The effect of peroral administration of toxic cyanobacteria on laboratory rats (*Rattus norvegicus* var. *alba*)

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Abstract

OBJECTIVES: The toxic cyanobacteria are a serious problem for water supply systems, recreation, and agriculture. Cyanobacteria produce numerous bioactive compounds including microcystins – the most studied cyanobacterial hepatotoxins. Only rare studies addressed realistic situation, i.e. impact of MCs accumulated in the fish tissues on the overall physiology. The aim of the present study was to provide a model simulation of the simple food chain for evaluation of impacts of cyanobacteria on the rat physiology under different exposure scenario.
METHODS: Experimental rats were fed with food with fish meat, which contained external additions of isolated microcystins as well as toxic cyanobacteria *Microcystis*, nontoxic cyanobacteria *Arthrospira* and green alga *Chlorella*. Subgroups of the animals were also challenged with a model antigen KLH to investigated immune-related parameters. We studied parameters of oxidative stress in the liver as levels of lipid peroxidation and glutathion levels. Series of hematological, biochemical and immunological parameters were also investigated.
RESULTS: Although considerable amounts of microcystins were administered to rats, all levels of MCs were under the detection limit (1 ng/g fresh weight) in the rat tissues using tandem LC/MS. Only some conjugates of microcystins with cystein and glutathion were detected in the rat liver exposed to *Microcystis* biomass (values were around the detection limit). Statistically significant depletion of body and liver weight was observed in groups with microcystin addition in comparison with all other groups. Rats exposed to MCs had stimulated immune

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system (showed higher antibody answer on administered antigen). Also modulation of some lymphocyte subpopulations was recorded with the most interesting observation of stimulated NK cell numbers in groups exposed to isolated toxins (but not to biomass containing the same toxin amount).

CONCLUSIONS: Our study demonstrates that oral exposure to microcystins in the diet may induce some detoxification responses and modulation of some hematological and immunological parameters.

Abbreviations:

ALT - alanine aminotransferase
ALB - albumin
ALP - alkaline phosphatase
ACP - acid phosphatase
AMS - amylase
AST - aspartate aminotransferase
BIL - total bilirubin
BW - body weight
CE - collision energies
CHE - cholinesterase
CHOL - cholesterol
CK - creatine kinase
CRE - creatinine
ESI - electrospray ionization
FCR - food conversion rate
FITC - fluorescein isothiocyanate
GLU - glucose
GMT - glutamyltransferase
GSH - glutathion
Hb - haemoglobin
HPLC - high performance liquid chromatography
HIS - hepatosomatic index
KLH - keyhole limpet hemocyanin
LACT - lactate
LC/MS - liquid chromatography with mass spectrometry
LDH - lactate dehydrogenase
LIP - lipase
LOD - limit of detection
MC, MCs - microcystin, microcystins
MCH - mean corpuscular haemoglobin
MCHC - mean corpuscular haemoglobin concentration
MCV - mean corpuscular volume
MDL - method detection limit
MRM - multiple reaction monitoring mode
MS/MS - tandem mas spectrometry
PBS - phosphate buffer saline
PLT - thrombocyte count
PP - protein phosphatase
PVC - haematocrit
RBC - erythrocyte count
TP - total serum protein
TRIG - triglycerides
UA - urea acid
UREA - urea
TBA - thiobarbituric acid
TBARS - reactive species
WBC - leukocyte count

