1980s (Đukić et al., 1991a) and also in recent years (Institute of Public Health, Subotica, 2015). In the 1990s blooms of *Aphanizomenon flos-aquae* Ralfs ex Bornet & Flahault were observed (Dulić and Mrkić, 1999), as well as those of *Anabaena flos-aquae* Brébisson ex Bornet & Flauhault and *Anabaena spiroides* Klebahn (Dulić, 2002; Seleši, 2006). After 2000, additional cyanobacterial species bloomed in Lake Ludoš, including *Microcystis flos-aquae* (Wittrock) Kirchner, *Microcystis wesenbergii* (Komárek) Komárek ex Komárek, *Lyngbya limnetica* Lemmermann, *Planktothrix agardhii* (Gomont) K. Anagnostidis & J. Komárek (Simeunović, 2009; Institute of Public Health, Subotica, 2015), *Microcystis viridis* (A. Braun) Lemmermann, and *Pseudanabaena limnetica* (Lemmermann) Komárek (Sedmak and Svirčev, 2011). Recently, two invasive species started to appear in the lake: the potentially toxic tropical/subtropical cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya & Subba Raju (Institute of Public Health, Subotica, 2012, 2013, 2014, 2015) and *Sphaerospermopsis aphanizomenoides* (Forti) Zapomelová, Jezberová, Hrouzek, Hisem, Reháková & Komárková which has extended its distribution in Serbia and appeared in the lake during 2014 (Jovanović et al., 2016).

Systematic research on water quality and on the abundance and composition of the plankton community has shown that Lake Ludoš was, and still is, in a perpetual eutrophic state (Đukić et al., 1991b; Dulić, 2002; Đurđević, 2007). As such, the lake supports extensive cyanobacterial growth, and the formation of cyanobacterial blooms, scums and mats. One of the detrimental outcomes of this phenomenon is the production of cyanotoxins, secondary metabolites with diverse structures and modes of toxicity.

Toxic cyanobacteria and their metabolites can present acute and chronic health risks to plants, animals and humans (MacKintosh et al., 1990; Falconer, 1998; Codd, 2000; Azevedo et al., 2002; Saqurane et al., 2009; Svirčev et al., 2009, 2015, 2016; Metcalf and Codd, 2012; Drobac et al., 2011, 2013, 2017). Numerous animal and human intoxications reported worldwide have been causally-associated with cyanobacteria (Kuiper-Goodman et al., 1999; Carmichael et al., 2001; Soares et al., 2006; Stewart et al., 2008; Svirčev et al., 2017a, 2017b). In addition, there is a possibility of cyanotoxin accumulation in aquatic organisms (Magalhães et al., 2003; Sabatini et al., 2011; Romo et al., 2012; Drobac et al., 2016) and their transport to the higher levels of food webs, ultimately endangering animal and human health (Xie et al., 2005; Mohamed and Al Shehri, 2009; Peng et al., 2010). The most commonly encountered cyanotoxins are the microcystins (MCs) (Chorus and Bartram, 1999; Codd et al., 2005). Moreover, while MCs have long been recognized as potent tumour-promoters and hazards to human health (Metcalf and Codd, 2012; Svirčev et al., 2013a, 2014a), they may also be potential carcinogens (Ito et al., 1997; Hernandez et al., 2009; Svirčev et al., 2010; Gan et al., 2010; Žegura et al., 2011). Investigations of cyanobacteria in aquatic ecosustems show that MCs have been widely present in various waterbodies in Serbia (Svirčev et al., 2014b, 2017c).

Lake Ludoš has become a focus of cyanobacterial research because it is almost constantly blooming, and in the same time the lake is important as a Ramsar site. The aims of the present study were to: (1) assess the eutrophic state of the water, determine the qualitative and quantitative structure of the cyanobacterial community and compare them with the summarized historicaly reported data on qualitative and quantitative structure of the cyanobacterial community; (2) assess cyanobacterial biomass toxicity and specific cyanotoxins in water samples by the *Artemia salina* bioassay, protein phosphatase inhibition (PPI) and enzyme-linked immunosorbent assay (ELISA); (3) analyze the presence of cyanotoxins in various tissues of aquatic plants and fish by LC-MS/MS; (4) study possible cyanotoxin effects on fish tissues by histopathological examination and comet assay; (5) carry out a preliminary in vitro assessment of the efficiency of hydrogen peroxide in decreasing the viability of cyanobacteria present in Lake Ludoš; and (6) discuss whether there is enough justification for the preservation of such degraded aquatic ecosystems.

Table 1
Historical overview of cyanobacterial occurrence and blooming since the 1970s in Lake Ludoš.

Date	Blooming* and dominant cyanobacteria	Microcystins (μg/L in water)	Other associated records	Reference
1970–1981	*Microcystis aeruginosa**	n.a.	n.a.	Seleši, 1981
	Aphanizomenon flos-aquae			
	Lyngbya limnetica			
	Oscillatoria tenuis			
1971	*Chroococcus minutus*	n.a.	Eutrophic	Futo, 1972
	Merismopedia glanca			
	Microcystis flos-aquae			
	Oscillatoria limnetica			
	Oscillatoria planctonica			
	Oscillatoria tenuis			
	Anabaena spiroides			
1981–1990	*Microcystis aeruginosa**	n.a.	Chlorophyll *a* 17.94–255.80 mg/m^3	Đukić et al., 1991b
	*Aphanizomenon flos-aquae**		(mesopolytrophic)	
			Cyanobacteria (23%)	
			Xanthophyta (1.2%)	
			Pyrrophyta (1.2%)	
			Chrysophyta (1.2%)	
			Bacillariophyta (14.6%)	
			Euglenophyta (9.8%)	
			Chlorophyta (37.8%)	
March–May 1992	*Microcystis aeruginosa*	n.a.	Phytoplankton:	Branković and Budakov, 1994
	Oscillatoria tenuis		Chlorophyta (44.9%)	
	Spirulina maior		Bacillariophyta (26.3%)	
			Euglenophyta (11.9%)	
			Cyanophyta (11%)	
			Pyrrophyta (5.1%)	
			Xantophyta (0.8%)	
May–October 1993	*Anabaena flos-aquae*	n.a.	Phytoplankton:	Novak, 1994
	Anabaena spiroides		Chlorophyta (41.2%)	
	Aphanizomenon flos-aquae		Cyanophyta (27.5%)	
	Holopedia irregularis		Bacillariophyta (17.6%)	
	Lyngbya limnetica		Euglenophyta (11.8%)	
	Merismopedia punctata		Xantophyta (1.9%)	
	Microcystis aeruginosa			
	Microcystis flos-aquae			
	Microcystis minutissima			
	Nostoc commune			
	Oscillatoria chalybea			
	Oscillatoria putrida			
	Oscillatoria princeps			
	Oscillatoria tenuis			
10th July 1996	*Aphanizomenon flos-aquae**	n.a.	n.a.	Sedmak and Svirčev, 2011
20th August 1997	*Aphanizomenon flos-aquae**	n.a.	n.a.	Sedmak and Svirčev, 2011
1998	*Aphanizomenon flos-aquae**	n.a.	n.a.	Dulić and Mrkić, 1999
	Oscillatoria chlorina			
	Microcystis aeruginosa			
	Microcystis flos-aquae			
	Anabaena flos-aquae			
20th August 1998	*Microcystis incerta*	n.a.	n.a.	Sedmak and Svirčev, 2011
	Microcystis aeruginosa			
1997–2000	*Microcystis aeruginosa**		Chlorophyll *a* 50.7–77.4 mg/m^3	Dulić, 2002
(north part of the lake)	*Microcystis flos-aquae**		(eutrophic)	
	*Merissimopedia tenuissima Lyngbya limnetica**		Phytoplankton:	
	Oscillatoria chlorina		Cyanobacteria (23.3%)	
	Oscillatoria tenuis		Xanthophyta (0.6%)	
	Anabaena circinalis		Pyrrophyta (0.6%)	
	Anabaena flos-aquae		Bacillariophyta (24.6%)	
	*Anabaena spiroides**		Euglenophyta (9.8%)	
	*Aphanizomenon flos-aquae**		Chlorophyta (41.1%)	
30th August 2000	*Microcystis aeruginosa**	n.a.	n.a.	Sedmak and Svirčev, 2011
8th August 2002	*Microcystis aeruginosa**	n.a.	n.a.	Sedmak and Svirčev, 2011
1st September 2003	*Microcystis viridis**	n.a.	n.a.	Sedmak and Svirčev, 2011
2005–2007	*Anabaena circinalis*	PPI	Chlorophyll *a* (mg/m^3)	Simeunović, 2009
	Anabaena flos-aquae	2005	2005	
	Anabaena planctonica	Summer: 80.59	Summer: 329.30	
	Anabaena spiroides	Autumn: 362.68	Autumn: 124.03	
	*Aphanizomenon flos-aquae**	2006	2006	
	Aphanizomenon ovalisporum	Winter: 4.21	Winter: 9.35	
	*Microcystis aeruginosa**	Spring: 268.07	Spring: 224.28	
	*Microcystis flos-aquae**	Summer: 603.61	Summer: 195.75	
	*Microcystis wesenbergii**	Autumn: 176.30	Autumn: 116.81	
	Oscillatoria sp.	2007	2007	
	Phormidium sp.	Winter: 144.87	Winter: 85.44	

(continued on next page)

Table 1 (*continued*)

