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Protected Freshwater Ecosystem with Incessant Cyanobacterial Blooming Awaiting a Resolution

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Abstract: For 50 years persistent cyanobacterial blooms have been observed in Lake Ludoš (Serbia), a wetland area of international significance listed as a Ramsar site. Cyanobacteria and cyanotoxins can affect many organisms, including valuable flora and fauna, such as rare and endangered bird species living or visiting the lake. The aim was to carry out monitoring, estimate the current status of the lake, and discuss potential resolutions. Results obtained showed: (a) the poor chemical state of the lake; (b) the presence of potentially toxic (genera *Dolichospermum*, *Microcystis*, *Planktothrix*, *Chroococcus*, *Oscillatoria*, *Woronichinia* and dominant species *Limnothrix redekei* and *Pseudanabaena limnetica*) and invasive cyanobacterial species *Raphidiopsis raciborskii*; (c) the detection of microcystin (MC) and saxitoxin (STX) coding genes in biomass samples; (d) the detection of several microcystin variants (MC-LR, MC-dmLR, MC-RR, MC-dmRR, MC-LF) in water samples; (e) histopathological alterations in fish liver, kidney and gills. The potential health risk to all organisms in the ecosystem and the ecosystem itself is thus still real and present. Although there is still no resolution in sight, urgent remediation measures are needed to alleviate the incessant cyanobacterial problem in Lake Ludoš to break this ecosystem out of the perpetual state of limbo in which it has been trapped for quite some time.

Keywords: cyanobacteria; blooms; microcystin; Lake Ludoš

1. Introduction

In the very north of Serbia there is an old and unusual lake, Lake Ludoš, with beautiful open water landscapes surrounded by reeds, wetlands and steppe. The environment is rich in many plant and animals species. European pond turtles, various amphibians, otters, moles, rabbits, foxes and roe deer have found their home there. What makes Lake Ludoš especially famous, and validates the name originating from the Hungarian word "lud" meaning goose, is that there are more than 200 bird

species, including rare and endangered species, nesting or resting during their migration. Because of all this, Lake Ludoš is recognized and protected as a special nature reserve on the list of Ramsar sites.

One part of the lake is often visited by fishermen, but their catch mostly consists of the very resilient and adaptable Prussian carp (*Carassius gibelio* (Block, 1782)) which are quite small in size. This may be related to the fact that the water of Lake Ludoš has an intense green color throughout the year caused by cyanobacteria (e.g., [1]). Their extensive growth and blooming causes many problems in freshwater ecosystems, including this one. Cyanobacteria can produce cyanotoxins that affect other organisms, including valuable flora and fauna, especially aqueous organisms such as fish [2]. Cyanobacteria also present a threat to humans, such as fishermen, who may be exposed to cyanotoxins through contaminated food, inhalation and direct contact [3,4]. In addition to human health, the health of this important ecosystem is also jeopardized. Furthermore, this "disease" could also be transmissible, since there is a possibility that water birds visiting the lake during their migration path can disseminate toxic cyanobacteria [5].

Lake Ludoš is only one of many aquatic ecosystems in Serbia where cyanobacteria are present and blooming [6]. What sets this ecosystem apart from many others is that it has been known for perpetual blooming in the last 50 years. Previous research has shown that the lake is in a poor ecological state which leads to the question of whether the protection of this natural habitat in a bad ecological state is justified. The cyanobacterial problem, which can potentially affect every living being in the proximity of this ecosystem, has also been preserved as measures to improve the water quality have not been undertaken on the lake [5]. The problem of cyanobacteria in Lake Ludoš has been addressed during our previous research when potentially toxic cyanobacterial species, cyanotoxins in water, macrophytes and fish tissues were detected, as well as histological alterations and DNA damage in fish tissue (see [5]). Six years later further monitoring was carried out in order to estimate the current state of this ecosystem. Therefore, several investigative steps have been taken: monitoring of physical and chemical parameters of the lake; assessment of qualitative and quantitative analyses of cyanobacteria; the first survey of the cyanotoxin coding genes; determination of cyanotoxins in water and fish; analysis of histopathology of different fish organs; and discussion of potential health risks and resolutions.

Freshwater ecosystems throughout the world have similar problems in connection to cyanobacterial blooming and cyanotoxin production. Recent publication of a global geographical and historical overview of cyanotoxin distribution demonstrated the presence of well-known cyanotoxins in each continent (including 520 lakes) and their harmful consequences on human health [4]. Hence, this issue is of global concern. The present investigation aims to assist in making appropriate decisions and measures for the remediation of not only this, but many other old and rapidly aging and protected lakes.

2. Materials and Methods

2.1. Sampling Site and Sampling of Water and Fish

Lake Ludoš (Figure 1) is a one of the few preserved shallow lakes in the region. It has a maximum depth of 2.25 m, is 4.5 km long, and it represents a remnant of the Pannonian Sea. In most places the depth does not exceed 1 m, and it may be frozen for more than three months a year. It is located in the north part of Serbia, near the city of Subotica. The lake and the associated wetland ecosystem is highly valued due to the great biological diversity, and as such the area is classified among wetlands of international significance. The quality of the lake's water is of great importance for the preservation of the flora and fauna connected to this marshland ecosystem.

The lake is supplied with water from aquifers and Kereš River. However, in the northern part, Lake Ludoš receives water from the canal Palić-Ludoš which is the recipient of wastewaters from the Palić settlement. Water treatment of these wastewaters is still inadequate and the canal water is characterized by a high level of organic pollution, high concentrations of salt and very high nutrient concentrations. The inflow of untreated and partially purified waters in Lake Ludoš contributes to the deterioration of the water quality and the increase of the sludge quantity [7–11].

Figure 1. Pier view of the blooming Lake Ludoš in July 2018.

Water samples were collected from the surface water layer within the littoral zone (pier next to the visitor center) (46.103207 N, 19.821360 E) and from the center of the lake (46.102159 N, 19.821149 E) in March, May, July and September of 2018. Samples of Prussian carp were collected from the center of the lake before and after the summer (March and September 2018) with gillnets of various mesh sizes and a standard electrofishing device.

2.2. Analyses of Physical and Chemical Parameters

Multi-parameter WTW probes were used for carrying out in situ measurements in the field and the following physical and chemical parameters were determined: temperature, pH, 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 in the laboratory conditions with a Pastel Ultraviolet (UV) Secomam.

2.3. Qualitative and Quantitative Analyses of Cyanobacteria

The phytoplankton samples for the cyanobacterial qualitative analysis were collected by sweeping a plankton net (netframe 25 cm ø, net mesh 23 µm). All samples were immediately preserved in a Lugol solution. Taxonomic identifications of cyanobacteria were made according to several taxonomic keys [12–15] and were done under a light microscope Motic BA310 using a Bresser (9MP) digital camera and Micro Cam Lab software. For the quantitative analysis of phytoplankton, the 15 L of water was collected by sweeping a plankton net at the depth of 0.3 m in March, while in May, July and September only 200 mL of water were collected directly from the lake as a result of high bloom density. The quantitative analysis was made by using the Utermöhl method [16] under a Motic AE 2000 inverted microscope. The phytoplankton individuals were sedimented and cyanobacteria quantified on the chamber (i.e., transects) with an inverted microscope at different magnifications depending on their size (100×, 400×) and expressed as the number of cells per mL.

