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Modulation of biochemical indices in common carp (*Cyprinus carpio* L.) under the influence of toxic cyanobacterial biomass in diet

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Abstract Cyanobacteria are producers of potent and environmentally abundant microcystins, representing an emerging global health issue. In the present study, we investigated the impact of cyanobacterial biomass on biochemical indices of common carp (*Cyprinus carpio* L., average weight of 246 ± 73 g) under laboratory conditions. The fish were fed a diet containing cyanobacterial biomass with microcystins in high concentration (0.4 mg/kg of fish weight and day) for 28 days. Statistical evaluation of the influence of the cyanobacterial biomass in food on the biochemical indices of the juvenile carp showed only minor differences. The activity of aspartate aminotransferase value and the urea concentration were significantly reduced compared to control group. The biochemical parameters of fish blood plasma significantly rose during the experiment in the control group as well as in the experimental group. This state was probably influenced by the environmental conditions and the fish diet. A significant rising value was established in calcium creatinine, total protein, phosphorus, lactate, urea and natrium. The present study demonstrates that the oral exposure of toxic cyanobacterial biomass has a minor influence on the biochemical indices of common carp and that the effect of other factors, e.g., nutrition is more visible.

Keywords Cyanobacteria · Fish · Experimental exposure · Biochemistry · Microcystin

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Introduction

Toxic cyanobacteria pose a serious problem for water supply systems, recreation and agriculture. Cyanobacteria, as photosynthesizing organisms, produce various biologically active compounds that affect aquatic organisms and also the physico-chemical characteristics of water (Chorus et al. 2000). Cyanobacteria and fish coevolved in same habitats, and thus, the question arises whether cyanotoxins-containing cyanobacteria given via the natural exposure route as a component of fish diet might affect fish physiology e.g., growth and cause toxin accumulation in fish. The influence of cyanotoxins on fish following experimental intoxication or the impact of an environment containing cyanotoxins on fish has been studied using clinical, morphological, histological, ultrastructural, hematological and biochemical methods (Ibelings and Chorus 2007; Ibrahem et al. 2012; Bieczynski et al. 2013). One of the most common results of cyanobacterial blooms

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is the production of hepatotoxic microcystins (*Microcystis*) that can occasionally occur in high concentrations in shallow waters where cyanobacteria can accumulate and may induce injury to fish. A complete summary about the effects of microcystins on fish was provided by Malbrouck and Kestemont (2006).

The majority of the MCs in fish are taken up via the gastrointestinal tract, whereas uptake through the skin or gills is negligible (Tencalla et al. 1994). Intraperitoneal or peroral exposures to MCs (or lysates of cyanobacterial biomass) caused significant changes in the numbers of blood cells and biochemical indices of liver damage such as elevated alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activities (Rabergh et al. 1991; Navratil et al. 1998; Zhang et al. 2008).

Toxic effects following the oral administration of MCs are approximately 10-times weaker in comparison with an intraperitoneal application (Carbis et al. 1997). The long-term impact of cyanobacteria-containing microcystins at lower concentrations or by food is relatively difficult to observe in individual fish; therefore, measurements from more individuals of a fish population are desirable. The measurement of aminotransferases (ALT, AST), bilirubin, albumin, cholesterol, lactate and electrolytes from blood serum has been recommended (Carbis et al.1996; Kopp et al. 2011).

The aim of this work was to study the effects of feeding fish by food with toxic cyanobacterial biomass on the biochemical indices of juvenile carp, *Cyprinus carpio* L. in laboratory conditions. The dose of MCs in fish feed was chosen to correspond to the extreme values, which can be reached in the natural environment.

Materials and methods

Fish

Fish with an average weight of 246 ± 73 g were obtained from Pohořelice Fishery. Fish were placed in laminated circular tanks, with their own recirculation, with a volume of 1 m^3. The acclimatization lasted 14 days. Fish were fed with commercial granulated food. Fish were exposed to a 12-h light/12-h dark photoperiod, and the tank water was changed daily.