INTRODUCTION

Cyanobacteria occur worldwide and are annually present in many Europeans reservoirs. They are able to produce wide range of bioactive toxins including hepatotoxic microcystins (MCs). Many of the cyanobacterial toxins have not been still indentified and might represent a cancer risk (Blaha *et al.* 2010). MCs are cyclic heptapeptides consisting of five common amino acids including two variable amino acids marked e.g. L-leucine, R-arginine Y-tyrosine. The structure of MC contains one unusual amino acid ADDA responsible for toxic effect. So far, more than 90 structural analogues of MC have been identified, and the most toxic, also the most common congener is microcystin-LR (MC-LR) (Pearson *et al.* 2010). It has been documented, that microcystins can cause intoxication and lethality in wildlife, livestock, and can cases of human illness (Dawson 1998). The toxicity of MC is associated with the highly specific inhibition (IC_{50}: 0.2 nM) of serine:threonine phosphatases, namely protein phosphatases-1 and -2A (PP-1 and PP-2A) (MacKintosh *et al.* 1990). Acute exposure to MC-LR could lead to severe liver damage, including massive intrahepatic hemorrhage and liver swelling. Chronic uptake of microcystins results in generalised hepatocyte degeneration with necrosis, progressive fibrosis and mononuclear leukocyte infiltration (Chorus *et al.* 2000).

Although toxicological properties of some cyanobacterial toxins (e.g. microcystins, anatoxins, cylindrospermopsins) are well described, there is lack of data in complex cyanobacterial biomass toxicity (Welker & von Dohren 2006).

Several studies with laboratory rodents addressed toxicity of isolated toxins but in most cases, injection applications were studied (Gupta 2003; Rao 2005). Much less is known about effects of orally-exposed toxins (Ito 2000) and to our knowledge, there are only limited studied on possible effects in more complex exposures.

In the present study, we aimed to investigate a simple model of food web transfer of cyanotoxins. Experimental rats were fed with food with fish meat, which contained external additions of isolated microcystins as well as toxic cyanobacteria *Microcystis*, nontoxic cyanobacteria *Arthrospira* and green alga *Chlorella*. Subgroups of the animals were also challenged with a model antigen KLH to investigated immune-related parameters. Using the purified hepatotoxin microcystin and complex biomass, we evaluated and compared the series of physiological hematological, biochemical and immunological parameters.

MATERIALS AND METHODS

Animals and experimental design

Experimental rats (Wistar Albino, males, 30 days old) were purchased from commercial breeding company Anlab s.r.o. (Prague, Czech Republic), and acclimated for one week in the laboratory conditions prior to the study. Animals were kept in the experimental facility (23 °C, 12h light /12h dark, 60% humidity) and were fed ad libitum with optimal diet for rats (mixture of wheat, starch, mixture of vitamins and minerals, lysine

and sunflower oil). As we have aimed to investigate impacts of microcystins by simulating simple food web model, 20% (based on wet weight) of fish meat (carp) was added to the home-made feed. Preliminary experiments showed that 20% content of fish meat did not affected food consumption by experimental animals. Rats (N=7 per exposure) were kept in the cages each containing 7 animals, they were fed ad libitum and provided with drinking water. Food consumption was recorded on the daily basis. The study lasted for 28 days. At the end, animals were weighted, sacrificed by chloroform anesthesia, and tissues and blood collected for further analyses. Weight of the major organs was recorded. Experimental conditions were the same as describe for the normal maintenance.

11 different exposure variants were investigated, some groups were also stimulated by model antigen KLH:

1. optimal food + no fish meat + placebo
2. optimal food + fish meat (20%) + placebo
3. optimal food + fish meat (20%) + KLH
4. optimal food + fish meat (20%) + biomass of *Microcystis* (1%) (5 variants of microcystins, total concentration 2.698 mg/g dry mass (MC-RR 1462 μg/g, MC-LR 1088 μg/g, MC-YR 96 μg/g and 2 non identified 43 a 9 μg/g) + placebo
5. optimal food + fish meat (20%) + *Microcystis* (1%) + KLH
6. optimal food + fish meat (20%) + *Arthrospira* (1%) + placebo
7. optimal food + fish meat (20%) + *Arthrospira* (1%) + KLH
8. optimal food + fish meat (20%) + *Chlorella* (1%) + placebo
9. optimal food + fish meat (20%) + *Chlorella* (1%) + KLH
10. optimal food + fish meat (20%) + MCs (the same concentrations as in 4.+.5) + placebo
11. optimal food + fish meat (20%) + MCs (the same concentrations as in 4.+.5) + KLH