2.4. Cyanotoxin Coding Gene Analyses

2.4.1. Samples—Reference Strains for Polymerase Chain Reaction (PCR) Analysis

Reference strains were obtained from Pasteur Culture Collection (PCC), National Institute for Environmental Studies Microbial Culture Collection (NIES), Australian National Algae Culture Collection (CS), and Finnish Environment Institute (SYKE). They consisted of:

- microcystin (MC) producers: PCC7820 (*Microcystis aeruginosa*), NIES-107 (*Microcystis wesenbergii*);

- cylindrospermopsin (CYN) producers: CS-505, CS-506 (*Cylindrospermopsis raciborskii*), SYKE-966 (*Anabaena lapponica*);
- saxitoxin (STX) producers: CS-337/01, CS-537/13 (*Dolichospermum circinale*);
- anatoxin-a (ATX) producer: ANA123 (*Dolichospermum circinale*).

2.4.2. DNA Extraction

Depending on the bloom density, 30–400 mL of water samples were filtered (pore size 2–3 µm), and filtride was freeze-dried. Approximately 10 mg of freeze-dried biomass was used for DNA extraction from reference strains. Genomic DNA from biomass of the reference strains and filtrides was extracted with the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions, with minimal modifications for the extraction from filtrides (double amount of Buffer AP1, RNase A and Buffer P3 was added to fully suspend the samples). During the initial steps of extraction, samples were homogenized using zirconia/silica disruption beads (0.5 mm) and by vortexing for 1 min. The quality was assessed spectrophotometrically (NanoDrop ND-1000, Thermo Scientific, Waltham, MA, USA), where A260/A280 ratio varied between 1.22 and 2.04.

2.4.3. Qualitative PCR

Qualitative PCR was run to analyze samples for the presence of MC (*mcyE*), CYN (*cyrJ*), STX (*sxtA, sxtG, sxtS*) and ATX (*anaC*) synthetase genes. PCR reaction mixtures were prepared in a total volume of 20 µL containing 1× Phire Reaction Buffer, 0.4 µL Phire II HotStart polymerase (Thermo Scientific), 0.2 mM deoxyribonucleotide triphosphates (dNTPs) (Thermo Scientific), 0.5 µL forward and reversed primers (Table 1), 2 µL of template and sterile deionized water. PCRs were run on a C1000 Touch Thermal Cycler (Bio-Rad, Helsinki, Finland) according to the following protocols: initial denaturation for 30 s at 98 °C; 40 cycles of 5 s at 98 °C, 5 s at 61 °C (HEPF, HEPR, sxtA1480_R stxA855_R), or 62 °C (cyrJ_F, cyrJ_R, sxtG432_F, sxtG928_R, sxtS205_F, sxtS566_R), or 52 °C (anaC-genF, anaC-genR) and 10 s at 72 °C; and a final extension of 1 min at 72 °C. To examine the potential inhibition of PCRs, an exogenous amplification control template was prepared containing 1 µL:1 µL (reference:sample). Following strains were used as a reference in the control template: PCC7820 for *mcyE*, CS-506 for *cyrJ*, CS-537/13 for *sxtA, sxtG, sxtS*, and ANA123 for *anaC*. Visualization of PCR products was performed on a 1.5% Top Vision agarose gel (Thermo Scientific) dyed with SYBR® Safe DNA gel stain. The observed bands were documented on Gel Doc™ XR (Bio-Rad) using Quantity One software (v. 4.6.9).

Table 1. List of primers used for qualitative polymerase chain reaction (PCR).

Gene	Primer	5′–3′ Sequence	Reference
mcyE	HEPF	TTTGGGGTTAACTTTTTTGGGCATAGTC	[17]
	HEPR	AATTCTTGAGGCTGTAAATCGGGTTT	
cyrJ	cyrJ_F	TTCTCTCCTTTCCCTATCTCTTTATC	[18]
	cyrJ_R	GCTACGGTGCTGTACCAAGGGGC	
sxtA	stxA855_F	GACTCGGCTTGTTGCTTCCCC	[19]
	sxtA1480_R	GCCAAACTCGCAACAGGAGAAGG	
sxtG	sxtG432_F	AATGGCAGATCGCAACCGCTAT	[19]
	sxtG928_R	ACATTCAACCCTGCCCATTCACT	
sxtS	sxtS205_F	GGAGTATTDGCGGGTGACTATGA	[20]
	sxtS566_R	GGTGGCTACTTGGTATAACTCGCA	
anaC	anaC-genF	TCTGGTATTCAGTCCCCTCTAT	[21]
	anaC-genR	CCCAATAGCCTGTCATCAA	

2.5. Cyanotoxin Analyses

2.5.1. Preparation of Water Samples for Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS)

Depending on the bloom density, 30–100 mL of water samples were filtered (pore size 2–3 μm). Biomass on the filter was then freeze-dried. Afterwards, filtrides were placed in glass tubes and the toxin was extracted with 3 mL of 75% MeOH and ultrasonication. The extracts were centrifuged for 10 min at 10,000× *g* and two 1 mL aliquots of the supernatant were evaporated to dryness (50 °C nitrogen flow) in glass tubes. For MC analysis the sample was redissolved in 75% MeOH in 200 μL, and for CYN analysis the sample was redissolved in 200 μL H_2O. The samples were then filtered (0.2 μm GHP ACRODISC 13 Pall Life Sciences, Ann Arbot, MS, USA) into inserts and were ready for liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis.

The extracellular MC content was concentrated by solid-phase extraction (SPE) on Waters Oasis HLB (30 mg). The samples eluted with 5 mL 90% MeOH were placed into glass tubes, evaporated using nitrogen flow and redissolved in 200 μL of 75% MeOH. Subsequently, they were filtered (0.2 μm GHPACRODISC 13 Pall Life Sciences) into inserts and were ready for LC–MS/MS analysis.

2.5.2. Preparation of Fish Tissue Samples for LC–MS/MS

Prussian carp from Lake Ludoš was analysed for MCs in fish liver, gills, kidney, intestine, gonads (testis and ovaries), spleen, and muscle samples by LC–MS/MS. A total of 30 individuals (TL: 16.63 ± 2.24 cm, SL: 14.58 ± 1.68 cm, 18 female/12 male) were collected during two separate sampling surveys—spring (March) and autumn (September) of 2018. Different fish tissues were separately homogenized and freeze-dried. Samples of the same organ of all the individuals were pooled together. Before further preparation, several fish tissues were spiked in order to test the preparation method. Freeze-dried fish tissue samples (100 mg) were placed into glass tubes and 5 mL of 75% MeOH was added for extraction of cyanotoxins. Homogenization was performed on ice for 30 second, and the samples were then ultrasonicated in a bath sonicator for 15 min and further extracted with a probe sonicator. Samples were then centrifuged for 10 min at 10,000× *g* followed by an addition of 1 mL of hexane to 2 mL of the supernatants obtained. The hexane (lipid) layer was removed using glass pipettes and the remaining samples were evaporated (50 °C nitrogen flow) in glass tubes. Finally, samples were redissolved in 300 μL 75% MeOH and filtered (0.2 μm GHPACRODISC 13 Pall Life Sciences) into inserts. The fish tissue samples were then ready for LC-MS/MS analysis.

2.5.3. LC–MS/MS

Toxin analyses were performed by LC–MS/MS [22]. The analytical targets consisted of nine MC variants (MC-dmRR, MC-RR, MC-dmYR, MC-YR, MC-dmLR, MC-LR, MC-LY, MC-LW and MC-LF) and CYN.

2.6. Analyses of Fish Histology

Captured Prussian carp from Lake Ludoš used for cyanotoxin detection were also used for histological analyses. Liver, kidney, gill, intestine, spleen, gonad and muscle samples were dissected from each fish and fixed in 4% formaldehyde. Additionally, 6 individuals of common carp (*Cyprinus carpio* L.) (TL: 18.35 ± 6.24 cm, 3 females/3 males) were obtained from the Department of Aquaculture, Szent István University, Hungary. These fish were kept in a recirculation system (Sentimento Kft., Hungary), under a 12 h light/12 h dark cycle at 24 ± 0.2 °C and served as a control group in this study.