Date	Blooming* and dominant cyanobacteria	Microcystins (μg/L in water)	Other associated records	Reference
	Phormidium autumnale *Planktothrix agardhii** *Spirulina maxima*	Spring: 238 Summer: 527.25 Autumn: 55.81	Spring: 124.60 Summer: 213.60 Autumn: 224.28	
6th June 2006	*Microcystis flos-aquae** *Pseudanabaena limnetica**	n.a.	n.a.	Sedmak and Svirčev, 2011
May 2007	*Anabaena spiroides**	238 (PPI)	n.a.	Svirčev et al., 2013b
September 2007	*Microcystis* sp.*	55.81 (PPI)	n.a.	Svirčev et al., 2013b
2011–2012	*Microcystis flos-aquae**	3.7.2011. 84.77 (ELISA) 29.3.2012. 15.35 (ELISA) 23.8.2012. 33.62 (ELISA)	n.a.	World Bank Report DM 4307, 2011;
2011	*Anabaena* sp. *Lyngbya* sp. *Microcystis* sp. *Oscillatoria* sp.	n.a.	Chlorophyll *a* 1.75 g/m^3 (highest in September)	Institute of Public Health, Subotica, 2011
2012	*Anabaena* sp. *Anabaenaopsis* sp. *Cylindrospermopsis* sp. *Lyngbya* sp. *Microcystis* sp. *Oscillatoria* sp.	n.a.	Chlorophyll *a* 1.40 g/m^3 (highest in August)	Institute of Public Health, Subotica, 2012
2013	*Anabaena* sp. *Anabaenaopsis* sp. *Cylindrospermopsis* sp. *Lyngbya* sp. *Microcystis* sp. *Oscillatoria* sp. *Cylindrospermopsis raciborskii**	n.a.	Chlorophyll *a* 1.46 g/m^3 (highest in September)	Institute of Public Health, Subotica, 2013
2014	*Cylindrospermopsis* sp. *Lyngbya* sp. *Microcystis* sp. *Oscillatoria* sp. *Lyngbya limnetica* *Microcystis aeruginosa* *Oscillatoria putrida* *Cylindrospermopsis raciborskii**	n.a.	Chlorophyll *a* 807 mg/m^3 (highest in September and October)	Institute of Public Health, Subotica, 2014
2014 spring	*Sphaerospermopsis aphanizomenoides*	n.a.	n.a.	Jovanović et al., 2016
2015	*Cylindrospermopsis* sp. *Lyngbya* sp. *Microcystis* sp. *Oscillatoria* sp. *Microcystis aeruginosa* *Microcystis flos-aquae* *Oscillatoria agardhii** *Oscillatoria putrida* *Lyngbya limnetica* *Cylindrospermopsis raciborskii*	n.a.	Chlorophyll *a* 672 mg/m^3 (highest in September and October)	Institute of Public Health, Subotica, 2015

n.a. - not analyzed; PPI - protein phosphatase inhibition assay; ELISA- enzyme-linked immunosorbent assay.

2. Materials and methods

2.1. Sampling site

Ludaš or Lake Ludoš is a shallow lake in the province of Vojvodina in northern Serbia, near the city of Subotica. The alluvial basin of Lake Ludoš is located in the sandy terrain between the Danube and the Tisa rivers, at the borderline of the Bačka loess plateau. The lake is 4.5 km long and covers 328 ha. The low and wide northern coast of the lake is swampy, while the narrow southern coast is submerged into loess. The lake is supplied with water from aquifers, from the Kereš River, and since 1981 with partially purified water from Lake Palić via the Palić-Ludoš channel. The maximum depth of the lake is 2.25 m, although in most places the depth does not exceed 1 m. Shallow water may be frozen for more than three months a year, and in the summer the water temperature may rise up to 30 °C.

Water samples were collected from the surface water layer within the littoral zone (pier next to the visitor center) (46°06′12.0″N 019°49′17.3″E) and from the center of the lake (46°06′01.5″N 019°49′19.8″E) during 2011 (July and August) and 2012 (March and August). Whole aquatic plants were sampled using knife in March 2012 from the center of the lake (water lilies) and the pier (reed and cattail), and fish samples were collected with gillnets of various mesh sizes and a standard electrofishing device in July 2011 and March 2012 from the center of the lake. For the hydrogen peroxide in vitro experiments, a water sample was taken from the pier in July 2014.

2.2. Cyanobacterial assessment

2.2.1. Chlorophyll a analyses

Water samples were collected for Chlorophyll *a* determination from a depth of 0.3 m and concentrated by filtering 0.2 L of water through a 0.45 μm membrane filter. Chlorophyll *a* was extracted with 90% acetone at 4 °C in darkness overnight. The extracts were centrifuged at 1500 ×g for 10 min and the Chlorophyll *a* concentration in the supernatant was measured spectrophotometrically (APHA, 1995). Measurements were done in duplicate and the results are expressed as means. Trophic state determination was assessed according to Felfoldy (1980).

2.2.2. Qualitative and quantitative analyses of cyanobacteria

For the analysis of the phytoplankton community, about 1 L of water was collected from a depth of 0.3 m. The samples were collected by sweeping the plankton net (netframe 25 cm ø, net mesh 22 μm) from the lake bottom to the surface and samples were transferred to the laboratory within 6 h from the sampling time and kept cool in the dark. Selected samples were preserved in a 4% (v/v) solution of formaldehyde according to the European standard EN 15204 (EN 15204, 2006). Taxonomic identifications of cyanobacteria were made according to widely used taxonomic keys (Komárek, 2013; Komárek and Anagnostidis, 1998, 2005). Quantitative analyses of phytoplankton were done using the Utermöhl method (Utermöhl, 1958) with a Leica inverted microscope and observations of taxa expressed as the number of cells per mL.

2.3. Toxicity and cyanotoxin assessment

2.3.1. Artemia salina bioassay of water samples

In order to distinguish between the potential intracellular versus extracellular content of cyanotoxins, 100 mL water samples were filtered and the extracellular fraction (filtrate) was stored at 4 °C until further analysis. The filters retaining cyanobacterial biomass (intracellular fraction) were dried overnight at 28 °C. The next day, 75% methanol (MeOH) was used to extract cyanotoxins for 24 h. The extracted filters were then ultrasonicated and centrifuged. Supernatants were stored at 4 °C until further analysis.

The toxicity of water samples was assessed using *Artemia salina* (L.) larvae according to Kiviranta et al. (1991). *Artemia salina* was grown in ASW medium (Artificial Sea Water), illuminated and gently sparged with air. 0.6 g of dried brine shrimp eggs were added to 100 mL sterile medium and incubated at 30 °C, under illumination for 24–36 h. For toxicity tests, 96-well microtiter plates were used. Samples prepared with MeOH were evaporated overnight to remove the MeOH, and the aqueous residues of the samples were used the next day. About 20 larvae were transferred to one microtiter plate well and then incubated with the cyanobacterial extracts for 24 h under low ambient light at 30 °C. ASW with *Artemia salina* was used as a control, and as a further control, 75% (v/v) evaporated MeOH in ASW with *Artemia salina* was used, showing a mortality below 5%. Mortality was recorded after 24 h and the surviving animals were killed by the addition of 100 μL of MeOH to each well and counted. Toxicity was expressed as the percentage of dead larvae minus the mortality in control samples. Triplicate aliquots of each sample were analyzed.

2.3.2. Protein phosphatase 1 inhibition assay (PPI) of water samples

An optimized method (Simeunović, 2009) derived from An and Carmichael (1994) was used for MC detection in water samples. The applied method is a colorimetric assay of the inhibition of protein phosphatase 1. The detection of MCs was performed in three replicates and the results were expressed as mean values.

2.3.3. Enzyme-linked immunosorbent assay (ELISA) in water samples

Water samples were freeze-thawed and ultrasonicated in order to ensure the release of cyanobacterial intracellular contents including cyanotoxins, if present. Samples were then centrifuged (NF 800 R, Nüve, Turkey) at 2348 ×*g* for 15 min, and the supernatants used for cyanotoxin detection. Two ELISAs were used for analyses: microcystins-ADDA and saxitoxin. The Abraxis Microcystins-ADDA ELISA (Microcystin/Nodularin ADDA ELISA, Abraxis, USA) is an immunoassay for the quantitative congener-independent detection of MCs and nodularin (NOD). This kit also responds to a range of MC detoxication products (Metcalf et al., 2002). The Saxitoxin ELISA (Saxitoxin (PSP) ELISA, Abraxis, USA) is an immunoassay for the quantitative detection of saxitoxins (STXs). The ELISA plates were read using a microplate reader (Asys Expert Plus UV, Biochrom, UK). According to the ELISA kit manufacturer, the detection range for the microcystins-ADDA ELISA assay, based on MC-LR, was from 0.10 to 5 ppb (μg/L), and for STX from 0.015 to 0.4 ppb (μg/L).

2.3.4. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) of plant tissue samples

Samples of different tissues (rhizome, shoot, mature leaves and flowers) from reed *Phragmites communis* (Cav.) Trin. ex Steud., cattail *Typha latifolia* L., royal blue water lily *Nymphaea elegans* Hook. and yellow water lily *Nuphar luteum* L. were analyzed by LC-MS/MS.

The tissues were prepared by adding from 5 to 20 mL of 100% MeOH to 3–5 g wet weight of plant material for 24 h. The samples were sonicated in an ultrasonic bath for 15 min at room temperature and then clarified by centrifugation. The supernatants were separated and evaporated under nitrogen flow at 40 °C. The dried samples were dissolved in 0.5–1 mL 20% MeOH, ultrasonicated for 5 min and filtered through 0.2 μm PVDF syringe filters. The prepared extracts were analyzed by LC-MS/MS (Zervou et al., 2017). Standards of 12 MC variants ((D-Asp^3)-MC-RR, MC-RR, MC-YR, MC-HtyR, (D-Asp^3)-MC-LR, MC-LR, MC-HilR, MC-WR, MC-LA, MC-LY, MC-LW and MC-LF), nodularin, anatoxin-a, cylindrospermopsin and phenylalanine-d5 were used for the detection of cyanotoxins in plant tissues.

2.3.5. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) of fish tissue samples

Prussian carp *Carassius gibelio* (Block) from Lake Ludoš was used for cyanotoxin detection in fish liver, gills, kidney, intestine, gonads, heart, spleen and muscle samples by LC-MS/MS. 12 individuals (TL:24.01 ± 1.99 cm, SL:19.38 ± 2.39 cm, W:197.91 ± 13.56 g) in 2011, and 7 (TL:19.24 ± 4.56 cm, SL:15.87 ± 3.5 cm, W:132.14 ± 51.02 g) in 2012 were sampled.