Experimental design

Fish were divided into two groups, each with 40 specimens. Fish in the control groups were fed with a commercial food Dibaq Carpio Plus (Spain, 35 % proteins, 9 % fat, 29.5 % NFE, BE 24.4 MJ/kg). The second group was fed with the same food with the addition of 1 % lyophilized toxic cyanobacterial biomass of natural origin (monospecific population of *Microcystis aeruginosa*, 43.3 % proteins, 2.3 % fat). The concentrations of the total microcystins in the cyanobacterial biomass were 2,698 μg/g dry weights. Three dominant microcystin variants were present in the cyanobacteria with the following average concentrations: microcystin-RR 1462, microcystin-YR 96 and microcystin-LR 1,088 μg/g dry weights. The concentrations of microcystins in the biomass were determined by reverse phase high-performance liquid chromatography with UV/VIS detection using established methods (Bláha and Maršálek 2003). The whole amount of microcystins was 27 mg/kg of food, i.e., 0.4 mg/kg of fish weight and day. Feeding was twice daily with the whole amount totaling 1.5 % of fish stock. An alteration to the feed ration was made by week on the basis of actual fish weight.

The exposure lasted 28 days. Immediately after the capture of the fish, samples of blood were collected from 16 specimens (eight from the experimental and eight from the control group). Inadequate and hemolytic specimens of blood were discarded. Sampling of fish was conducted at the beginning of the experiment and every 7th day during and after exposure (T0, T7, T13, T20, T28). The experiment was performed in compliance with the laws for the protection of animals against cruelty as approved by the Ethical Committee of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic.

Water quality analyses

A series of physico-chemical parameters [temperature, dissolved oxygen (DO), pH, conductivity, nitrite nitrogen ($N\text{-}NO_2$), nitrate nitrogen ($N\text{-}NO_3$), ammonia nitrogen ($N\text{-}NH_4$) and phosphate phosphorus ($P\text{-}PO_4$)] were determined every week. Water parameters during the experiments were as follows (given for the experimental and control groups, respectively): water temperature 19.0 ± 0.3, 19.0 ± 0.4 °C; dissolved oxygen 92.5 ± 4.6, 87.3 ± 6.5 %; pH 7.8 ± 0.1, 7.6 ± 0.1;

conductivity 58.6 ± 13.7, 63.6 ± 2.5 mS/m; ammonia 0.13 ± 0.04, 0.18 ± 0.12 mg/l N-NH_4; nitrate 15.2 ± 0.8, 15.2 ± 1.5 mg/l N-NO_3; nitrite 0.025 ± 0.0.020, 0.126 ± 0.167 mg/l N-NO_2; and phosphate 0.38 ± 0.05, 0.47 ± 0.15 mg/l P-PO_4. Water saturation by oxygen, pH and temperature were measured using the portable Oxi 340i meter (WTW, Weilheim, Germany). Conductivity measurements were taken by conductivity meter HI 98129 (HANNA Instruments, USA), and other chemical parameters were determined using standard methods (APHA 1998).

Sampling and measurement of biochemical parameters

Fish blood was collected by cardiac puncture using heparinised syringes. Heparin at a concentration of 50 I.U. per 1 ml was used for blood stabilization. The blood was centrifuged at 400 g for 15 min at 4 °C, and the resulting plasma was stored at −80 °C until the day of the analyses. Biochemical analyses were performed by the automatic clinical chemistry analyzer KONELAB T20xt (Thermo Fisher Scientific, Finland) using commercially available reagents. Serum enzymatic activities were determined at 37 °C. Alanine aminotransferase (ALT) activity determination was based on the kinetic assessment of NADPH consumption coupled with the generation of pyruvate. Aspartate aminotransferase (AST) activity was determined by the kinetic measurement of NADH consumption coupled with the formation of oxaloacetate (Expert panel on enzymes of the IFCC 1976). Lactate dehydrogenase (LDH) was determined as the formation of NADH during the conversion of L-lactate to pyruvate (Hajzer and Jagelkova 1988). Alkaline phosphatase (ALP) was determined by a modification of the enzymatic method using an AMP (adenosine monophosphate) buffer (Tietz et al. 1980).