After fixation of at least three days, samples were processed by standard histological procedure. Gill and muscle samples were decalcified beforehand. For tissue processing, samples were dehydrated in graded series of ethanol, cleared in xylene and subsequently embedded in paraffin wax blocks. Three five-μm-thin sections per tissue per individual were cut and placed onto glass slides, and

stained with haematoxylin and eosin (H&E) dyes. Sections were examined under a Nikon Eclipse 600 microscope and photographed using a QImaging Micro Publisher 3.0 digital camera.

3. Results

3.1. Physical and Chemical Parameters of Water Samples

The recent investigations of Lake Ludoš during 2018 corroborated the continuously poor chemical state of the lake. pH levels, saturation with O_2 as well as electrical conductivity were high during the investigated period (Table 2).

Table 2. Physical and chemical parameters of water from Lake Ludoš in 2018.

Physical and Chemical Parameters	March	May	July	September
temperature (°C), in situ	10.5	20.3	25.9	17.5
pH, in situ	8.3	8.9	8.9	8.6
concentration O_2, in situ (mg/L)	16.2	9.5	26.9	8.5
saturation O_2, in situ (%)	146.7	99.7	>300	90.5
conductivity, in situ (µS/cm)	873.5	915	874.5	967.5
TSS (mg/dm^3)	43	104.5	46.8	48.4
TOC (mg/dm^3)	8.45	10.4	9.5	13.2
NO_3 (mg/dm^3)	≤0.5	≤0.5	≤0.5	≤0.5
detergents (mg/dm^3)	2.05	2.5	2.1	3.1
COD (mgO_2/dm^3)	23.85	31.8	27.7	38
BOD (mgO_2/dm^3)	12	15	13.5	18.9

3.2. Presence of Cyanobacterial Species in Water Samples

Most dominant cyanobacterial species were *Limnothrix redekei* (Van Goor) Meffert and *Pseudanabaena limnetica* (Lemmermann) Komárek (Table 3, Figure 2). Furthermore, both *Microcystis* species, *Microcystis aeruginosa* (Kützing) Kützing and *Microcystis wesenbergii* (Komárek) Komárek, were numerous during the whole investigated period. At the end of the summer, an invasive *Raphidiopsis raciborskii* (Woloszynska) Aguilera, Berrendero Gómez, Kastovsky, Echenique and Salerno (basionym *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju) also started to occur. Usually, more cells per mL were found in the pier samples compared to the center samples; however, the same species were present in both sampling sites.

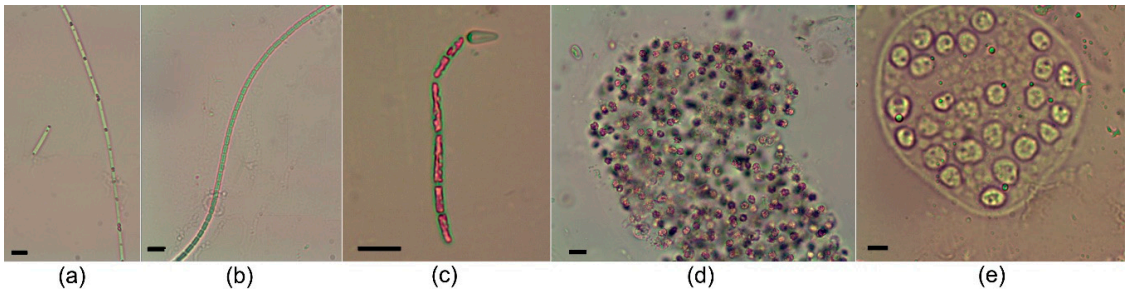

Figure 2. Dominant cyanobacterial species from Lake Ludoš in 2018: (**a**) *Limnothrix redekei*; (**b**) *Pseudanabaena limnetica*; (**c**) *Raphidiopsis raciborskii*; (**d**) *Microcystis aeruginosa*; (**e**) *Microcystis wesenbergii*; Scale bars: 10 µm.

Table 3. Qualitative and quantitative composition of cyanobacteria from Lake Ludoš in 2018.

Sampling Period	March		May		July		September	
	Center cells/mL	**Pier cells/mL**	**Center cells/mL**	**Pier cells/mL**	**Center cells/mL**	**Pier cells/mL**	**Center cells/mL**	**Pier cells/mL**
Cyanobacteria								
Chroococcus limneticus Lemmermann	13,980	15,100	13,210	17,650	31,000	53,700	68,100	82,300
Raphidiopsis raciborskii (Woloszynska) Aguilera, Berrendero Gómez, Kastovsky, Echenique and Salerno	–	–	–	–	–	–	48,000	65,000
Dolichospermum flos-aquae (Brébisson ex Bornet and Flahault)	–	–	–	–	86,500	58,300	21,000	187,300
Limnothrix redekei (Van Goor) Meffert	10,140,000	9,855,000	10,983,000	10,287,000	11,180,000	13,100,000	9,983,100	10,150,000
Microcystis aeruginosa (Kützing) Kützing	205,410	320,210	198,310	285,400	185,600	232,160	185,300	213,100
Microcystis wesenbergii (Komárek) Komárek	104,670	200,930	163,750	210,820	321,700	485,300	1,504,100	1,756,100
Oscillatoria sp. Vaucher ex Gomont	–	–	+	+	+	+	+	+
Planktothrix agardhii (Gomont) Anagnostidis and Komárek	43,000	74,600	51,000	62,600	62,700	70,200	85,400	91,300
Pseudanabaena limnetica (Lemmermann) Komárek	12,785,000	14,521,000	13,956,000	15,281,000	15,813,000	17,323,000	13,685,000	15,321,000
Woronichinia sp. A. A. Elenkin	–	–	+	+	+	+	+	+
Σ	23,292,060	24,986,840	25,365,270	26,144,470	27,680,500	31,322,660	25,580,000	27,866,100

Legend: (**+**) present taxa with abundance less than 0.1% of total; (**–**) not present in sample.

3.3. Presence of Cyanotoxin Coding Genes in Biomass Samples

Biomass samples were tested for the presence of cyanotoxin coding genes, including MCs, STX, CYN and ATX (Table 4).

Table 4. The prevalence of *mcyE*, *sxtA*, *sxtG*, *sxtS*, *cyrJ* and *anaC* PCR products in Lake Ludoš.

Sampling Period	March		May		July		September	
PCR Products	**Center**	**Pier**	**Center**	**Pier**	**Center**	**Pier**	**Center**	**Pier**
mcyE	+	+	+	+	+	+	+	+
sxtA	/	/	/	/	−	−	−	−
sxtG	+	−	−	+	+	+	+	+
sxtS	−	−	−	−	+	+	+	+
cyrJ	−	−	−	−	−	−	−	−
anaC	−	−	−	−	−	−	−	−

Legend: (**+**) amplified; (**−**) not amplified; (*/*) not analysed.

MC coding gene *mcyE* (472 bp, Figure 3a) was amplified in all samples. STX coding genes *sxtG* (519 pb, Figure 3c) and *sxtS* (382 bp, Figure 3d) were amplified in a total of 6 and 4 samples, respectively. The *sxtG* gene was observed in all sampling seasons, while *sxtS* gene was observed only in samples collected in August and September. Saxitoxin coding gene *sxtA* (648 bp, Figure 3b), CYN coding gene *cyrJ*, and ATX coding gene *anaC* were not amplified in this study. Significant inhibition of the PCR reaction was not observed in exogenous amplification control templates.