Freeze-dried fish tissue samples (400 mg) were placed into glass tubes and 3 mL of 100% MeOH was added. Ultrasonication was carried out four times for 30 min. After each treatment, the supernatant was decanted into plastic tubes and then centrifuged for 10 min at 3300 ×*g*. The supernatants were placed in glass vials (40 mL), 6 mL of hexane added to extract lipids, and placed in a shaker for 15 min. The layers of hexane were removed with glass Pasteur pipettes. The samples were evaporated, and 5 mL 10% MeOH was added into samples which were ultrasonicated in a waterbath for 15 min. Cartridges (Waters HLB Cartridge, 200 mg) were used for cleaning of the samples. The elution of processed cartridges was done with 3 mL of 100% MeOH. The samples were placed into glass tubes, evaporated, dissolved with 200 μL of 75% MeOH and vortexed for about 30 s to 1 min. Subsequently, they were filtered (0.2 μm GHP ACRODISC 13 PALL Corporation) and centrifuged for 10 min at 10,000 ×*g*. Supernatants were then diluted ten-fold with 75% MeOH to ensure good dissolution of the analytes and to avoid problems with ion suppression in the MS instrument.

For the identification of MCs, two reference samples were used: cyanobacterial extract from *Microcystis* NIES-107 (National Institute for Environmental Studies, Tsukuba, Japan) which included dm-MC-RR MC-RR, dm-MC-YR, MC-YR, dm-MC-LR and MC-LR variants (Meriluoto et al., 2004), and an extract from *Microcystis* PCC 7820 (Institut Pasteur, Paris, France) which contained MC-LR, MC-LY, MC-LW and MC-LF (Lawton et al., 1994). MCs were extracted according to Meriluoto and Spoof (2005a, 2005b). The extracts were dissolved in 75% MeOH and diluted to appropriate concentrations for analysis.

The instrument consisted of an Agilent 1290 Infinity Binary LC system (Agilent Technologies) coupled to an Agilent 6460 triple-quadrupole mass spectrometer. The toxins were quantified on a Supelco (Bellefonte, USA) Ascentis C18 HPLC column; 50 mm × 3 mm I.D., 3 μm particles, protected by a 4 × 2 mm C8 guard column. The mobile phase consisted of solvents A: 99% water - 1% acetonitrile - 0.1% formic acid and B: acetonitrile - 0.1% formic acid (Fluka, Switzerland). Data acquisition was done with MassHunter Quantitative Data Analysis Software (Agilent Technologies). For tissue samples the amount of MCs was calculated and reported as ng of toxin variant per mg dry weight of tissue.

2.4. Effects of the cyanobacterial bloom on fish tissues

2.4.1. Fish histopathology

A total of 16 individuals of Prussian carp (TL: 24.26 ± 1.87 cm, W: 204.37 ± 24.99 g) were caught. Liver, gill, kidney, spleen, intestine, heart, gonad and muscle tissues of the fish were sampled and fixed in 4% formaldehyde. The samples were processed by a standard histological procedure (Humason, 1979). Five μm-thin sections were cut and placed onto glass slides which were stained using standard haematoxylin and eosin (H&E) technique. Sections were examined under a Primo Star light microscope (Carl Zeiss, Germany) and photographed with an AxioCam MRc 5 digital camera (Carl Zeiss, Germany).

2.4.2. Comet assay

Four specimens of Prussian carp (TL: 22.63 ± 0.75 cm, W: 183.25 ± 18.55 g) were used in this assay. Prior to blood collection fish were anesthetized in clove oil solution, than sacrificed by overdosing by clove oil and dissected for tissue sampling. Blood was collected with a syringe directly from the heart and added to microtubes with 0.5 mL cooled Heparin-PBS (phosphate-buffered saline) solution to prevent clotting. Liver and gills were obtained after dissection and each organ was excised and chopped separately 10 times in 0.2 mL of HBSS (Hank's Balanced Salt Solution) using two scalpel blades in a scissor-like movement in a Petri dish, washed off gently into a 15 mL centrifuge tube with a further 2.5 mL HBSS and 0.3 mL of trypsin (final conc. 0.05%). The contents were gently shaken for 10 min at room temperature, after which 10 mL of HBSS was added and the suspensions passed through a sieve to remove any large fragments that remained. After centrifugation (475 ×*g* for 10 min), the supernatants were discarded and the pellets carefully resuspended in 0.5 mL of HBSS.

The alkaline comet assay procedure was performed according to Singh et al. (1988) with some modifications (Sunjog et al., 2016). Microscope slides were precoated with two layers of 1% NMP (Normal Melting Point) agarose after which 30 mL of cell pellet suspension was gently mixed with 70 mL of 1% LMP (Low Melting Point) agarose and pipetted on the supportive layer of 1% NMP agarose. Slides were then placed into cold lysis buffer for 1 h. To allow DNA unwinding, slides were put into the electrophoresis chamber containing cold alkaline electrophoresis buffer for 20 min. Electrophoresis was performed at 0.75 V/cm at 4 °C for 20 min. After electrophoresis, slides were placed into freshly made neutralizing buffer for 15 min. Staining was performed with 20 μL per slide of EtBr (2 μg/mL). Microscopic images of comets were scored using Comet IV Computer Software (Perceptive Instruments, UK). Images of 50 cells were captured from each slide per sample and among the parameters available for analyses the Tail Intensity (TI) and Olive Tail Moment (OTM) were chosen as parameters to assess the DNA damage. Values and observations from several other studies, not including cyanobacteria or cyanotoxins, were used as control (Velma and Tchounwou, 2013; Zhao et al., 2015; Sunjog et al., 2016).

2.5. Hydrogen peroxide treatment of water in vitro

The Chlorophyll *a* content was determined at the beginning of the hydrogen peroxide experiments in order to estimate the initial amount of phytoplankton biomass used in the experiments. 100 mL samples were filtered on Whatman (Maidstone, UK) GF/C glassfibre filters (pore size 1.2 μm) and the pigment extraction was performed with 3 mL 100% MeOH in darkness at 4 °C, followed by ultrasonication, centrifugation (1400 ×*g*, 10 min) and spectrophotometric reading (Nicolet Evolution 100, Thermo Electric Corporation, UK) of absorbance at 663 nm (Mackinney, 1941). The measurements were performed in triplicate and the result is expressed as the mean value.

Hydrogen peroxide (H_2O_2) treatment of lake water samples was performed under laboratory conditions. The water was divided into 5 L incubation flasks and kept at ambient room light and temperature. H_2O_2 was obtained from Grund d.o.o. (Irig, Serbia). The stock H_2O_2 (35% w/w) was pre-diluted with lake water and added at appropriate quantities to achieve the following desired concentrations of H_2O_2 in the incubation flasks: 0 mg/L (control), 2 mg/L, 5 mg/L and 20 mg/L. The water was mixed prior to sampling. Three parallel 100 mL subsamples for each concentration and timepoint (0 h, 12 h, 48 h) were filtered on glassfibre filters and used for the quantitative analysis of cell-bound MCs.

Further, 50 mL samples were taken to observe the influence of hydrogen peroxide on the cyanobacterial colonies and filaments. The samples were fixed by adding 1% (0.5 mL) of acidified Lugol's iodine. Cyanobacterial abundance and morphological changes, as a consequence of exposure to different hydrogen peroxide concentrations, were assessed by microscopic observation (Olympus BX 51) at ×200 and ×400 magnification. Microphotographs were taken using an Olympus DP 26-DKTB digital camera and processed with Olympus cellSens Entery CS-EN-V1,7 software. Six microscope view-fields of precipitated biomass were examined for each sample.

2.5.1. Extraction of filters from hydrogen peroxide experiments for MC analysis

The filters were air-dried, frozen and lyophilized, then extracted with 3 mL of 75% MeOH in borosilicate glass tubes in an ultrasonic bath (Bandelin RK 156, Germany) for 15 min. Extraction was further enhanced by probe ultrasonication for 1 min (Bandelin Sonopuls HD 2070 with a 3 mm microtip probe, 30% pulse, 30% energy). The samples of the extracts (1.5 mL) were then centrifuged for 15 min at 10,000 ×*g*. Supernatants (1 mL) were then evaporated to dryness at 50 °C under nitrogen gas. The dry residues were re-dissolved in 300 μL 75% MeOH and the samples filtered into chromatographic inserts using Pall Corporation (Port Washington, USA) 0.2 μm GHP Acrodisc 13 filters. The LC-MS/MS for MC detection was the same as used for fish tissues.

3. Results

3.1. Cyanobacterial presence and toxicity

Several analyses on Lake Ludoš water samples were performed, including Chlorophyll *a* quantification to determine the trophic status of the water (Felfoldy, 1980), bioassay using *Artemia salina* to assess the intracellular and extracellular toxicity, PPI assay (MC-LR equiv.) and ELISA assays (MC/NOD and STX) to detect and quantify cyanotoxins. Furthermore, concentrations of MC variants (MC-LR, -dmLR, -LY, -LW, -LF, -RR, -dmRR, -YR, and -dmYR) were analyzed in various fish tissue samples of Prussian carp from Lake Ludoš by LC-MS/MS (Table 2).

According to the Chlorophyll *a* concentrations in 2011, the trophic state of Lake Ludoš was highly eutrophic. The highest concentration of Chlorophyll *a* was recorded in biomass from the pier in July 2011. A high mortality rate of *Artemia salina* was also found in bioassays of the July 2011 water samples (intracellular), while in August a very low mortality rate was recorded. The toxicity of extracellular content overall was very low in both sampling periods. The PPI assay indicated that very low concentrations of MC-LR equivalents occurred extracellularly (i.e. dissolved in the water phase). The highest intracellular MC concentration was detected in the biomass of the water sample taken in July 2011. MC concentrations indicated by the ADDA ELISA kit were similar to those indicated by PPI assay in water samples from the same period, with the highest concentrations recorded in the biomass from July 2011. Only two MC variants (MC-LR and MC-RR) were found using LC-MS/MS in the fish tissue samples from Lake Ludoš, and the highest concentration was detected in the gills of Prussian carp.