Analyses of MCs and extraction procedure

Analyses were based on Liquid Chromatography Mass Spectrometry (MS/MS) with MRM (multiple reaction monitoring mode) using the HPLC apparatus Agilent 1200 series (Agilent Technologies, Waldbronn, Germany), which consisted of a vacuum degasser, a binary pump, an autosampler, and a thermostatted column compartment kept at 30 °C. The column was a Supelcosil ABZ+Plus RP-C18 endcapped (5 μm) 150 × 4.6 mm i.d. (Supelco). A SecureGuard C18 (Phenomenex, Torrance, CA, USA) guard column was used. The mobile phase consisted of 5 mM ammonium acetate in water, pH 4 (A) and acetonitrile (B). The binary pump gradient was linear (increase from 20% B at 0 min to 53% B at 30 min, then 90% B for 15 min); the flow rate was 0.4 mL/min. 20 μL of individual sample was injected for the analyses. The mass spectrometer was an Agilent 6410 Triple Quad mass spectrometer (Agilent Technologies, Waldbronn, Germany) with electrospray ionization (ESI). Ions were detected in the positive mode. The ionization parameters were as follows: capillary voltage, 5.5 kV; desolvation temperature, 350 °C; desolvation gas flow, 11 L/min. The transitions from the protonated molecular ion to a fragment of amino acid Adda (unusual aminoacid present only in microcystins and related nodularins – (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) at m/z 135.2 and fragment at m/z 127.1 were monitored in multiple reaction monitoring (MRM) mode. Collision energies (CE, V) used for fragmentation were 50 V for MC-RR and respective conjugates, and 40 V for MC-YR and -LR and respective conjugates). Quantification of analytes was based on external standards of MC-RR, MC-YR, MC-LR in matrix (final extract of microcystin-free fish tissue). Method detection limit (MDL; per gram of tissue, fresh weight) was 3 ng/g in MRM mode.

Tissue (frozen sample; 0.5 g fresh weight) was homogenized three times with methanol (3 mL), sonicated in an ultrasonic bath for 30 min, and centrifuged at 4 000 g for 10 min. Supernatants were pooled and extracted repeatedly (three times) with hexane (1 mL) to remove lipids. Extract was evaporated at 50 °C, and the residue was dissolved in 300 μL of 50% aqueous methanol (v/v).

Determination of lipid peroxidation and GSH

The liver tissues were homogenized on ice in phosphate buffer saline (PBS, pH 7.2) using mechanical homogenizer, 100 mg of tissue in 1 mL of PBS; postmitochondrial supernatant was collected after centrifugation (15 min at 10 000 g at 4 °C) and stored frozen at −80 °C until biochemical analyses. All biochemicals and enzymes were purchased from Sigma-Aldrich (Prague, CR), other chemicals used for preparation of buffers were of the highest commercial grade available. Concentration of glutathione (GSH) was determined according to the method described by Ellmann (Ellmann 1959) using 5,5-dithiobis-2-nitrobenzoic acid as a substrate. The level of lipid peroxidation in tissues was assessed as total thiobarbituric acid (TBA) reactive species (TBARS) (Livingstone *et al.* 1990). The extracts were mixed with trichloroacetic acid (TCA, 6% w/v) and butylated hydroxytoluene (0.6% w/v) and centrifuged (1 500 g for 20 min). Supernatant was further mixed with 0.06 N HCl and 40 mM TBA prepared in 10 m MTRIS (pH 7.4). The mixture (total volume 250 μL) was boiled in water bath for 45 min and then cooled to room temperature. Absorbance of the sample was measured at 550/590 nm and the concentration of TBARS (nmol TBARS per milligram protein) was calculated according to the standard calibration curve generated with malondialdehyde prepared by acidic hydrolysis of 1,1,3,3-tetraethoxypropane. The protein concentrations were determined by the method using Folin–Ciocalteu

phenol reagent that forms with proteins red-coloured complex measurable at 680 nm (Lowry *et al.* 1951) . Bovine serum albumin was used as a standard for protein calibration. The GENios microplate reader (Tecan Group, Switzerland) was used for measurement of absorbance in all spectrophotometric assays.