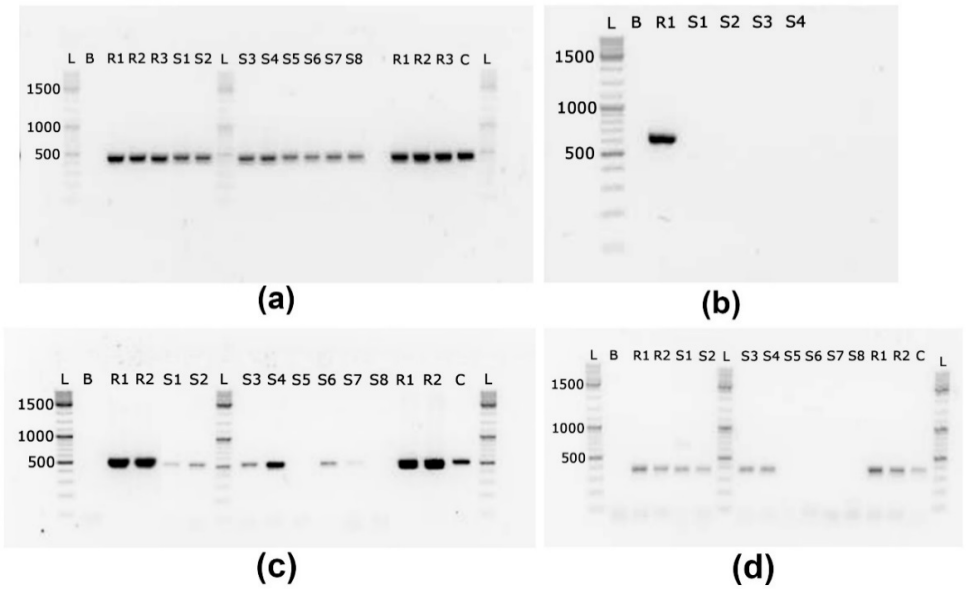

Figure 3. Visualization of PCR products on agarose gel: (**a**) *mcyE*; (**b**) *sxtA*; (**c**) *sxtG*; (**d**) *sxtS*. Legend: L—ladder; B—blank; R1, R2—reference strains; C—exogenous amplification control; S1—sample September—center; S2—sample September—pier; S3—sample July—center; S4—sample July—pier; S5—sample May—center; S6—sample May—pier; S7—sample March—center; S8—sample March—pier.

3.4. Presence of Cyanotoxins in Water and Fish Samples

Biomass and extracellular content of water samples were tested for the presence of cyanotoxins. Several MC variants were noted in the total content (Table 5) including most commonly occurring MC-LR and MC-RR, however, their concentrations were rather low during the whole investigated period. CYN was not detected during the investigated period in Lake Ludoš, even though there were some cyanobacterial species that could potentially produce this cyanotoxin.

Table 5. Presence and highest concentrations of microcystin (MC) variants in Lake Ludoš during 2018.

Microcystin Variants (µg/L)	March	May	July	September
MC-LR	–	0.1	0.022	0.285
dmMC-LR	0.023	–	–	–
MC-RR	–	–	–	0.006
dmMC-RR	0.003	–	0.002	–
MC-LF	–	–	–	+

Legend: (**+**) present in sample; (**–**) not present in sample.

Analyses of several fish tissue samples did not show a presence of investigated MC variants in spring nor autumn samples.

3.5. Histological Alterations in Fish Samples

During both investigated periods, spring (before) and autumn season (during and after the bloom), most affected fish organs were liver, kidneys and gills. Liver samples of individuals from the control group displayed typical organization of the hepatic parenchyma, with cord-like formations of hepatocytes interspaced with sinusoids and radially arranged around blood vessels (Figure 4a). Hepatocytes were polygonal, with a clearly visible cell membrane and large round nuclei with distinguishable nucleoli. In contrast, microscopic examination of fish from Lake Ludoš revealed severe alterations of liver histology which were observed in both March and September sampling groups. Livers of these fish showed changes in architectural structure, with less prominent cord-like organization and sinusoid capillaries no longer clearly distinguishable (Figure 4b). Loss of shape and rounding of hepatocytes was most characteristic in the March group, with large groups of cells displaying ball-or onion-like shape (Figure 4c). Altered hepatocytes typically had darker nuclei with condensed chromatin and no discernable nucleoli. Signs of kariopyknosis, predominantly in the September sampling group, were indicative of necrosis (Figure 4d). Most prominent alterations present in all examined samples were glycogen depletion and vacuolization of hepatocytes. Many cells had a completely clear cytoplasm, which suggest hypervacuolization (Figure 4e).

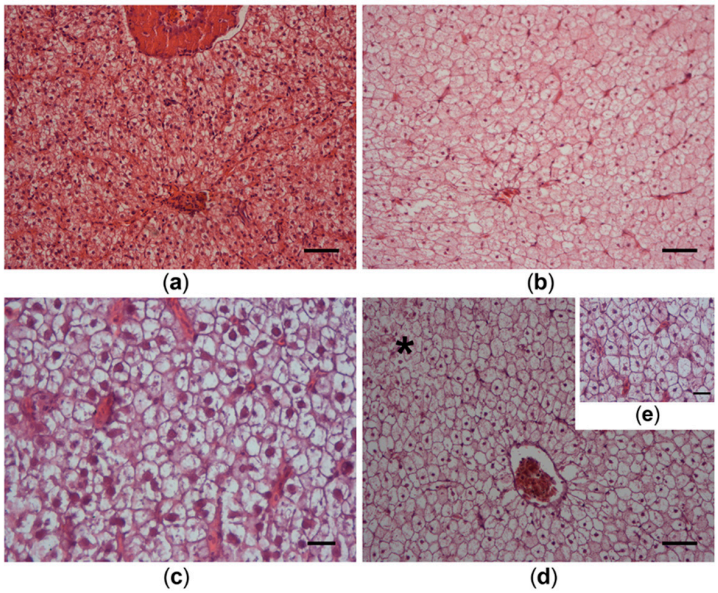

Figure 4. Histopathological alterations in the liver of Prussian carp *Carassius gibelio* from Lake Ludoš, 2018: (**a**) control fish; (**b**) loss of the cord-like parenchymal structure; (**c**) rounding of hepatocytes; (**d**) necrotic fields (asterisk) with hepatocytes displaying karyopyknosis; (**e**) hepatocytes displaying vacuolization and glycogen depletion. Haematoxylin and eosin (H&E) staining. A, B and D–50 µm; C and E–20 µm.

Renal corpuscles in the kidneys of the control individuals were round in shape and had relatively large glomeruli. The Bowman's capsule was continuous with thin intercapsular space. Both proximal and distal renal tubules had one layer of columnar epithelial cells with proximal segments having basal nuclei, and distal segments having central nuclei and less intensive cytoplasmic stain (Figure 5a). Even in controls, slight clogging of tubules and slight vacuolization was observed.

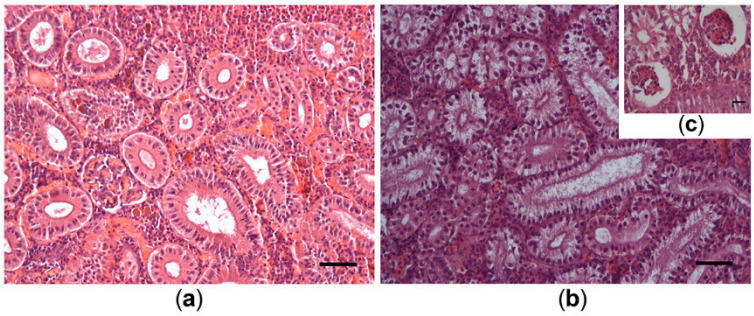

Figure 5. Histopathological alterations in the kidney of Prussian carp *Carassius gibelio* from Lake Ludoš, 2018: (**a**) control fish; (**b**) degeneration of tubules including vacuolization and separation of epithelial layer from the basal lamina; (**c**) reduction of glomeruli size and intense dilatation of Bowman's capsule. H&E staining. A and B–50 μm; C–20 μm.