Research on the lake continued in the following year. In the spring of 2012 seven cyanobacterial species/genera were found in the bloom material (Table 3). The dominant cyanobacteria were *Limnothrix redekei* (van Goor) Meffert and *Pseudanabaena limnetica*.

Table 2
Chlorophyll *a*, toxicity assessment by bioassay, and cyanotoxin analysis of Lake Ludoš water and fish samples in 2011.

Location and date	Chlorophyll *a*		Bioassay *A. salina* (%)[b]		PPI assay water samples (μg MC-LR equiv./L)			ELISA water samples (μg/L)		LC-MS/MS fish tissues (ng/g dw)	
	Conc. Chl *a* (mg/m^3)	Trophic state[a]	IC	EC	IC	EC	Risk level[c]	Total MC/NOD	Total STX	MC-RR	MC-LR
3rd July 2011											
Pier 1	190	EPT	95	5	828	1.1	H	119	0.03	n.a.	n.a.
Pier 2	87	ET	100	10	218	30.8	H	43	0.03	n.a.	n.a.
Pier 3	61	ET	22.5	5	120	1.3	H	n.a.	n.a.	n.a.	n.a.
17th August 2011											
Pier	37	MET	5.6	0	18.4	1.5	M	8.2	0.04	0.06 MU	0.27 I
Center	52	ET	4.2	3.5	6.5	1.8	L	3.9	0.04	0.25 G	

IC-intracellular, EC-extracellular; dw-dry weight; EPT-Eu-polytrophic, ET-eutrophic, MET-Meso-eutrophic; H-high, M-medium, L-low; n.a.-not analyzed; MU-muscle, G-gills, I-intestine.
[a] Trophic state according to Felfoldy (1980).
[b] Control was 0%, and results are presented as mean values.
[c] Risk level-water for recreation (WHO, 1998).

Cyanobacterial taxa constituted 33% of the total phytoplankton, the remainder were Chlorophyta (47%), Bacillariophyta (11%), Euglenophyta (5.6%), and Chromophyta (2.8%), however, Cyanobacterial taxa were the most abundant. Other analyses from 2012 included ELISA on water samples, as well as LC-MS/MS analyses of plant and fish tissues for the presence of cyanotoxins (Table 4).

MC/NOD ADDA ELISA results of water samples during 2012 showed very low concentrations of MCs and/or MC-detoxification products in the spring, and concentrations increased during the summer at both sampling sites. Concentrations of STX according to ELISA were very low during both investigated periods in the lake. Plant tissue analyses showed the presence of MC-RR in rhizomes of reed, cattail and royal blue water lily. MC variants were also found in fish tissues: MC-RR was found in muscle and gonad tissues, and slightly higher concentrations of MC-LR were found in kidney and intestine tissues of Prussian carp.

3.2. Effects of the water bloom on fish tissues

Histopathological analyses of Prussian carp from Lake Ludoš in 2011 showed histopathological alterations in their tissues compared to the normal tissue structures (Fig. 1).

Loss of the typical cord-like parenchymal structure and dissociation of cells was observed in the liver (Fig. 1A). Hepatocytes had lost their polyhedral shape and were onion-shaped with serrated membranes (Fig. 1B). Hepatocyte cytoplasm displayed a loss of glycogen and granular structure. Intense vacuolization was observed, with cells having a completely clear cytoplasm (Fig. 1B). Intense condensation of chromatin and karyorrhexis was also observed, as well as several anucleated cells (Fig. 1C). Pycnotic nuclei were indicative of necrosis. Sporadic melanin aggregates were also observed.

In the kidney, glomerulopathy with intense dilatation of Bowman's capsule and glomerular atrophy were evident (Fig. 1D). Both proximal and distal renal tubules were highly vacuolized and clogged (Fig. 1E), while tubular cells were degenerated. Several tubular cells displayed pyknosis and karyrrhexis. Nephrocalcinosis was also observed as well as severe macrophage infiltration (Fig. 1F).

Gills were also severely damaged. The proliferation of interlamellar cell mass had led to complete fusions of lamellae (Fig. 1G). Oedema and epithelial lifting were observed (Fig. 1H), as well as hypertrophy of epithelial cells and several necrotic lamellae. Proliferations of chloride cells were intense (Fig. 1G).

In the intestines, edematous alterations in lamina propria were observed (Fig. 1J), with its subsequent dilation. Necrosis and subsequent desquamation of enterocytes (Fig. 1K) was indicative of necrotic enteritis, mostly in the apical parts of the villi. Hypertrophies of goblet cells were also observed (Fig. 1L).

Table 3
Presence and abundance of phytoplankton from Lake Ludoš in 2012.

Taxon	Pier cells/mL	Center cells/mL
Cyanobacteria		
Anabaenopsis V.V.Miller sp.	−	1320
Chroococcus limneticus Lemmermann	12,160	19,280
Limnothrix redekei (van Goor) M.E.Meffert	11,665,960	28,967,680
Merismopedia Meyen sp.	−	2240
Microcystis aeruginosa (Kützing) Kützing, *Microcystis flos-aquae* (Wittrock) Kirchner, *Microcystis wesenbergii* (Komárek) Komárek	192,080	478,960
Oscillatoria Vaucher ex Gomont spp.	40,000	20,000
Planktolyngbya Anagnostidis & Komárek sp.	−	25,520
Planktothrix agardhii (Gomont) Anagnostidis & Komárek	135,520	314,160
Pseudanabaena limnetica (Lemmermann) Komárek	1,142,000	2,755,000
Woronichinia compacta (Lemmermann) Komárek & Hindák	+	+
Σ	**13,187,720**	**32,584,160**
Chromophyta		
Peridinium Ehrenberg sp.	160	480
Σ	**160**	**480**
Bacillariophyta		
Cyclotella (Kützing) Brébisson sp.	27,680	21,640
Navicula Bory de Saint-Vincent spp.	800	1240
Nitzschia acicularis (Kützing) W.·Smith C	3280	3200
Nitzschia palea (Kützing) W.·Smith	+	+
Σ	**31,760**	**26,080**
Euglenophyta		
Euglena clavata Skuja	240	960
Phacus pyrum (Ehrenberg) W.·Archer	320	800
Σ	**560**	**1760**
Chlorophyta		
Actinastrum Lagerheim sp.	−	2320
Ankistrodesmus Corda sp.	1920	5440
Coelastrum Nägeli sp.	−	1760
Cosmarium Corda ex Ralfs sp.	400	1680
Dictyosphaerium tetrachotomum Printz	10,240	14,240
Golenkinia radiata Chodat	80	240
Hyaloraphidium Pascher & Korshikov sp.	3280	6880
Kirchneriella irregularis (G.M.Smith) Korshikov	1040	1360
Micractinium pusillum Fresenius	1920	6880
Monoraphidium komarkovae Nygaard	560	680
Monoraphidium Komárková-Legnerová sp.	5200	14,000
Pediastrum Meyen spp.	3200	6240
Scenedesmus acuminatus (Lagerheim) Chodat, *Scenedesmus opoliensis* P.G.Richter, *Scenedesmus* Meyen sp., *Scenedesmus quadricauda* Chodat	110,560	210,520
Tetraedron Kützing sp.	−	520
Σ	**138,400**	**272,760**
Total phytoplankton Σ	**13,358,600**	**32,885,240**

+ – present in the sample; − – not present in the sample.

Table 4
Cyanotoxin analysis of Lake Ludoš water, plant and fish samples in 2012.

Location and date	ELISA water samples (μg/L)		LC-MS/MS plant tissues (ng/g dw)	LC-MS/MS fish tissues (ng/g dw)	
	Total MC/NOD	Total STX	MC-RR	MC-RR	MC-LR
29th March 2012					
Pier	1.52	0.02	0.22 R	0.06 MU	0.07 K
Center	1.55	0.03	0.05C 0.02 RW	0.06 GO	0.08 I
23rd August 2012					
Pier	20.9	0.25	n.a.	n.a.	n.a.
Center	22.2	0.26	n.a.	n.a.	n.a.

R-reed, C-cattail, RW-royal blue water lily; dw-dry weight; MU-muscle, GO-gonads, K-kidney, I-intestine; n.a.-not analyzed.

No significant alterations were observed in the heart, spleen and muscle tissues. Slight oedema and vacuolization were noted in the heart tissue. Large areas of macrophage aggregates were observed in the spleen.