Sampling and measurement of hematological and biochemical parameters

Blood was obtained from rats by cardiopuncture using syringes. The blood samples (about 3 ml) were rapidly collected in a beaker containing 0.1 ml of 500 mM EDTA. The Animal Blood Counter Veterinary (ABC Vet, Horiba ABX, France) was used for our blood analysis. It is fully automated (microprocessor controlled) haematology analyser used for the in vitro diagnostic testing of blood specimens. Values of haemoglobin (Hb), haematocrit (PCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), erythrocyte (RBC), leukocyte (WBC) and plateled count (PLT) were determined. 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 analyses. Biochemical analyses were performed by the ADVIA 1650 automatic analyser (Siemens, USA) using commercially available reagents. Serum enzymatic activities were determined at 37 °C. Alanine aminotransferase (ALT) activity determination was based on the kinetic assessment of NADH consumption coupled with the generation of pyruvate (Expert panel 1976). Aspartate aminotransferase (AST) activity was determined by kinetic measurement of NADH consumption coupled with the formation of oxaloacetate (Expert panel 1976). Lactate dehydrogenase (LDH) was determined as the formation of NADH during conversion of L-lactate to pyruvate (Hajzler & Jagelkova 1988). Alkaline phosphatase (ALP) was determined by a modification of the enzymatic method using AMP (adenosine monophosphate) buffer (Tietz *et al.* 1980). Acid phosphatase (ACP) activity was determined by an enzymatic method with 1-naphtylphosphate (Hillmann 1971). Cholinesterase (CHE) was determined by a modification of the kinetic test with butyrylthiocholine (Gary 1971). The γ-Glutamyltransferase (GGT) method is based on the procedure described by Shaw *et al.* (1983), modified by IFCC. The creatine kinase (CK) method is based on the procedure described by Szasz *et al.* (1976), modified by IFCC.

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 & Trinder 1972). Phosphorus (P) was determined by an endpoint method with sample blanking using ammonium molybdenate reagent (Kratochvila & 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 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 (Roeschlau 1974). Electrolyte levels (Na, Cl) were analysed by ion selective electrodes (Eisenman 1967). Amylase (AMS) was determined by the method using ethylidene blocked p-nitrophenyl-maltoheptaoside as a substrate (Jensen & Wydeveld 1958). The triglycerides (TRIG) were assessed by the Fossati three-step enzymatic reaction with a Trinder endpoint (Fossati & Prencipe 1982). The lipase (LIP) activity was determined by the enzymatic colorimetric assay according to Neumann *et al.* (1987). The uric acid (UA) method is based on the Fossati enzymatic reaction using uricase with a Trinder-like endpoint (Fossati *et al.* 1980). The creatinine (CRE) was determined by the Jaffe kinetic method without deproteination (Jaffe 1886).

Immunization of rats and determination of anti-KLH antibodies

For determination of the ability to mount a specific antibody response to an antigen, rats were immunized 3 weeks prior the end of the experimental period with KLH (Hemocyanin from *Megathura crenulata*, Sigma-Aldrich). PBS used as medium was sterilely filtrated (0.1 μm filtr) and with KLH (200 μL containing 200 μg KLH/rat) injected into abdominal cavity. Animal antibodies against KLH were measured using commercial ELISA assay Anti KLH (TDAR) Rat-IgG ELISA kit, SHIBAYAGI, Japan.