Total serum protein (TP) was determined by the biuret reaction (Doumas et al. 1981). Glucose (GLU) concentration was determined by the glucose hexokinase method at 37 °C with an endpoint reading at 340 nm (Barham and Trinder 1972). Calcium (Ca) and magnesium (Mg) concentrations were determined by the modified colorimetric methods with arzenazo III (Michaylova and Ilikova 1971; Skavrada 1999). Phosphorus (P) was determined by an endpoint method with sample blanking using an ammonium molybdenate reagent (Kratochvila and Garcic 1977). Total bilirubin (BIL) was determined by the oxidation reaction with potassium ferricyanide (O'Leary et al. 1993). Iron (Fe) was determined by the photometric method with ferene (ferroin-type reagent) without deproteination (Higgins 1981). The concentration of lactate (LACT) in plasma was measured by the enzymatic method according to Shimojo et al. (1989). Albumin (ALB) was determined by the photometric method with bromocresol green (Doumas et al. 1971). Urea (UREA) concentrations were determined by the kinetic enzymatic method with urease (Roch-Ramel 1967). The cholesterol (CHOL) was determined by the CHOD-PAP method after enzymatic hydrolysis and oxidation (Roschlau et al. 1974). Electrolyte levels (Na, K, Cl) were analyzed with ion selective electrodes (Eisenman 1967) by an electrolyte analyzer EasyLyte Plus (Medica, USA). The triglycerides (TRIG) were assessed by the Fossati three-step enzymatic reaction with a Trinder end point (Fossati and Prencipe 1982). The creatinine (CREA) was determined by the Jaffe kinetic method without deproteination (Jaffe 1886).

Statistical analysis

Statistica for Windows® 7.0 (StatSoft, Tulsa, OK, USA) was used to compare differences among treatment groups using a one-way analysis of variance (ANOVA) and post hoc analysis of means by the Scheffé test. The homogeneity of variances was tested by Levene's test. In these cases, the nonparametric Kruskall–Wallis and Mann–Whitney tests were used for the comparison of treatment groups. Values of $p < 0.05$ and $p < 0.01$ were considered statistically significant and highly significant, respectively, for all tests.

Results

The results of the study are presented in Table 1. The biochemical parameters affected by cyanobacterial water bloom or time are presented in Fig. 1. The total statistical evaluation of the influence of the cyanobacterial biomass in diet on the biochemical indices of the juvenile carp showed a significant decrease in activity of AST and concentration of urea compared to the controls. The significance of differences between fish groups was analyzed on every 7th day of the experiment and did not always correspond to the total

Table 1 The biochemical indices in the blood plasma of common carp (average ± SD) during the period studied (eight fish from the experimental group and from the control were analyzed at each sampling)

Indices	Unit	Group	T0	T7	T14	T21	T28
ALB	g/l	C	12.28 ± 1.49	12.62 ± 1.60	11.38 ± 1.40	13.31 ± 1.38	11.96 ± 1.65
		E	12.76 ± 1.31	12.17 ± 1.17	11.18 ± 2.10	12.57 ± 1.19	12.88 ± 2.13
ALP	μkat/l	C	0.68 ± 0.44	0.46 ± 0.19	0.58 ± 0.23	0.43 ± 0.38	0.45 ± 0.39
		E	0.65 ± 0.25	0.52 ± 0.29	0.51 ± 0.75	0.46 ± 0.21	0.77 ± 0.57
ALT	μkat/l	C	0.80 ± 0.44	0.48 ± 0.15	0.76 ± 0.25	0.77 ± 0.77	1.08 ± 0.59
		E	0.55 ± 0.20	0.51 ± 0.20	0.53 ± 0.08	0.90 ± 0.65	0.64 ± 0.17
CHOL	mmol/l	C	3.90 ± 0.52	3.46 ± 0.75	4.06 ± 1.03	3.96 ± 0.55	4.19 ± 0.65
		E	3.90 ± 0.79	3.61 ± 0.41	4.06 ± 0.83	4.06 ± 0.27	3.89 ± 0.60
GLU	mmol/l	C	2.95 ± 0.59	3.66 ± 0.61	4.87 ± 1.05	3.10 ± 0.51	3.73 ± 0.68
		E	3.36 ± 0.93	3.57 ± 0.83	3.57 ± 0.73	3.75 ± 0.53	3.48 ± 0.69
Fe	μmol/l	C	3.51 ± 1.09	2.60 ± 1.12	3.01 ± 1.32	4.39 ± 1.52	4.05 ± 1.38
		E	3.26 ± 1.67	3.32 ± 1.56	2.88 ± 1.23	5.81 ± 4.90	5.11 ± 2.88
LDH	μkat/l	C	3.72 ± 1.95	1.38 ± 0.44	3.17 ± 1.91	1.58 ± 1.55	2.53 ± 1.81
		E	2.00 ± 1.17	1.54 ± 0.60	1.99 ± 1.57	1.57 ± 0.77	2.00 ± 1.07
Mg	mmol/l	C	0.78 ± 0.11	0.80 ± 0.05	0.77 ± 0.07	0.83 ± 0.05	0.87 ± 0.10
		E	0.82 ± 0.04	0.81 ± 0.07	0.89 ± 0.14	0.83 ± 0.05	0.93 ± 0.14
BIL	umol/l	C	0.46 ± 0.22	0.85 ± 0.29	0.81 ± 0.18	0.48 ± 0.19	0.69 ± 0.29
		E	0.51 ± 0.27	0.45 ± 0.23	0.82 ± 0.39	0.64 ± 0.20	0.70 ± 0.25
TRIG	mmol/l	C	3.28 ± 0.80	3.04 ± 0.96	3.28 ± 1.12	3.81 ± 0.59	4.03 ± 1.14
		E	3.54 ± 0.90	3.03 ± 0.56	3.44 ± 0.89	3.14 ± 0.63	3.98 ± 0.91
K	mmol/l	C	2.94 ± 0.07	2.84 ± 0.30	2.65 ± 0.39	3.21 ± 0.13	3.18 ± 0.04
		E	2.83 ± 0.36	3.06 ± 0.39	2.96 ± 0.40	3.55 ± 0.31	3.60 ± 0.49
Cl	mmol/l	C	116.5 ± 2.82	117.8 ± 3.34	113.2 ± 2.72	116.3 ± 2.81	123.5 ± 4.68
		E	122.5 ± 14.89	118.5 ± 6.47	120.2 ± 10.12	121.9 ± 3.79	123.6 ± 0.71