Furthermore, in 2012 the potential effects of cyanotoxins in fish tissue were investigated with the comet assay. The results indicated DNA damage in four specimens of Prussian carp expressed with two

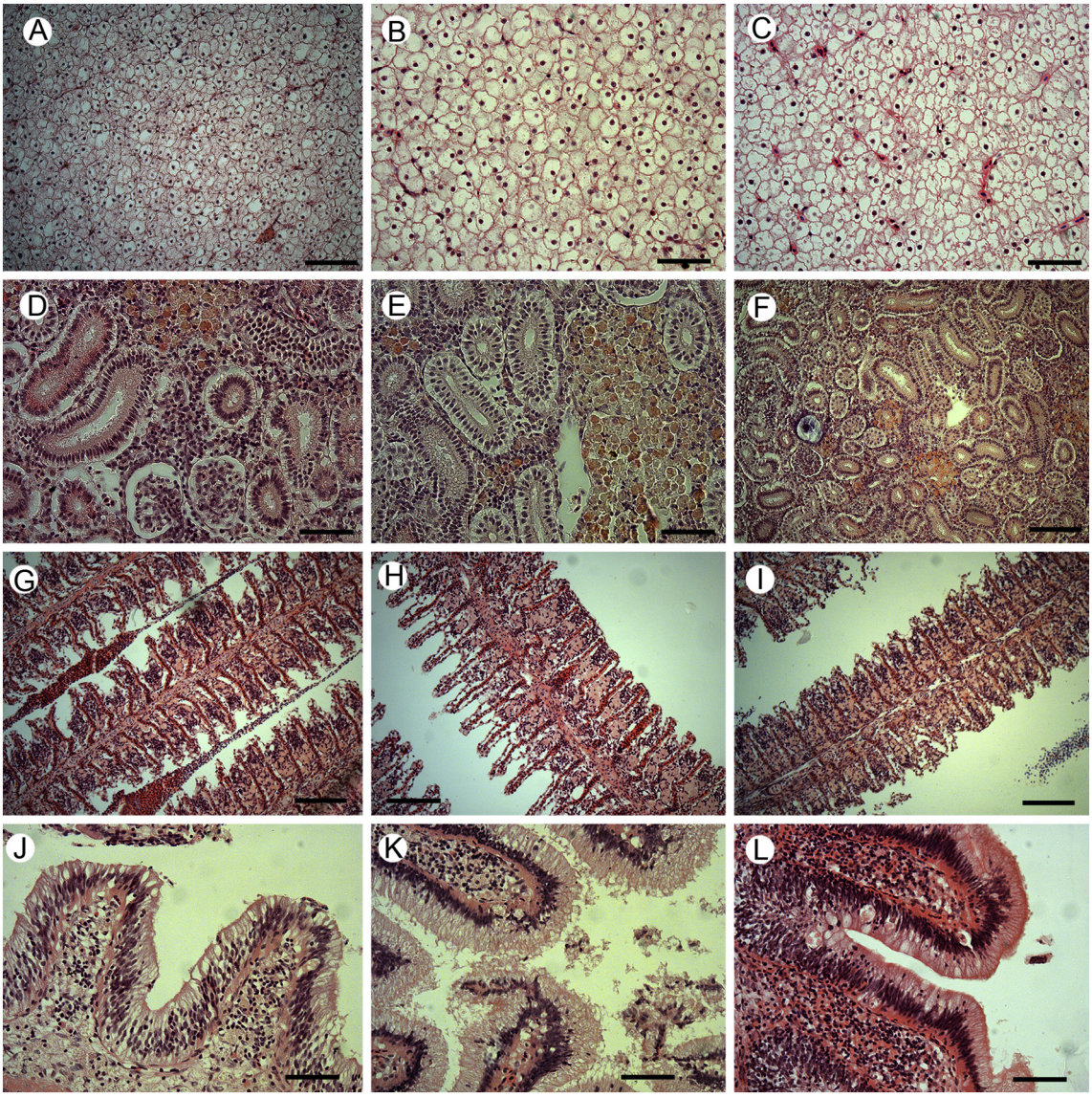

Fig. 1. Histopathological alterations of tissues of Prussian carp *Carassius gibelio* from Lake Ludoš, 2011. Legend: A–C) histopathological alterations in the liver: (A) loss of cordlike parenchymal structure; (B) onion-shaped hepatocytes with clear cytoplasm; (C) pyknosis (arrow) and fields of anucleated cells (asterisk). D–F) histopathological alterations in the kidney: (D) glomerulopathy with intense dilatation of Bowman's capsule (asterisk); (E) vacuolization of tubules (arrow) and macrophage infiltration (asterisk); F) macrophage infiltration. G–I) histopathological alterations observed in the gills: (G) fusions of lamellae; (H) oedema and epithelial lifting (arrow); (I) intensive proliferations of chloride cells. J–L) histopathological alterations in intestines: (J) intensive oedematous alteration in the lamina propria; (K) desquamation of enterocytes; (L) hypertrophies of goblet cells. H&E staining. Scale bars: A, F, G, I - 100 μm; B, C, D, E, J, K, L - 50 μm.

parameters, OTM and TI, and showed a difference in the degree of DNA damage in three tissues (blood, liver and gills). Mean values were: blood (OTM-0.71, TI-4.85), liver (OTM-1.32, TI-7.26), gills (OTM-1.12, TI-8.03) (Figs. 2 and 3). According to the results, the blood sustained the lowest level of DNA damage in comparison with cells of the liver and gills, which showed a similar response.

3.3. Hydrogen peroxide treatment of the lake water in vitro

In the hydrogen peroxide-dosing experiments, the total intracellular MC content in the Lake Ludoš water was 4.5 μg/L consisting of the following MC variants: MC-LR 32.9%, MC-YR 24%, MC-RR 20%, dmMC-RR 16%, dmMC-LR 6% and dmMC-YR 1% (Fig. 4).

The intracellular concentration of MCs was substantially reduced only after exposure for 12 or 48 h at the highest dosage concentration of 20 mg hydrogen peroxide per L. The lower dosages did not decrease the intracellular MC concentration as compared to the control (0 mg hydrogen peroxide per L), at least during 48 h of exposure.

The concentration of Chlorophyll *a* in the water used for the hydrogen peroxide trials was 104 mg/m^3, which indicates a highly eutrophic state of the lake. Qualitative analysis of the untreated water samples showed the presence of the cyanobacterial species *Aphanocapsa incerta* (Lemmermann) G. Cronberg & Komárek, *Microcystis wesenbergii*, *Planktothrix agardhii*, *Dolichospermum affine* (Lemmermann) Wacklin, L. Hoffmann & Komárek, *Anabaenopsis circularis* (G.S. West) Woloszynska & V. Miller, *Cylindrospermopsis raciborskii* and *Pseudanabaena limnetica*. Cyanobacterial cells constituted 80% of the phytoplankton community. The species *Microcystis wesenbergii* and *Planktothrix agardhii* represented around 95% of the cyanobacterial community. Microscopic comparison of untreated and treated cyanobacterial samples at given timepoints revealed certain morphological changes in the dominant cyanobacterial species *Microcystis wesenbergii* (Fig. 5) and *Planktothrix agardhii*. It is important to point out that described changes listed below did not refer to the observed samples in their entirety, but that the number of altered colonies and filaments increased with the exposure time and the increase of applied concentrations of hydrogen peroxide.

In the samples containing 2 mg H_2O_2/L, minor changes in the morphology of *Microcystis wesenbergii* colonies were observed 48 h after the hydrogen peroxide application (Fig. 5F). There was an increase in the number of individual cells released from the colonies compared to the corresponding control samples, and the outer borders of the mucilage had become less visible. The filaments of *Planktothrix agardhii* appeared to be undamaged.

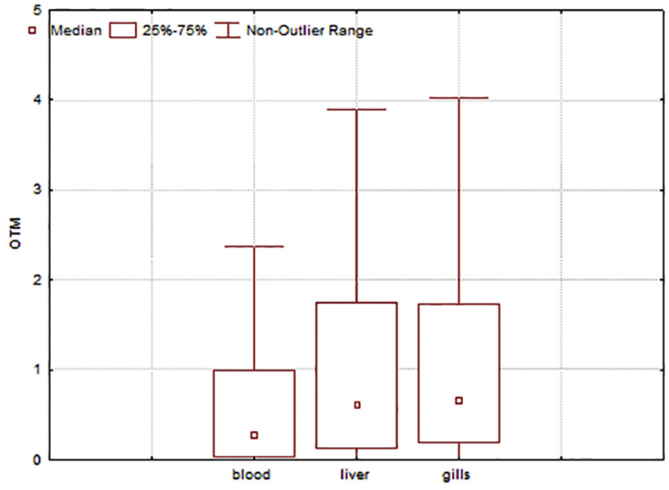

Fig. 2. DNA damage expressed by olive tail movement (OTM), showing the difference in the degree of DNA damage in three tissues of Prussian carp.

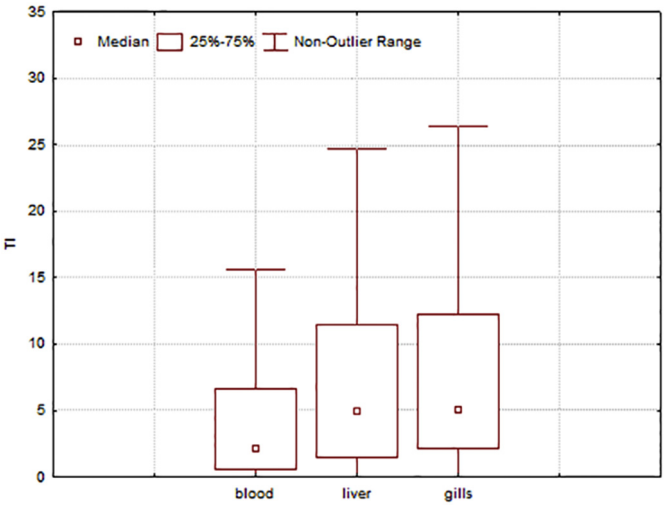

Fig. 3. DNA damage expressed by tail intensity (TI), showing the difference in the degree of DNA damage in three tissues of Prussian carp.

After 12 h at 5 mg H_2O_2/L, a clustering of cells was observed in the central part of *Microcystis wesenbergii* colonies (Fig. 5H). Biomass density decreased in treated water after 48 h. The most pronounced morphological changes were observed in many *Microcystis wesenbergii* colonies which appeared to be degraded. Larger colonies were fragmented into the smaller ones and the outer walls of the surrounding mucilage were less visible (Fig. 5I). Many shortened fragments of *Planktothrix agardhii* were also observed.

In the samples containing 20 mg H_2O_2/L an immediate influence of the oxidant was detected (Fig. 5J). Clusters of brighter cells were positioned in the central parts of *Microcystis* colonies. Filament fragmentation of *Planktothrix agardhii* was observed with the release of many individual cells from the filaments. After 12 h, the biomass density was significantly lower and bright clusters of cellular debris were dominant inside the colonies (Fig. 5K). A great number of individual cyanobacterial cells, both deformed and empty, were also present. The amount of unaffected biomass was even lower after 48 h (Fig. 5L).