Immunological parameters

Fifty micro liters of rat peripheral blood was lysed with an ammonium chloride solution (154.4 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, all from Sigma-Aldrich, St. Louis, USA), blood leukocytes were washed with cell washing solution (CWS, phosphate buffered saline containing 1.84 g/L EDTA, 1 g/L sodium azide and 4 mL/L gelatin, all from Sigma-Aldrich) and the final leukocyte count was ascertained. Total white blood cell count was ascertained using an auto hematology analyzer (BC-2800Vet, Shenzhen Mindray Bio-Medical Electronics, Shenzhen, People's Republic of China). The 1×10^6 leukocytes were stained with two different coctails of primary antibodies for 15 min at 4 °C. The T-lymphocytes, B-lymphocytes and NK cells were distinguished by R-PE-conjugated anti-CD3 (clone eBioG4.18, eBioscience, USA), FITC-conjugated anti-IgM (clone HIS40, eBioscience, USA), AlexaFluor647-conjugated anti-CD161 (clone 10/78, Biolegend, USA), FITC-conjugated

anti-CD8α (clone G28, Biolegend, USA) antibodies, APC-conjugated anti-CD4 (clone W3/25, Biolegend, USA), AlexaFluor647-conjugated anti-γδTCR (clone V65, Biolegend, USA). The cells were finally washed with CWS and measured immediately by FACS Calibur flow cytometer (Becton Dickinson, USA). At least 40,000 events were acquired. Postacquisition analysis of data was performed using Summit software (DAKO, Denmark). The T-cells were calculated as percentage of CD3+ cells from all lymphocytes. The percentage of CD4/CD8 cells was calculated as percentage from CD3+ cells. Gamma-delta T-cell subpopulations were calculated as percentage from all γδT-cells.

Statistical analyses

Differences between experimental groups were tested by Analysis of Variance followed by LSD test, and the results were controlled by non-parametric methods (Kruskal-Wallis ANOVA, Mann-Whitney U-test). $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Mean dose of MCs for exposed groups (4,5,10 and 11) were 3.2 mg MCs/kg BW/day, thus the dose of MCs is lower than lethal dose of the orally given MC-LR (10.9 mg/kg) estimated by Yoshida *et al.* 1997. The feed containing cyanobacterial biomass or pure microcystins had several harmful impacts on experimental rats. Statistically significant was the decrease of weight gain after 4 week of exposure in the group 10 and 11 exposed with MCs, compared with control groups 1 and 2 (Figure 1). Decreased weight gain corresponds with adverse food conversion ratio FCR (ratio between mass of the consumed food divided by the weight gain) for the same groups (10+11) (Table 1). All groups fed with fish in food (groups 2–11) had much higher FCR than controls without fish (group 1). Groups 10+11 (due to lower weight gains) had even higher food conversion rates. Calculation of hepatosomatic index (HSI) does not show significance difference from control among groups (Table 1) although MCs could increase liver weight as well as HSI (Gupta *et al.* 2003; Moreno *et al.* 2003). Lower weight gain and negative impact on food conversion ratio show adverse impact on metabolism of experimental rats. Decreased of mean body weight after exposure of cell extract of *M. aeruginosa* was pre-

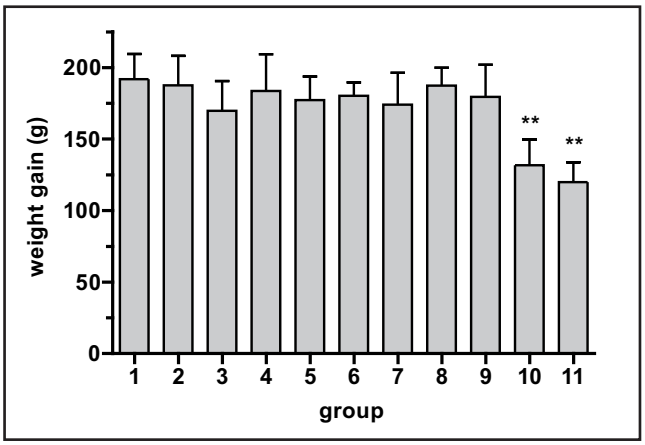

Fig. 1. Effect of different exposure on the weight gain of rats exposed daily for 28 days with *Microcystis* cell extracts (group 3,4) or microcystins (10,11). Data are expressed as mean ± SD, N=7. **Statistically different (Dunn's multiple comparison test) are groups 10,11 from the controls 1+2.

Tab. 1. Description of individual experimental variant, analysis of microcystin on liver, hepatosomatic index (HSI), weight gain, food consumtion and food conversion FCR (food consumtion/weight gain) after 28 days of subchronic exposure.