C control, *E* experimental, *T* days after start of the experiment

statistical evaluation Significant differences were established in only one sampling (AST, T14; UREA, T21), in the other cases the difference was not statistically significant.

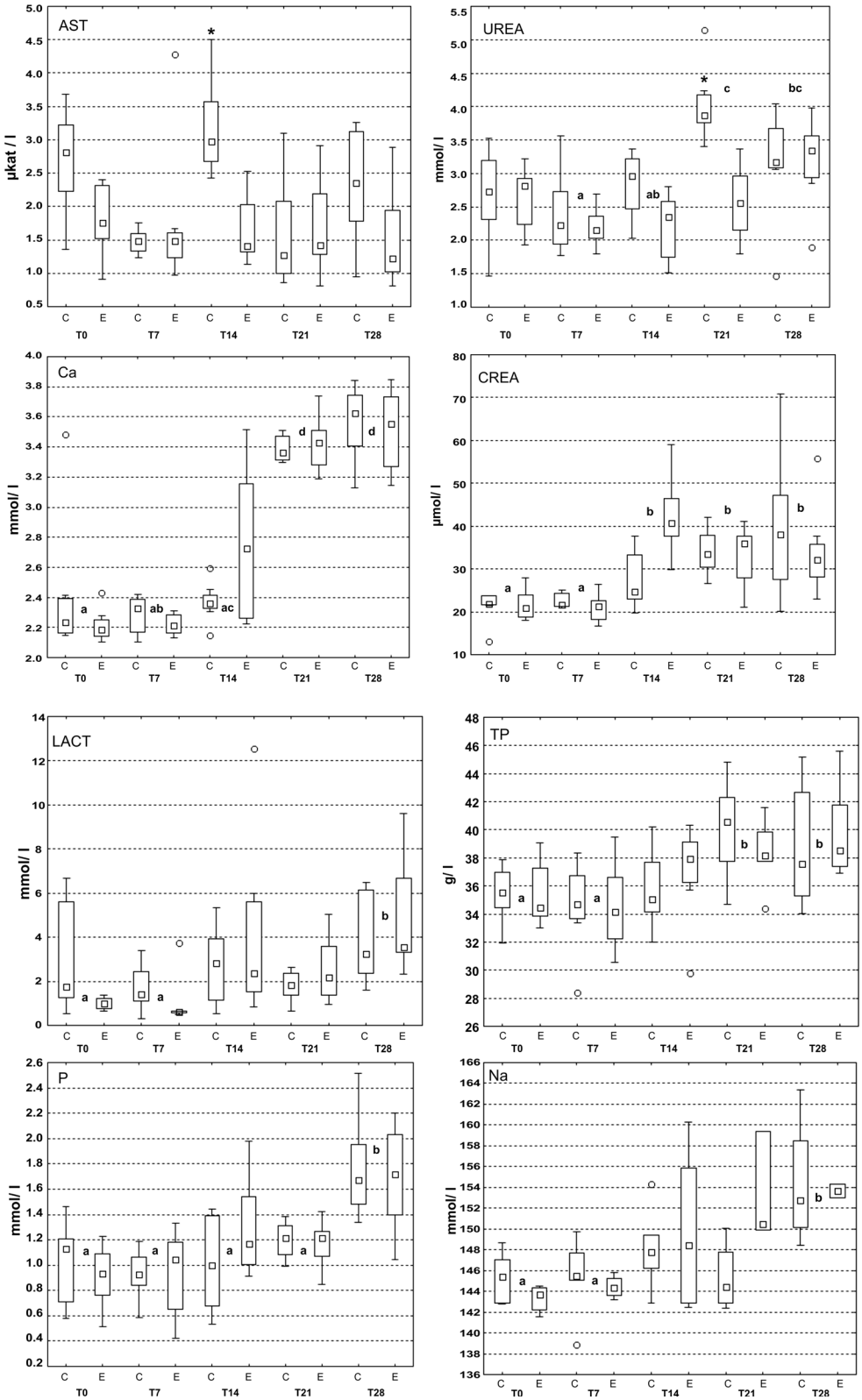
An increase in the value of some observed parameters, dependent on the duration of the experiment, was established. This increase was established in the controls as well as in the experimental fish group. The increase in calcium, creatinine, total protein, phosphorus, lactate, urea and natrium was statistically significant (Fig. 1).