Only individuals sampled in March had significant pathological alterations in the kidney. These included degeneration and loss of nephron formation, as well as interstitium structure. Renal corpuscles showed a reduction of glomeruli size, accompanied with dilatations of intercapsular space of the Bowman's capsule (Figure 5c). In tubules, epithelial cells showed intense vacuolization and in some cases the epithelial layer was separated from the basal lamina (Figure 5b). The number of tubules appeared clogged and in the process of necrosis. Cells in the necrotic area had pyknotic nuclei and displayed signs of cell membrane lysis, such as no discernable boundary between cells. In some fish, necrosis, hyalization of the interstitium and the presence of macrophage aggregates were evident. Certain alterations, such as vacuolization and tearing of the tubular epithelium, were also present in individuals from the September group; however, this was not as frequent and severe compared to the March group.

Gills of the control group showed no pathological changes (Figure 6a). Secondary lamellae regularly lined both sides of the primary lamellae (filament) and were covered with one layer of squamous epithelial cells. Contrarily, individuals from Lake Ludoš in both sampling periods (March and September) had noticeably altered gill structure. Several individuals displayed signs of hyperplasia, as well as hypertrophy of interlamellar cell mass, mainly epithelial and mucous cells. Such swelling of secondary lamellae and proliferation of interepithelial cells has led to a complete fusion of the secondary lamellae, especially noticeable in the September sampling group (Figure 6b). Other observed lesions included epithelial lifting and oedema, accompanied with hypertrophy of interepithelial chloride cells (Figure 6c). In some individuals, endothelial cells of the capillaries showed signs of telangiectasia (aneurysm), along with epithelial rupture and hemorrhage (Figure 6d).

Other examined organs of the bloom-exposed fish in the present study did not display histopathological alterations (not shown). Intestines of all groups showed normal histology, with villi regularly lining the lumen. A single layer of enterocytes with basal nuclei lined the surface of the vili, along with fewer goblet cells. Furthermore, sections of muscle tissue in all groups also showed no structural alterations. Spleen of all examined individuals displayed a normal structure, with aggregates of erythroid and lymphoid cells, surrounding blood vessels. Neither male nor female gonads had histopathological changes. Testes and ovaries had normal structural organization with germline cells present in different stages and numbers, which is dependent on the season and age of the fish.

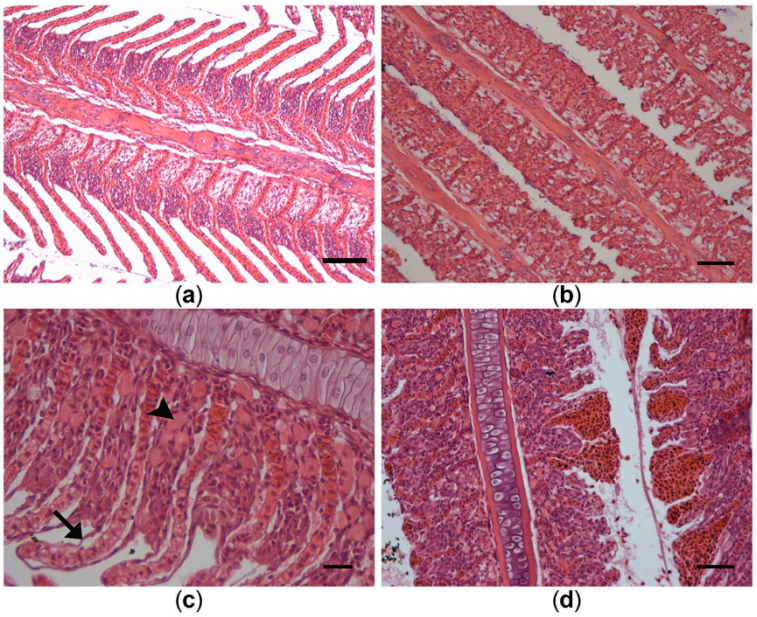

Figure 6. Histopathological alterations in the gills of Prussian carp *Carassius gibelio* from Lake Ludoš, 2018: (**a**) control fish; (**b**) fusion of the secondary lamellae; (**c**) intensive proliferations of chloride cells (arrowhead) and oedema (arrow); (**d**) telangiectasia. H&E staining. A and D–50 μm; B–100 μm; C–20 μm.

4. Discussion

4.1. Monitoring of the Water

4.1.1. Physical and Chemical Parameters in Lake Ludoš

The measurements of physical and chemical parameters showed several important findings:

- the lake water pH values were very high (almost 9), probably as a results of the activity of phytoplankton;
- O_2 saturation showed high values, and even supersaturation during July 2018, likely due to photosynthetic activity of phytoplankton;
- electrical conductivity was relatively high.

It is assumed that cyanobacteria are more resistant to solute increases compared to other phyla e.g., Chlorophyta [23]. Additionally, conductivity changes lead to decreased zooplankton—predators of phytoplankton, thus it is possible that a reduction in zooplankton would potentiate phytoplankton increase.

Regular monitoring showed similar findings in recent years (2013–2017) [7–11]. pH levels were between 8 and 9, O_2 saturation during the year was uneven with high values and supersaturation during summer and sometimes during autumn months, while high values of electric conductivity of the water seemed to be rising in the recent years. Additionally, COD was extremely high, similar to wastewaters, indicating a poor status of the lake and based on the measured parameters it is recommended that the lake should not to be used for any purpose [24]. BOD was uneven during the year, demonstrating the problem of instability of the system. The water of the Lake Ludoš is very rich in nitrogen compounds (mostly organically bound nitrogen), which led to increased biological production. Nitrates were uneven during the year and seemed to depend on the inflow from the Palić-Ludoš canal. Phosphates showed a similar trend as nitrates and their concentration seemed to be on the rise thus contributing to the deterioration of the water quality. Sediment analysis suggested rich deposits of nutrients which will contribute to the continual hypertrophic state of the lake [7–11].

The status of surface waters in terms of general quality can be shown by the Serbian Water Quality Index (SWQI). SWQI is based on 10 quality parameters (temperature, pH, conductivity, O_2 saturation, BOD for 5 days, suspended matter, total nitrogen oxides, orthophosphates, ammonia ions, coliform bacteria) that are aggregated into a composite indicator of the quality of surface waters, leading them to one index number [24]. SWQI of the water from Lake Ludoš through 2018 found it to be of either very low quality or low quality [25]. Similar findings were also noted for the period from 2013 to 2017 [7–11].

4.1.2. Cyanobacterial Community in Lake Ludoš

During 2018, the most dominant cyanobacterial species were *L. redekei* and *P. limnetica* (Table 3). Furthermore, both *Microcystis* species, *M. aeruginosa* and *M. wesenbergii*, were numerous during the whole investigated period. Same species were present in pier and center samples, although more cells were noted in the pier samples possibly due to lower depth, higher temperature, wave, and wind effects. In the recent years (2013–2017), several other cyanobacterial species were also frequently found: *Microcystis delicatissima*, *Oscillatoria agardhii* (current *Planktothrix agardhii*), *Oscillatoria putrida*, *Lyngbya limnetica* and *Anabaena spiroides* [7–11]. Furthermore, in our previous research during 2011 and 2012, similar cyanobacterial species were abundant: *L. redekei*, *P. limnetica*, *P. agardhii* and *Microcystis* spp. [5]. Most of the present species and genera are known as potential cyanotoxin producers.