Group number	Description	MC analyses**	HSI* [%]	Weight gain (sum of the all animals) [g]	Food consumption (sum for all animals) [g]	FCR
1	Control	na	4.7±0.4	1343.9	3844.9	2.86
2	Control+fish meat	na	4.1±0.7	1313.7	5698.8	4.34
3	Control+fish meat+KLH	na	4.3±0.4	1188.6	5571.0	4.69
4	Fish meat+*microcystis*	conjugates	4.6±0.4	1285.8	6227.6	4.84
5	Fish meat+*microcystis*+KLH	conjugates	4.8±0.2	1241.2	6136.7	4.94
6	Fish meat+*Arthrospira*	na	4.7±0.3	1261.5	5775.6	4.58
7	Fish meat+*Arthrospira*+KLH	na	4.2±0.3	1218.7	5790.1	4.75
8	Fish meat+chlorella	na	4.5±0.2	1311.8	5887.3	4.49
9	Fish meat+chlorella+KLH	na	4.4±0.1	1256.4	5733.0	4.56
10	Fish meat+MCs	< LOD	4.1±0.3	921.7	5417.3	5.88
11	Fish meat+MCs+KLH	< LOD	4.5±0.3	839.8	4834.7	5.76

* Data are expressed as mean ± SD

** na - tissues not analysed (animals not exposed to food potentially containing MCs); conjugates - no MCs were detected, only traces of conjugeates with cystein or GSH observed in some samples; < LOD - MCs or conjugates were not observed.

viously described in other studies with mice (Bu *et al.* 2006; Ding *et al.* 2006).

Our previous studies (Adamovsky *et al.* 2007; Kohoutek *et al.* 2010) demonstrated very low concentrations of MCs in other tissues than liver, which is a target organ for MCs. Thus, we investigated MC concentrations in the liver tissue. Animals, which were exposed to materials containing MCs were analysed by LC-MS/MS (groups 4+5 and 10+11). In none of the sample, individual toxins were detected above LOD, traces of MC-conjugates with cystein and glutathion were detected in the liver of animals exposed to *Microcystis* biomass (groups 4+5 – values were always around the detection limit). In groups 10+11 (exposed to isolated MCs) no conjugates were observed (Table 1). Generally, existing analytical method of MCs overestimates real concentration of MCs, but advanced analytical technique LC-MS/MS, used in our case, demonstrates that many analytes are not in fact microcystins (Kohoutek *et al.* 2010). Detailed information about concentration of MCs in rat's liver are rare, but studies prove MCs in liver with radioassay (Robinson *et al.* 1989).

We studied influence of *Microcystis* biomass and pure microcystin in diet on activation of detoxification system of rats using markers of oxidative stress – glutathione and lipid peroxidation (LPO) marker MDA. Conjugation with GSH is essential step in detoxification pathway of MCs (Fu & Xie 2006) while lipid peroxida-