Fig. 1 The effect of cyanobacteria on the biochemical indices of the common carp (*C* control, *E* experimental, *T* days after start of the experiment). *Box* includes the 25th to 75th percentiles, with the *middle point* representing the median and the spots showing the extremes. Significantly different indices compared to the control are marked by *asterisk* ($p < 0.05$). Significant differences among indices with time are marked by *letters* ($p < 0.05$)

Discussion

The values of biochemical indices obtained in this work do not correspond well with the results of other authors (Rabergh et al. 1991; Tencalla et al.1994; Carbis et al. 1996, 1997; Navratil et al.1998; Kopp and Heteša 2000a; Kopp et al. 2009, 2011; Sieroslawska et al. 2012). Some differences are probably mainly due to the different ways of administering the toxin and cyanobacterial cell biomass and to other factors e.g., nutrition and environmental condition.

Liver enzymes (ALT, AST and LDH) are the most frequently tested enzymes in fish for the indication of cyanobacterial toxicity. Rabergh et al. (1991) reported that the activity of blood plasma enzymes (ALT, AST


AST
µkat / l
UREA
mmol/ l
Ca
mmol/ l
CREA
µmol/ l
LACT
mmol/ l
TP
g/ l
P
mmol/ l
Na
mmol/ l
C E C E C E C E C E
T0 T7 T14 T21 T28

and LDH) was raised in the 2 h after an intraperitoneal injection of toxin as a consequence of hepatocyte necrosis. Tencalla et al. (1994) observed a decrease in their activity after 48 h and interpreted this as a result of damage to the majority of hepatocytes that were not able to release enzymes into the circulatory system. Significant increase in the activities of ALT, AST and LDH after the intraperitoneal or oral administration of microcystin-LR to carp has been reported (Bury et al. 1997; Navrátil et al. 1998); Malbrouck et al. 2003; Li et al. 2004). Zhang et al. 2008 observed high mortality (100 %) within 60 h post-injection of crude cyanobacterial extract in the dose μg MC-LR equiv kg^{-1} BW. Kopp and Heteša (2000a); Sieroslawska et al. (2012) stated that the activity of blood plasma enzymes was increased after 72–96 h exposure of carp to a natural cyanobacterial population. Carbis et al. (1996) noted a delay in toxic manifestation in fish exposed to water with dispersed microcystin. Serum activities of AST and ALT increased 7 days after the carp were exposed to water that contained microcystins. Feral carp from a lake, where toxic *Microcystis aeruginosa* was dominant, had higher activity of AST in the serum (Carbis et al. 1997). Malbrouck et al. (2003) reported that the activities of plasma enzymes (ALT, AST and LDH) completely recovered 21 days after the intraperitoneal injection of microcystin-LR.