4. Discussion

The status of Lake Ludoš has great ecological importance for the preservation of the flora and fauna diversity at this Ramsar site. Based on Chlorophyll *a* (Felfoldy, 1980), Lake Ludoš in 2011 was highly eutrophic. A historical overview of the Lake Ludoš demonstrated an annual presence of cyanobacteria since the 1970 (Đukić et al., 1991b; Dulić, 2002; Đurđević, 2007), and cyanobacterial blooms have been a frequent phenomenon in this lake (Table 1). The results from our investigation in

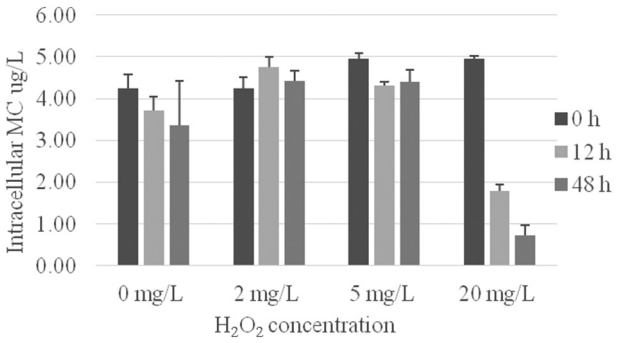

Fig. 4. Effect of exposure of Lake Ludoš plankton samples, including cyanobacteria, to hydrogen peroxide measured as intracellular MC concentrations.

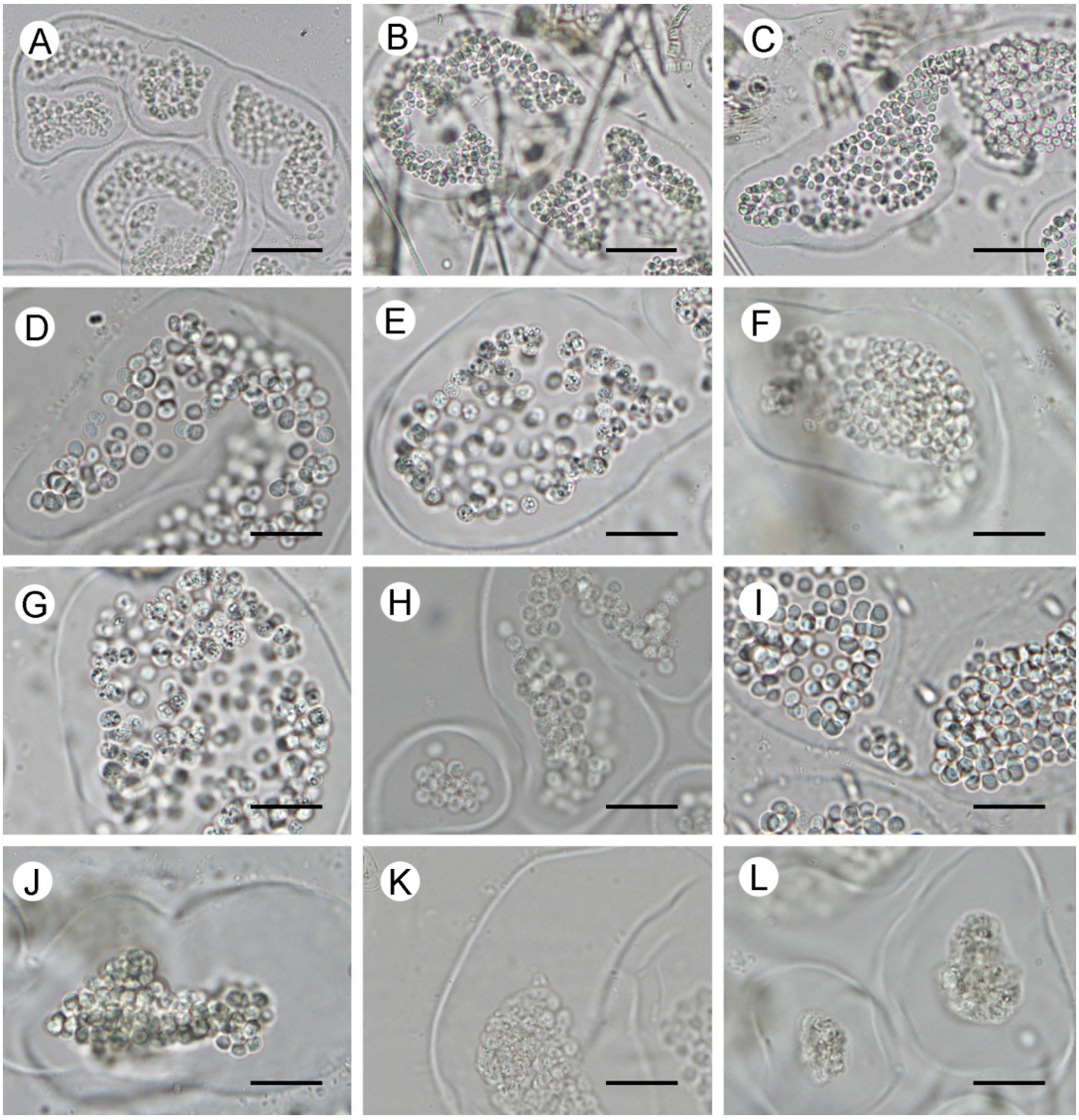

Fig. 5. Morphological changes of *Microcystis wesenbergii* colonies as a result of hydrogen peroxide treatment. Legend: A–C) Morphological changes at 0 mg/L H_2O_2, at 0 h, 12 h, 48 h, respectively. D–F) Morphological changes at 2 mg/L H_2O_2, at 0 h, 12 h, 48 h, respectively: (F) reduced visibility of outer borders of the mucilage. G–I) Morphological changes at 5 mg/L H_2O_2, at 0 h, 12 h, 48 h, respectively: (H) clusters of cells in the central part of the colonies; (I) reduced visibility of outer borders of the mucilage. J–L) Morphological changes at 20 mg/L H_2O_2, at 0 h, 12 h, 48 h, respectively: (J) clusters of brighter cells in central parts of colonies; (K) domination of brighter clusters in central parts of colonies; (L) domination of cellular outlines. Magnifications: A–C) - 200×; D–L) - 400×. Scale bars: A–C) - 100 μm; D–L) - 50 μm.

2012 have showed the same trend with several species blooming (≥10,000 cells/mL which according to Falconer (1998) is a criterion for blooming), with the most abundant cyanobacterial species being *Limnothrix redekei*, *Pseudanabaena limnetica*, *Planktothrix agardhii* and *Microcystis* spp. Most of these species were found blooming in the previous investigations of Lake Ludoš, as in the other investigated lakes (Palić lake, Gazivode lake, Sjeničko lake, Veliki Zaton lake) and further aquatic ecosystems in Serbia including rivers, canals, ponds, fishponds, and reservoirs used for irrigation and drinking water supply (Svirčev et al., 2014b). These cyanobacteria are frequently found practically all over the world. Among the species observed in Lake Ludoš (Table 1), *Cylindrospermopsis raciborskii* can be regarded as invasive and its distribution in Europe is expanding (Padisák, 1997; Svirčev et al., 2014b; Kokociński et al., 2017). For example, this species has been documented in Belarus (Micheeva, 1968), Poland (Burchardt, 1977), Bulgaria (Stoyneva, 1995), Germany (Stüken et al., 2006), Greece (Moustaka-Gouni et al., 2009), Italy (Barone et al., 2010), Hungary (Antal et al., 2011), Croatia (Mihaljević and Stević, 2011) and Spain (Romo et al., 2013). Another invasive species recently observed in Lake Ludoš, *Sphaerospermopsis aphanizomenoides*, has also been documented in other European countries including Czech Republic (Zapomelová et al., 2011; Gadea et al., 2013), the Netherlands (Veen et al., 2015), and Romania (Caraus, 2017).

Previous studies with blooms and isolates of *Limnothrix* (Bernard et al., 2011; Humpage et al., 2012), *Pseudanabaena* (Oudra et al., 2001; Maršálek et al., 2003; Nguyen et al., 2007; Gantar et al., 2009), *Planktothrix* (Lindholm and Meriluoto, 1991; Luukkainen et al., 1993; Fastner et al., 1999), and *Microcystis* (Kiviranta et al., 1992; Luukkainen et al., 1994) from other sources have shown that members of these genera can produce toxic compounds. Most of the total MC pool remains intracellular until bloom aging and lysis occurs (e.g. Chorus and Bartram, 1999; Griffiths and Saker, 2003). Intracellular toxicity in *Artemia salina* bioassay was detected only in July 2011, as well as a high MC-LR equiv. concentration according to PPI assay of samples from off the pier of Lake Ludoš (828 μg/L MC-LR equiv.). The same method was used for the first cyanotoxin analyses in Lake Ludoš in the summer of 2006 during the blooming of *Microcystis*, when a high concentration of MC-LR equiv. was also detected (603 μg/L), this value

being one the highest recorded in Serbia during the investigations of Simeunović (Simeunović, 2009; Simeunović et al., 2010). Furthermore, the MC results from the ELISA tests supported the PPI assay findings in 2011 (although the observed concentrations were much lower). The presence of MC/NOD and STX was also detected in the 2012 water samples.