Additionally, in the autumn of 2018, the invasive species *Raphidiopsis raciborskii* was also found. This invasive and potentially toxic species was first noted in Serbia in 2006 [26] and soon in Lake Ludoš as well [27], and since then it has been frequently found and blooming in the lake. The *R. raciborskii* presence is of particular concern due to its ability to expand its distribution rapidly (see [28]). Differences in toxin production are known among the strains: South American strains produce STX, Australian and Chinese strains produce CYN, and European and North American strains are considered to be non-toxic [28]. Furthermore, non-toxic and toxic strains can co-exist, and even co-occurring strains exhibit genomic variability [29]. Bearing in mind that this species is expanding its distribution in Europe, and in Serbia as well, it is necessary to establish whether this species is a greater threat than previously assumed, and how it succeeds in spreading so rapidly.

4.1.3. Presence of Cyanotoxin Genes and Cyanotoxins in Lake Ludoš

For the first time, Lake Ludoš was assessed for the presence of genes coding several frequently occurring cyanotoxins. During investigated months, the MC-coding gene *mcyE* was amplified in all the biomass samples. This is in accordance with the cyanobacterial species composition observed in the lake, and confirms the uniform distribution of MC-producing species throughout the year. Amplification of STX coding genes, *sxtG* and *sxtS* occurred in some of the samples. The *sxtG* gene was observed in all sampling seasons, while *sxtS* gene was observed only in samples collected in August and September. The STX *sxtA* coding gene was not amplified in this study. Since the specificity of *sxtA*, *sxtG* and *sxtS* primers has been previously validated by Savela et al. [19,20], the lack of targeted genes may be due to unexpected sequence dissimilarities between primers and target that can occur in natural populations. Large-scale gene mutations such as deletions and insertions resulting in non-functional gene clusters may also cause a lack of PCR amplification. Major deletions events in MC-coding gene cluster were previously observed in non-toxic *Planktothrix* strains [30], suggesting that similar events may occur in STX-producing strains. Similarly to this study, the lack of the some of the targeted STX genes was previously observed by Savela et al. (2017) in Polish lakes. As STXs were not measured directly, conclusions on correlation between gene presence/absence and toxin production cannot be made. However, since STXs have been detected in the lake Ludoš before [5], and a potential STX producer, *R. raciborskii*, was detected, additional studies are warranted to confirm potential production of STXs in the lake.

There are several publications that show MC presence in various water bodies in Serbia [6,31]. However, only a few publications demonstrate presence of cyanotoxins in Lake Ludoš. MCs were first

noted in 2006 by Simeunović [32,33], thereafter the presence of MCs and low concentrations of STXs in the lake was observed during our previous investigation in 2011 and 2012 [5]. In 2018, analyses of water and biomass samples for the presence of MCs and CYN were performed by LC–MS/MS and several MC variants were detected in low concentrations. CYN was not detected, although some investigations have shown that some of the present cyanobacterial species potentially could produce this toxin (e.g., [34,35]).

4.2. Uptake of Cyanotoxins and Bloom Effects

Fish are directly influenced by water blooms, which can cause health impairments and mortality [36]. Primary route of exposure is by ingestion, either directly or through the food web, and indirectly via epithelial absorption [37]. Consequently, liver and gills are often the most affected organs during cyanobacterial blooms [38,39]. Furthermore, there is evidence of cyanotoxin accumulation in fish tissue, which ultimately endangers the health of animals and even humans that use them as a food source (e.g. [40,41]). Multiple other stress factors co-occuring with the blooms can additionally threaten fish, damaging tissues and impairing their development and survival [42].

A previous investigation of Lake Ludoš by the authors showed cyanotoxin uptake by macrophytes and fish. Accumulation of MC-RR was detected in the rhizome of *Phragmites australis*, cattail *Typha latifolia* and royal blue water lily *Nymphaea elegans* [5]. Furthermore, the same investigation demonstrated accumulation of two MC variants (MC-LR and MC-RR) in the tissue samples of the Prussian carp from Lake Ludoš. During 2011 and 2012, MCs were found in the intestine (MC-LR) and muscle samples (MC-RR), however, no MCs were found in the liver samples. In 2011, the fish gills were also positive for MC-RR, and in 2012, kidneys and gonads were found to accumulate MC-LR [5]. In 2011, histopathological changes were observed in liver, kidney, gills and intestine samples of the tested individuals. Furthermore, DNA damage was detected in Prussian carp as revealed via comet assay in 2012. Three tissues were selected and assessed: blood had the lowest level of DNA damage, while liver and gills showed more damage [5]. In another study from fishponds in Serbia, MC-RR accumulation in muscle tissues was recorded and histopathological changes were noted in liver, kidneys, gills, intestines and muscles of the *Cyprinus carpio* tissues [41]. Many histological changes in different fish species and tissues after exposure to cyanobacterial bloom or cyanotoxins were presented in a review by Svirčev et al. [2].

In addition to histological changes and accumulation, cyanotoxins can affect fish growth, development, reproduction, and survival. Embryos and larvae are especially sensitive to the effects of MCs, and exposure to these cyanotoxins in the early life stages can disrupt embryos, reduce survival and growth rate, and cause disorders such as: small head, curvature of the body and tail, enlarged and opaque yolk sachet, hepatobiliary abnormalities and cardiac disturbances [43–45]. Furthermore, cyanobacteria and their metabolites can have a wide range of negative effects on the adults of fish. They affect locomotor activity and fish behavior, impair ingestion rate and growth, disrupt heart function, ion regulation, cause changes in serum composition, trigger oxidative stress and disturb the reproduction of fish. Alongside direct harmful effects caused by cyanotoxins, cyanobacteria can adversely affect fish through the alteration of environmental conditions during blooming. Decreased dissolved oxygen, increase in ammonia concentration, changes in pH value and water temperature, or a combination of all of these factors can have detrimental changes on fish [46].

4.2.1. Cyanotoxin Accumulation in Fish Tissues from Lake Ludoš

Several fish tissues of *Carassius gibelio* were examined for the presence of MCs. As already stated, in our previous study accumulation of two microcystin variants (MC-LR and MC-RR) was noted in several fish tissues: muscle, intestines, kidneys, gonads and gills of *Carassius gibelio* [5], however, accumulation in tissues was not found during this investigation. Although spiked samples demonstrated that the method for the preparation of the samples is adequate, it is possible that cyanotoxins remained covalently bound to protein phosphatases in the tissue [47–50] resulting in

negative results. Furthermore, it is possible that cyanotoxins were excreted and concentrations lowered by detoxification processes which vary between different species, organ, MC congener and metabolism [40,51–53], or even that concentrations in water were not high enough to be accumulated in high concentrations for detection.

4.2.2. Bloom Effects on Histopathology of Fish from Lake Ludoš

Histopathology has an important place as a biomarker of fish health status, as histological changes often occur in response to acute or chronic exposure of an organism to a pollutant or a hazardous chemical, as well as adverse conditions present in the water. Even though no accumulation of cyanotoxins were detected during this investigation, histological observation of tissues from Prussian carp taken from Lake Ludoš suggest serious health implications often attributed to water blooming and cyanobacteria-rich aquatic environments. In general, most affected organs during both the spring and autumn seasons were liver, kidneys and gills. Furthermore, presence of alterations in both sampling periods (spring and autumn) suggests that chemical state of the lake was poor and that harmful blooms probably occurred in the previous year.