Our results showed a significant decrease ($p < 0.05$) in the values of AST after exposure to cyanobacteria. Values of ALT and LDH did not significantly change during the experiment. A significant decrease in AST compared with controls was only on one occasion after 14 days of exposure. Qiu et al. (2009) and Kopp et al. (2010) similarly observed a significant decrease in AST level in the occurrence of cyanobacterial water bloom in silver carp (*Hypophthalmichthys molitrix* Val.). There were no significant changes established in the activity of AST values in experiments with common carp (Kopp et al. 2009, 2011), silver carp (Kopp and Heteša 2000b) and bighead carp (*Hypophthalmichthys nobilis* Rich.) (Qiu et al. 2009).

Even a daily high dose of cyanobacterial biomass containing microcystins intake by food to fish caused nonsignificant changes in serum, ALT, AST and LDH activities. The absorption of common concentrations of cyanobacterial biomass with microcystins in natural water through oral pathways may be limited in healthy fish. The toxic effect after the oral administration is approximately 10 times lower than after the intraperitoneal application (Carbis et al. 1996). Acute toxicity of microcystins is unlikely to occur in feral carp, and chronic injury will probably not be detected by changes of enzyme (AST, ALT and LDH) activity in the blood plasma.

Our results showed a significant decrease ($p < 0.05$) in the values of urea in blood plasma after exposure to cyanobacteria. The significant decrease in urea compared with controls was only recorded on one occasion after 21 days of exposure. Urea is not a commonly observed indicator in connection with toxic cyanobacteria metabolites. Significant changes in urea concentration were observed only in the intraperitoneal application of a microcystin extract to goldfish (*Carrasius auratus* L.). In this case, values in the experimental fish increased in comparison to control group of fish (Zhang et al. 2007). There were no significant changes in urea values established in the cyanobacteria natural habitat experiments with fish (Kopp et al. 2005; Qiu et al. 2009; Kopp et al. 2009, 2010, 2011).

The concentration of urea in blood plasma depends on the amount of protein intake in the diet, on the catabolism level of exogenous and endogenous proteins, on the ureosynthetic ability of hepatocytes, on the state of hydration and on the rate of kidney excretion. Urea is not a specific indicator for the detection of the cyanobacterial metabolites influence on fish organisms. Changes in the urea values are caused by other endogenous or exogenous factors, mainly by the diet.

Within the experiment, a significant increase in calcium, creatinine, total protein, phosphorus, lactate, urea and natrium values was detected. The value increase was established in the controls as well as in the experimental group of fish. The cause of this increase was probably a change in the physicochemical environmental conditions and the intake of quality diet during the experiment. Fish used in this experiment originated from a natural habitat without regular supplemental feeding. During the 14 day period, they were accustomed to a regular dose of feed and the higher temperature of the experimental system. The higher environmental temperature and regular dose of a good quality diet probably caused the increase in the values of some biochemical parameters. This hypothesis is supported by increasing the amount of total protein and urea whose concentration in blood plasma depends on the amount of protein intake in the diet.

Changes in the blood plasma biochemical indicators of common carp dependent on the nutrition and environmental conditions are described by Serpunin (1995), Lusková et al. (2002), Svobodova et al. (2006).

A relatively high dose of microcystins in the feeding ration of experimental fish produced only minimal changes in the observed biochemical indices of blood plasma during the 28 day experiment. Even at lower doses of MC's, significant changes were observed in the biochemical indices of blood plasma in the case of a carp experiment conducted without the direct application of MC's or cyanobacteria biomass (intraperitoneally or perorally) (Carbis et al. 1997; Navratil et al.1998; Kopp and Heteša 2000a; Kopp et al. 2009, 2011; Sieroslawska et al. 2012). A low ration of cyanobacterial biomass with a relatively high concentration of MCs used in our experiment gives an support assumptions that MCs are not principal factor of cyanobacterial biomass, which influence the parameters of fish organism. This hypothesis is supported by results of Palíková et al. (2007) who found a higher mortality of common carp embryos in the fractions of cyanobacterial biomass without MCs than in the fractions with MCs. More radical influence of the changes in fish nutrition was observed as well as environmental conditions on the fluctuation of biochemical indices of blood plasma. These results show that the microcystins in the diet have no fundamental influence on the changes in the biochemical indices of healthy fish kept in optimal environmental conditions.

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