Cyanotoxins can affect a wide range of aquatic and other organisms although there are few studies on MC accumulation in the macrophytes: reed, cattail and royal blue waterlily. During the investigation of aquatic plants, MC-RR was found in the rhizome (0.22 ng/g dw) of *Phragmites australis*. The Common Reed *Phragmites australis* (syn. *Phragmites communis*) is an emergent aquatic macrophyte of major ecological importance (Ostendorp, 1989; Ye et al., 1998; Pflugmacher, 2002; Mészáros et al., 2003), and the decline of its populations in Europe has attracted the attention of many researchers (e.g. Ostendorp, 1989; Armstrong and Armstrong, 2001; Mészáros et al., 2003). In the presence of cyanobacterial blooms and cyanotoxins in eutrophic lakes, some authors have observed a decrease in the abundance and diversity of macrophyte species (e.g. Harper, 1992). Observations include: reduced shoot length and dry weight, as well as deterioration of nutrient and oxygen absorption (Yamasaki, 1993), growth inhibition and histological alterations (Máthé et al., 2000; Máthé et al., 2007), and MC accumulation in stems, rhizomes and leaves (Pflugmacher et al., 2001). An accumulation of MC-RR in the rhizome of cattail *Typha latifolia* (0.05 ng/g dw) and royal blue water lily *Nymphaea elegans* (0.02 ng/g dw) from Lake Ludoš was observed.

Cyanotoxins can be accumulated in aquatic wildlife (Magalhães et al., 2003; Romo et al., 2012; Drobac et al., 2016), and transferred to higher trophic levels causing various negative effects on tissues of different organisms (Xie et al., 2005; Peng et al., 2010; Malbrouck and Kestemont, 2006). The present research showed accumulation of two MC variants (MC-LR and MC-RR) in the tissue samples of the omnivorous fish *Carassius gibelio* from the Lake Ludoš. It was notable that, in both investigated periods, MCs were found in the intestines (MC-LR) and muscle samples (MC-RR), but that no MCs were found in the livers. In 2011, the fish gills were also positive for MC-RR, and in 2012, kidneys and gonads were found to accumulate MC-LR. Previous research has shown that MCs can accumulate in various fish organs including liver, muscles, kidneys, intestine, gallbladder, spleen, gonads, blood and brain (Cazenave et al., 2005; Lei et al., 2008). However, different fish species can accumulate different concentrations of cyanotoxins in their tissues depending on their diet (Xie et al., 2005; Qiu et al., 2007; Zhang et al., 2009). The apparent absence of MC in the livers could be due to detoxification processes which vary depending on species, organ, MC congener and metabolism (Snyder et al., 2002; Xie et al., 2004; Malbrouck and Kestemont, 2006; Adamovský et al., 2007), but MC could also remain covalently bound to protein phosphatases in the tissue (MacKintosh et al., 1990; Williams et al., 1997a, 1997b; Ibelings et al., 2005). Accumulation of MC in the muscle can vary regardless of the concentrations of the cyanotoxins measured in the water (Mohamed et al., 2003; Deblois et al., 2008) due to detoxification processes (Mohamed and Hussein, 2006). It should also be noted that *Carassius gibelio* is used in the human diet, and ingested concentrations of these cyanotoxins should be below tolerable daily intake of 0.04 μg MC-LR equiv./kg body weight/day (Chorus et al., 2000).

The histology of tissues from Prussian carp from the lake indicated the fish livers to be the most severely damaged. This was probably due to the hepatotoxic nature of MCs (Metcalf and Codd, 2012). Loss of the typical cord-like parenchymal structure, dissociation of cells, loss of shape in hepatocytes, glycogen loss, vacuolization and necrosis with pyknosis are very common alterations after exposure of fish to MCs (reviewed in Svirčev et al., 2015). Loss of parenchymal structure, dissociation of hepatocytes and loss of hepatocyte shape may be attributed to binding of MCs to protein phosphatases (PP1 and PP2A), but also to the fact that MCs disrupt the protein expression of many cytoskeleton proteins (Li et al., 2011).

The present study also confirms that, although MCs are primarily hepatotoxins, they can also exert toxic effects on other organs. Recorded glomerulopathy with dilatation of Bowman's capsule and vacuolization of tubules from the fish in Lake Ludoš are the most frequently described histopathological alterations in kidneys after exposure to MCs (reviewed in Svirčev et al., 2015).

The most common alterations to the gills observed during this research and in general were intense hyperplasia with complete fusions of lamellae as well as oedema and epithelial lifting. These features were in agreement with the gill pathology of brown trout from Loch Leven, Scotland with a MC-containing bloom of *M. aeruginosa* (Rodger et al., 1994). These alterations are considered to be protective mechanisms which decrease the respiratory area and blood-to-water surface, thus decreasing xenobiotic uptake (Poleksić and Mitrović-Tutundžić, 1994; Lujić et al., 2015). Hyperplasia of chloride cells may be the result of a direct effect of MCs since Gaete et al. (1994) and Vinagre et al. (2003) observed inhibitory effects of these toxins on chloride cell ionic pumps. Due to the constant contact with the water, delicate structure and large surface area, gills are the first organs to be affected by waterborne xenobiotics (Poleksić and Mitrović-Tutundžić, 1994; Bernet et al., 1999; Lujić et al., 2015). However, the effects of MCs on fish tissue and organ structure and function in controlled exposure studies are poorly documented.

Since cyanobacteria are a part of the Prussian carp diet, histopathological alterations observed in the intestines may be a result of cyanotoxins. Necrotic enteritis with villi desquamation was also observed in tench *Tinca tinca* L. (Atencio et al., 2008), silver carp *Hypophthalmichthys molitrix* Valenciennes (Ferreira et al., 2010), common carp (*Cyprinus carpio* L. (Drobac et al., 2016), and medaka *Oryzias latipes* Temminck & Schleger (Trinchet et al., 2011).

The presence of cyanotoxins in the Lake Ludoš water could also explain the DNA damage detected in Prussian carp as revealed via comet assay in 2012. Based on the previous research, three tissues were selected: blood, liver and gill cells (Alink et al., 2007; Sunjog et al., 2013; Sunjog et al., 2014; Aborgiba et al., 2016; Sunjog et al., 2016; Kostić et al., 2016; Kračun-Kolarević et al., 2016). It is known that different tissues can accumulate pollutants to different degrees, depending on their biochemical characteristics (Suicmez et al., 2006; Sunjog et al., 2016). Blood is usually the first choice tissue in comet assay because of easy collection and without need for cellular dissociation. In this work the choice to use gill and liver cells, in addition to red blood cells, was that the gills represent the first organ which is in direct contact with water and, consequently, with the pollutants present in it, while the liver has an important role in xenobiotic metabolism and accumulation (Kilemade et al., 2004; Sunjog et al., 2014). According to the results, blood had the lowest level of DNA damage in comparison with liver and gills, which showed a similar response. Similarly, the results from our previous studies (Sunjog et al., 2013, 2014, 2016) also showed that gills and liver were often the most damaged tissue organs.

The pathological findings in the fish from Lake Ludoš, as well as the bulk of previous results, provide evidence that the ecological balance of this Ramsar site is impaired, which is the exact opposite of the desired intention. Although the goal is to preserve this important natural resource for numerous bird species, it appears that ideal conditions for extensive cyanobacterial growth are also being maintained. The persistent occurrence of cyanobacteria and cyanotoxins can lead to many detrimental effects on other aquatic organisms, and can consequently endanger birds (Fig. 6). The presence of cyanotoxins has been linked with bird mass mortalities (e.g. Krienitz et al., 2003; Ibelings and Havens, 2008; Stewart et al., 2008), histopathological alterations (Skocovská et al., 2007) and cyanotoxin accumulation in various bird organs (Henriksen et al., 1997; Sipiä et al., 2004, 2006, 2008; Pasková et al., 2008; Chen et al., 2009).

The Ramsar Convention supports and requires a wise use and protection of wetlands through a range of measures to ensure that the ecological character of Ramsar Sites is protected and preserved. As

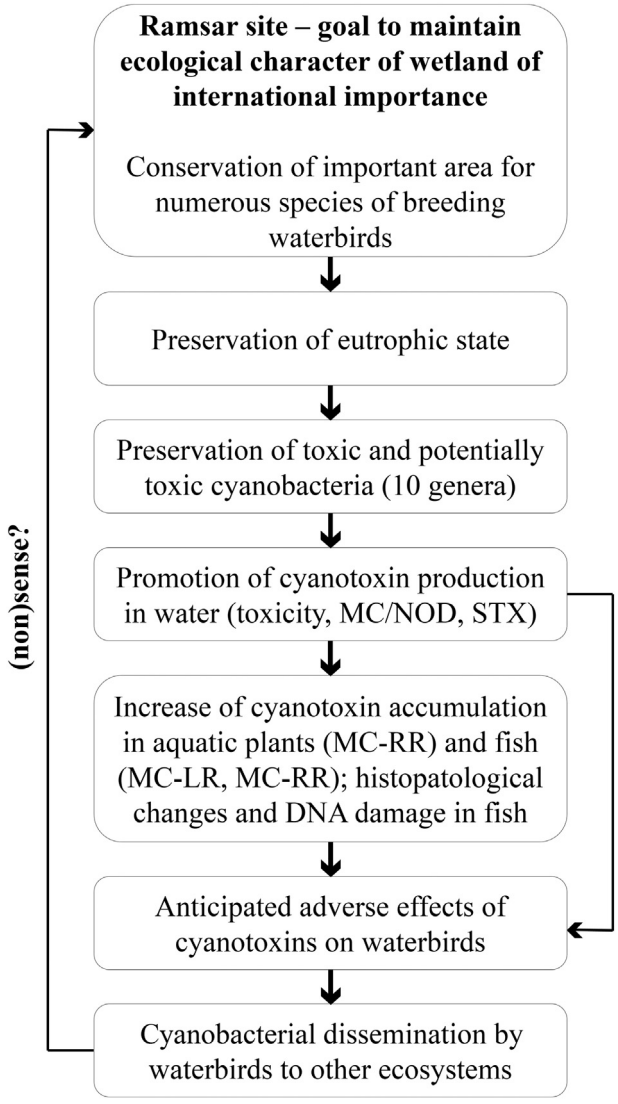

Fig. 6. Schematic representation of the complexity of preserving the eutrophic state of Lake Ludoš. Conservation of the lake in order to protect the waterbirds (without urgent control of eutrophication) actually endangers them and the rest of the biota in this wetland habitat, and possibly other ecosystems through anticipated dissemination of toxigenic cyanobacteria by waterbirds.

wetlands offer a whole range of goods and ecosystem services (de Groot et al., 2002), these systems have a relatively high priority in restoration. Furthermore, several authors have suggested measures for water quality improvement at this lake, including the identification of source water with lower nutrient content for maintaining the volume of the lake, sediment removal (Dulić, 2002; Seleši, 2006), and sediment phytoremediation (Radić et al., 2013).