Structural alterations of the liver observed in this study are similar to those found by other authors after the exposure of fish to cyanotoxins or extracts of cyanobacterial strains and blooms [54–60]. Most prominent changes were loss of parenchymal structure, rounding of cells, glycogen depletion, vacuolization and pyknosis, all of which were so far reported after exposure of fish to MCs [2]. These alterations, specifically vacuolization and increase of lipid content, were also observed in mammalian livers in reaction to MC [61–63]. Hepatotoxicity of MC might be attributed to its affinity to bind and inhibit eukaryotic serine-threonine protein phosphatases 1 and 2A (PP1 and PP2), enzymes that are important in maintaining cell homeostasis and tumor suppression signaling pathways [64–66]. Studies in mammalian models have shown that inhibition of PP1 and PP2 can cause cytoskeletal damage [67,68] and may lead to loss of cell-to-cell contact, rounding of hepatocytes, condensation of chromatin and nuclear pyknosis.

Kidneys may be good indicators of environmental stress since they receive the majority of postbranchial blood. Additionally, kidney tubule cells possess a transport mechanism similar to that of hepatocytes (the multispecific bile acid transport system) which is responsible for MC uptake into the cell [69]. A study undertaken by Fischer and Dietrich [56] has shown that due to this efficient uptake of toxins in carp, MC-induced kidney pathologies in carp develop rapidly and at lower toxin concentrations. Such a finding supports the results of this study, as the kidneys exhibited the strongest histopathological changes, particularly in the fish that were taken during the spring when the levels of cyanotoxins are lower. Histological changes observed in the present study supports previous research on the effects of MCs on fish [39,55,56]. Glomerulopathy, with dilatations of the Bowman's capsule, and vacuolization of tubules are the most common alterations observed after exposure of fish to MCs [70,71]. Necrosis and impairment of renal tubules can affect ion and water regulation in the kidney, thus damaging the survival capabilities of fish [72].

Fish gills constitute over 50% of the total surface area of the animal and are in direct contact with water, which makes them sensitive to pollutants and toxic chemicals. A number of histopathological alterations were detected in fish from Lake Ludoš, which can be associated with conditions during water blooms, mostly the presence of cyanotoxins [54,57,73]. Lifting of the lamellar epithelial cells caused by the fluid penetration and epithelial hyperplasia were the most common lesions. Along with epithelial hyperplasia, these are considered defensive mechanisms of gills, both of which reduce the uptake of xenobiotics [74,75]. Other persistent alterations, such as oedema and telangiectasia from the secondary lamellae could be attributed directly to MC toxicity. Gill ion pumps (Na^+ and K^+ ATPases) could be inhibited by MCs [76,77], leading to a decline in blood Na+ and Cl− concentration and ion exchange imbalance. This could result in swelling of secondary lamellae as well as proliferation of interepithelial chloride cells. Intensive hyperplasia decreases the space between lamellae and causes fusion, which increases the thickness of the water–blood barrier and decreases the oxygen uptake.

These lesions can cause capillary hemorrhage and significantly hinder gill functions, such as respiration, ion regulation, acid-base regulation and nitrogenous waste excretion [78]. The molecular actions of cyanotoxins in fish liver and gills involve increased generation of reactive oxygen species (ROS) which randomly attack all cell components, including proteins, lipids and nucleic acids [79,80]. An imbalance between the generation and removal of ROS in these tissues results in oxidative stress and extensive cellular tissue damage in these organs [81,82].

Previous research on fish from Lake Ludoš by the authors showed similar findings. The liver showed a loss of cordlike parenchymal structure; presence of onion-shaped hepatocytes with clear cytoplasm as well as pyknosis and fields of anucleated cells. Changes in the kidney were glomerulopathy with intense dilatation of Bowman's capsule as well as vacuolization of tubules and macrophage infiltration. Furthermore, histopathological alterations observed in the gills showed fusions of lamellae; oedema and epithelial lifting and intensive proliferations of chloride cells. Alterations were also found in intestines where intensive oedematous alteration in the lamina propria; desquamation of enterocytes; and hypertrophies of goblet cells was noted [5]. Although more than five years have passed since the previous research, problems found in fish tissues remain present and could be a result from the constant cyanobacterial blooming in this lake. The aforementioned alterations can severely impact the life quality of fish and consequently disturb the whole food chain.

It should also be noted that fishing at Lake Ludoš by local fisherman is frequent, and therefore *Carassius gibelio* is sometimes included in the human diet. It is necessary to monitor concentrations of cyanotoxins in water and fish to make sure that ingested concentrations of MCs are below tolerable daily intake of 0.04 µg MC-LR equiv./kg body weight/day [83], so that any health consequences could be prevented.

4.3. Potential Health Risks Caused by Cyanobacterial Blooms

4.3.1. Transfer to Protected Water Birds

Cyanotoxins can be transmitted through the food web to different consumers, such as various animals living in and around the lake, including birds. In the case of Lake Ludoš, this is particularly important since one of the main reasons for protection of this wetland is because it is a habitat for water birds. In addition to the contaminated food, birds can come into contact with cyanotoxins via direct contact with the blooming water. There are few papers dealing with the effect of cyanotoxins on birds. Early reports, such as that from Storm Lake in Iowa associated with *Anabaena flos–aquae* blooms, include estimated deaths of 5–7000 gulls, 560 ducks, 400 coots, 200 pheasants, 50 squirrels, 18 muskrats, 15 dogs, 4 cats, 2 hogs, 2 hawks, 1 skunk, 1 mink, plus "numerous" songbirds. It seems that neurotoxicity resulted in prostration and convulsions preceded death; milder cases displayed restlessness, weakness, dyspnoea and tonic spasms. Furthermore, 57 weak and partially paralyzed mallards were recovered following gastric lavage [84,85]. In Denmark in 1993, two grebes and a coot died during cyanobacterial bloom and *Anabaena lemmermannii* was found in the stomach contents all three birds together with low levels of anatoxin-a(S)-like compounds [86,87]. Death of 20 ducks in Shin–ike pond (Japan) were described after evident *M. aeruginosa* bloom and presence of MC was confirmed, indicating MC intoxication as a possible cause of death [88]. Cyanobacteria-related mortalities have been reported in three flamingo species, both wild and captive [89]. Four MC congeners and ATX found in cyanobacterial mats and stomach contents of dead lesser flamingos as well as faecal pellets collected from shorelines of Lake Bogoria (Kenya) [90]. Similar Lesser Flamingo poisonings have been reported from alkaline lakes in Tanzania, with toxic *Arthrospira fusiformis* implicated [91]. The presence of MCs in several organs was detected in the domestic duck (*Anas platyrhynchos*) and in the black-crowned night heron (*Nycticorax nycticoraxs*) (alongside fish and turtle) from Lake Taihu (China) during toxic *Microcystis* blooms [92]. After experimental exposure to *Microcystis* biomass containing MCs, histopathological changes were observed in the form of cloudy swelling of hepatocytes (shrunken nuclei with ring-like nucleoli, cristolysis within mitochondria and vacuoles with pseudomyelin

structures), vacuolar dystrophy, steatosis, hyperplasia of lymphatic centers and vacuolar degeneration of the testicular germinative epithelium in male Japanese quails (*Coturnix coturnix japonica*) [93].