In this investigation, preliminary in vitro tests of the effectiveness of hydrogen peroxide treatment were performed. This treatment has been proposed as a method to selectively kill cyanobacterial populations in lakes and successful in-lake trials have taken place elsewhere, e.g. in the Netherlands (Matthijs et al., 2012; Weenink et al., 2015). However the generally recommended doses of hydrogen peroxide (2–5 mg/L, Matthijs et al., 2012) did not kill the cyanobacteria present in Lake Ludoš water samples in laboratory trials. In these tests only a slight inhibitory effect on the viability of the cyanobacteria was observed at 5 mg hydrogen peroxide per L when the viability of the cyanobacterial populations was measured as remaining cell-bound MC (leakage of MC would indicate cell damage). Further, it appears that the doses 2 mg per L and 5 mg per L may even have had a slight stimulatory effect on MC production (Fig. 4) but requires further investigation. Microscopic analysis of samples exposed to these concentrations (2–5 mg per L) confirmed a certain degree of hydrogen peroxide influence including the disintegration of *Microcystis* colonies and mucilage and shortening of *Planktothrix* filaments (Fig. 5). An increase of cell-bound MC content co-occurred with some of the observed morphological changes at 2–5 mg H_2O_2/L which may be explained by the physiological role of MCs in cyanobacteria. The doses of 2 and 5 mg H_2O_2/L appeared to be too low in the laboratory exposure trials to exert cyanobacteriocidal potential, but still high enough to provoke the cells to accumulate cell-bound (intracellular) MCs, whose presumed roll, among possible other roles, may be to protect the photosynthetic apparatus and prevent photo inhibition caused by oxidative stress (Downing et al., 2015), in this case caused by the addition of hydrogen peroxide. This hypothesis is supported by the study performed by Wood et al. (2011), which claimed that the increase in total intracellular MCs per cell, together with the up-regulation of *mcyE* gene expression, may be due to some external or internal stimuli. While the higher dose of hydrogen peroxide (20 mg/L) reduced the viability of the cyanobacteria, it is known that the viability of zooplankton is also compromised at hydrogen peroxide concentrations higher than 5 mg/L (Weenink et al., 2015). Thus, in terms of safety margins, it would be very questionable to use the more efficient 20 mg/L hydrogen peroxide dose in order to combat the dense cyanobacterial population in the lake.

According to current best practice, hydrogen peroxide should be primarily used to treat an emerging cyanobacterial population during spring and early summer when the likely MC concentrations in the lake are still relatively low and a sudden extracellular release of the toxins due to cell lysis is unlikely to cause concern (Matthijs et al., 2012). Chlorophyll *a* remained fairly high in Lake Ludoš in the investigated year, so the required hydrogen peroxide concentrations would thus be high. Hence, it is possible that Lake Ludoš is not amenable to a successful hydrogen peroxide treatment in its present state with dense cyanobacterial populations. However, in-situ trials with hydrogen peroxide would be necessary to determine unequivocally whether this treatment would be of assistance in reducing cyanobacterial populations. It is recommended that the total capacity of the lake (including the high load of organic matter that consumes applied hydrogen peroxide) should be dramatically lowered through nutrient removal before other treatments targeting cyanobacteria are being applied.

So far, an effective and long-term solution to reduce cyanobacterial blooms in Lake Ludoš has not been attained. However, there is some consensus among researchers: an unsatisfactory state of water quality has persisted in the lake for almost 50 years, and the need for intervention is clearly apparent (Table 5).

The eutrophication problem that affects Lake Ludoš is not an isolated one of local concern, but a global issue which is becoming more and more pervasive. Wetlands in Europe have been modified, drained and destroyed during earlier centuries. Currently, <20% of original, pristine wetland areas remains (Verhoeven, 2014). These ecosystems also play an important role in the life cycle of migrating birds. Whether bird migration contributes to the geographical dissemination of toxigenic cyanobacteria, including alien and invasive species (Kokociński et al., 2017), requires investigation, but this scenario appears likely. Schlichting (1960) found 86 viable species, including cyanobacteria, from the feet, 25 from the feathers, 25 from the bills, 14 from the gullets, and 12 from the faecal material of the birds. When species of phytoplankton passed through the gut apparently undamaged, only a few remained in a viable condition (Atkinson, 1971). Other studies also demonstrate that many organisms can potentially be dispersed by waterbirds (e.g. Figuerola and Green, 2002). Futhermore, cyanobacteria compose a major part of the diversity of airborne microalgae (Sahu and Tangutur, 2014), which can originate from aquatic environments (Lee and Eggleston, 1989).

Cyanobacterial dispersal could lead to unexpected biogeographic expansion into freshwater ecosystems (Lebret et al., 2013), which

Table 5
Historical overview of published findings and recommendations concerning the water quality of the Lake Ludoš (text in *italic* was translated from Serbian original publications).

Findings and suggestions	Reference
The quantity of submerged plants is drastically reduced due to algal abundance, and thus the number of floating bird nests is also reduced. It is necessary to take serious measures in order to preserve this natural reserve.	Futo, 1972
We believe that the Lake Ludoš ecosystem is very vulnerable and that without rapid and radical intervention for its rehabilitation, this lake would vanish in a very short time.	Đukić et al., 1991b
Very unfavorable ecological condition of Lake Ludoš imposes the need for radical measures in order to slow down the aging process of the lake. Protection should be given priority over the use of this area, because of its value - Lake Ludoš deserves it.	Dulić, 2002
Lake Ludoš is today a very serious patient, and in the process of succesion into a pond.	Seleši, 2006
Consequently, the achievement of good ecological status of Lake Ludoš is needed, and the aim in the next decade is to develop protection status and ecohydrological management.	Nemeš and Matavulj, 2006
Negative trend in increasing the amount of organic matter in the water needs to be placed under control in order to preserve and enhance the ecological status of Lake Ludoš.	Đurđević, 2007
Algal and cyanobacterial bloom episodes in surface waters of Vojvodina region, including Lake Ludoš, deserves immediate attention by the authorities in charge for preserving the environmental quality. Detected presence in mass development of the potentially toxic and toxic species represents latent and real threat to human and animal health, but also an importan indicatior of the rapid water quality deterioration.	Svirčev et al., 2008
Organic loading results in a significantly higher number of saprophytic heterotrophs, and less autochthone oligotrophs thereby reducing the capacity for water self-purification, pointing to the rapid process of eutrophication in Lake Ludoš.	Rudić et al., 2015
Serious eutrophic processes are present in Lake Ludoš due to agricultural runoff and untreated wastewater. Both water quality and microbiological results have confirmed the highly eutrophic status of Lake Ludoš.	Grabić et al., 2016
Dibenzo[*a*,*h*]anthracene (DahA) and fluorene (Flo) were found at levels indicative of causing adverse effects to biota. The historical pollution at this Ramsar site shows the importance of taking care in the design of future monitoring programmes.	Grba et al., 2017

introduces competition between new and already present microorganisms (De Meester et al., 2002), and changes community dynamics (e.g., Genitsaris et al., 2011a). Furthermore, airborne microalgae and cyanobacteria were shown to be associated with numerous health issues such as allergy, skin irritation, hay fever, rhinitis, sclerosis and respiratory problems when aerosolized and inhaled (Genitsaris et al., 2011b).

Based on the presented arguments, an important question arises: is a policy where natural habitats should be protected, but are in a bad ecological state, justified? Or are we, in this manner, preserving the problem which can potentially affect every living being in the proximity of this ecosystem? Because of the biodiversity and ecosystem services which wetlands can provide, many governmental and non-governmental organizations strive for wetland protection and restoration, supported by the Ramsar Convention, EU directives and national legislation for nature protection (Verhoeven, 2014). The present investigation indicates that a more systematic and detailed monitoring of the water quality and environmental toxicology of Lake Ludoš is necessary, together with the most appropriate management strategy to achieve a better ecological state and minimize further degradation of Lake Ludoš.

5. Conclusion

Results obtained in this research including Chlorophyll *a* concentrations, as well as qualitative and quantitative analyses of cyanobacteria, indicate that the ecological condition and water quality of Lake Ludoš are highly eutrophic. Cyanobacteria were blooming and there is a risk of recurrent cyanotoxin production and release. High mortality of *Artemia salina* in bioassays of lake water samples from July 2011 indicated the presence of toxic compounds. This was confirmed with PPI and ELISA for MCs, with these cyanotoxins being identified in fish and plant species from the lake using LC-MS/MS. As a potential solution for the reduction of the cyanobacterial population in Lake Ludoš, the potential efficiency of hydrogen peroxide treatment was examined. Although further research, including in-situ hydrogen-peroxide incubations in the lake, is required, the initial laboratory results showed that this method may not be readily applicable, which further affirms the low water quality and ecological state of the lake. Conservation of the lake in order to protect the waterbirds (without urgent control of eutrophication) actually endangers them and the rest of the biota in this wetland habitat, and possibly other ecosystems. Since Lake Ludoš is on the Ramsar list of wetlands of international importance, the health of this ecosystem is of great concern. Therefore, continued monitoring of cyanobacterial and cyanotoxin occurrence, as well as further studies of cyanotoxin effects and consequences on the ecosystem are necessary. Most importantly, a rapid intervention aiming at the restoration of the good ecological status in Lake Ludoš should be of highest priority, so that preservation of this Ramsar site would be fully justified.

Conflicts of interest

The authors declare no conflict of interest.

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