4.3.2. Transfer to Humans as a Result of Fish Consumption

Humans at the top of the food chain may also be endangered. Lake Ludoš is regularly frequented by the local fishermen, and year-long and day-long fishing permits are available for this protected freshwater ecosystem. Based on the available data it was found that fishermen with prolonged and cumulative exposure through contaminated water (direct contact, inhalation, oral) and food may suffer a type of poisoning with a long-term complications. Such an outcome was demonstrated through biochemical alterations of liver damage biomarkers in fishermen and children that consumed (in addition to water) ducks, fish, shrimps, snails, and other aquatic organisms grown in blooming lakes in China [94,95]. Serum analysis of 35 fishermen who worked and lived on fishing ships on the blooming Lake Chaohu (China) for over 5 years, drank the lake water and ate fish, shrimps, and snails, showed the presence of MC [94]. The values of serum enzymes were significantly higher in the children that were exposed to MCs for over 5 years through drinking MC contaminated water and food e.g., carp and duck in the Three Gorges Reservoir Region in China. Additionally, 0.9% of parents of high-exposed children self-reported a cancer diagnosis (9 of 994, including four with hepatic carcinoma) compared to 0.5% of parents of low-exposed children (1 of 183), and none of the parents of children in the unexposed group [94]. To confirm this, in south-west China where MC-LR was detected in water, fish, and ducks, people with abnormal indicators of renal function had a much higher mean level of MC-LR exposure than those with normal indicators [96]. The foregoing indicates that the blooming phenomenon should not be taken lightly, but rather should receive more scientific attention.

4.4. A Potential Resolution?

By some estimates, we lost 50% of the world's wetlands in the 20th century [97]. So far an effective and long-term solution to reduce cyanobacterial blooms worldwide has not been attained. Cyanobacterial blooms have been recorded in the wetlands of the Perth region (Australia), with *Microcystis aeruginosa* and *M. flos-aquae* being the most ubiquitous bloom-forming cyanobacteria. Furthermore, for the first time *Nodularia* blooms have been recorded in such low salinity waters in Australia, and hepatotoxins, microcystin and nodularin, were associated with the analysed blooms [98]. Lake Ludoš has been notoriously known for consistent cyanobacterial blooming. Wetland ecosystems need help in dealing with this issue.

Mitigation of the global expansion of cyanobacterial harmful blooms, together with a variety of traditional (e.g., nutrient load reduction) and experimental (e.g., artificial mixing and flushing, omnivorous fish removal) approaches has been presented in a review by Pearl et al. [99]. Virtually all mitigation strategies are influenced by climate change, which may require setting new nutrient input reduction targets and establishing nutrient-bloom thresholds for impacted waters. Physical-forcing mitigation techniques, such as flushing and artificial mixing, will need adjustments to deal with the ramifications of climate change. Current mitigation strategies need to be examined and the potential options for adapting and optimizing them in a world facing increasing human population pressure and climate change [99]. A notable approach on large-scale wetland restoration has been proposed in Ohio (USA). Through restoration of the Great Black Swamp, cyanobacterial blooming in Lake Erie should be mitigated [97]. The goal would be to restore rare and declining plant communities and species and to provide nutrient load reduction to Lake Erie, specifically total phosphorous. With the creation of the 20,000 of wetlands in "hot spots" of the former Great Black Swamp, removal of 18% of the 2617 metric tons/year of phosphorus loading by the Maumee River to Lake Erie would be expected [100]. This would lead to a cleaner lake and a more sustainable landscape in that region [97].

In order to reduce cyanobacterial population in Lake Ludoš, the potential efficiency of hydrogen peroxide treatment was examined in vitro. Although further research is needed, the initial laboratory results showed that this method may not be readily applicable, since the dense cyanobacterial

population and the high load of organic matter (that consumes hydrogen peroxide) would require the use of harmfully high doses of hydrogen peroxide in order to fight the cyanobacteria [5]. Another recent study performed in Lake Ludoš demonstrated the use of H_2O_2 and the MC-degrading capacity of the enzyme MlrA. Results showed that the treatment decreased the abundance of the dominant cyanobacterial taxa and reduction of the intracellular concentration of MC by H_2O_2, but the reduction of the extracellular MC was not accomplished in combination with MlrA. Since H_2O_2 was found to induce the expression of *mcyB* and *mcyE* genes involved in MC biosynthesis, the use of H_2O_2 as a safe cyanobacteriocide still requires further investigation [101]. Several other authors have also suggested measures for water-quality improvement of this lake, including the identification of source water with lower nutrient content for maintaining the volume of the lake, sediment removal [102,103], and sediment phytoremediation [104]. Even if restoration of the endangered ecosystem were to be realized, continuous monitoring would still be preferable. Such system (South Florida Wetland Monitoring Network (SFWMN)) has been created in the Everglades (USA), with three real-time hydrologic, water-quality, and meteorological field stations. Besides research, data from these monitoring stations assist in a better understanding of wetlands dynamics and function [105].

Lake Ludoš, a significant ecosystem and a habitat for several endemic and relict plant species, is preserved at the moment, however, the problematic ecological state of the lake is also perpetuated, so justification for such action was questioned. Current research strongly supports the earlier findings that the ecological balance of this Ramsar site is impaired. By preserving the lake and its cyanobacterial problem the water birds and their habitat are not really protected and, quite the contrary, the lake and nearby ecosystems are put at risk. There is a likely probability that the birds visiting the contaminated lake during their migrations carry viable cyanobacterial cells on their feet, feathers, bills, gullets, and faecal material, thus contributing to the spreading of cyanobacteria and hence expanding the problem [5].

The future of Lake Ludoš is still unclear and a potential resolution is still not in sight. How long will this vital ecosystem stay in a state of limbo? Based on this research, and as an extension and confirmation of the previous investigations, it is possible to predict the continuation of cyanobacterial blooms in Lake Ludoš, degradation of protected habitat and negative effects on aquatic organisms. Food items derived from Lake Ludoš also present one path of human exposure to cyanotoxins, which together with the direct contact and ingestion of contaminated water, as well as inhalation, signifies a health risk. Therefore, it is necessary to continue the monitoring of this lake, and work on finding an effective treatment that will help this ecosystem, but also many others that suffer from the same problem. Recently, it was published that there are over 1000 recorded identifications of major cyanotoxins in more than 800 aquatic ecosystems from over 60 countries worldwide [4].

It is time to solve this problem but most if not all the options being considered are limited and/or inconsequential [97]. This paper should be seen as an invitation to scientists, engineers, competent authorities, policy makers and anyone else who can contribute to solving the problems of Lake Ludoš and finally ending the lake's perpetual state of limbo. Potential solutions should be comprehensive and holistic, and lead to a sustainable management of this marshland ecosystem and its services.

5. Conclusions

This investigation regarding the presence of cyanobacteria and cyanotoxins, together with the observations of effects on aquatic organisms, in Lake Ludoš during 2018 has resulted in the following findings:

- the poor chemical state of the lake based on the physical and chemical parameters;
- the presence of potentially toxic (genera *Dolichospermum*, *Microcystis*, *Planktothrix*, *Chroococcus*, *Oscillatoria*, *Woronichinia* and dominant species *L. redekei* and *Pseudanabaena limnetica*) and invasive cyanobacterial species *R. raciborskii*;
- the detection of MC and STX coding genes in biomass samples;

- the detection of several MC variants MC-LR, MC-dmLR, MC-RR, MC-dmRR, MC-LF) in low concentrations in water samples;
- histopathological alterations in fish liver, kidney and gills.

Additional emphasis has to be placed on the detection of MC and STX coding genes, which was performed for the first time in Lake Ludoš indicating these toxins as the main threat, as well as identification of demetylated forms of well-known toxins (MC-dmLR, MC-dmRR), and a more rare variant MC-LF in the water. Although present MC variants have a different toxicity, nonetheless, they contribute to possible adverse effects.

The results presented indicate that the potential threat to many organisms in the ecosystem, including birds and humans, is real and present. The persistent alarming condition of Lake Ludoš poses a great health risk and a "ticking (cyanobacterial) bomb" that could lead to the collapse of this special nature reserve. Urgent remediation measures are needed to alleviate the incessant cyanobacterial problem in Lake Ludoš and to break this ecosystem out of the perpetual state of limbo in which it has been trapped for quite some time.

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