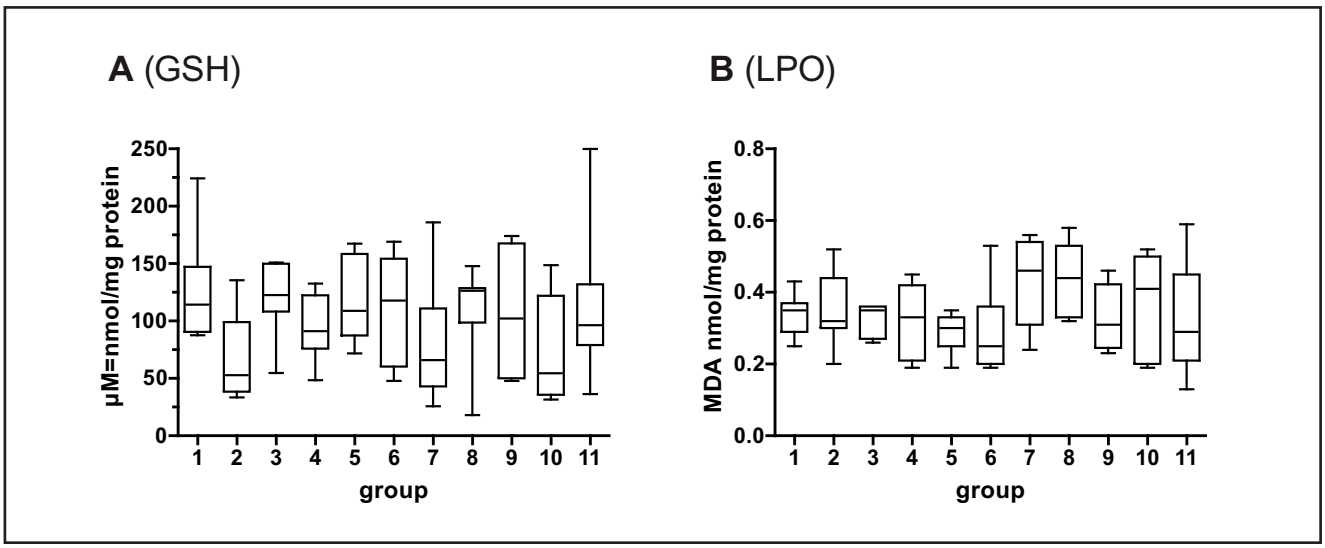

Fig. 2. Level of glutathione (**A**) and lipid peroxidation (**B**) in the livers of rats after 28 days of exposure. Each group is consisted of 7 individuals. Graphs represent min to max box blot, with median.

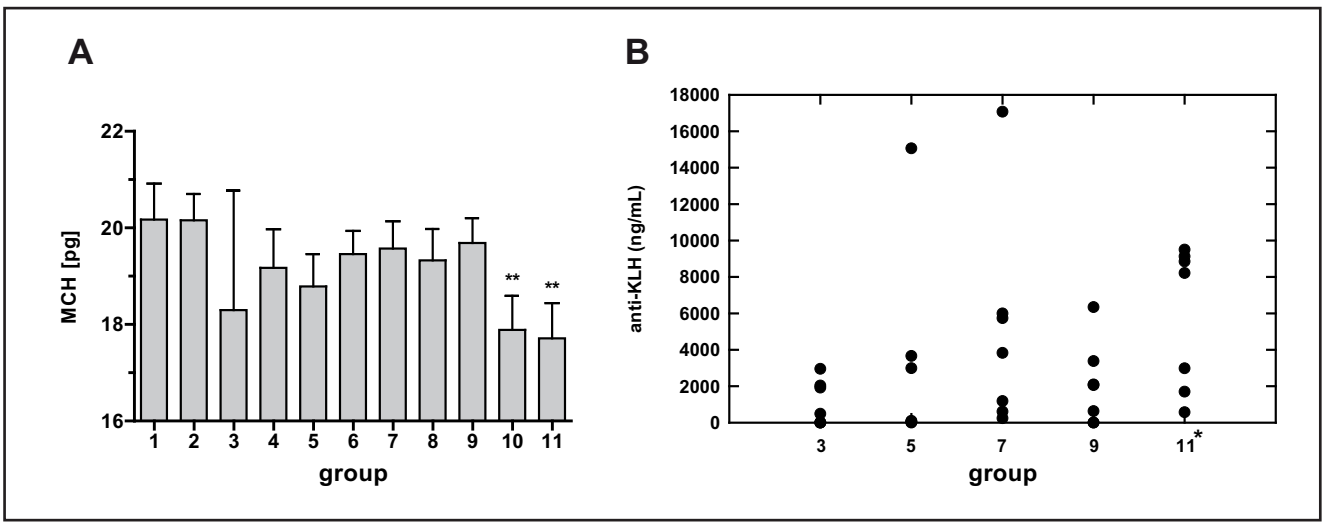

Fig. 3. (**A**) Effect of different exposure on the mean corpuscular hemoglobin (MCH) of rats exposed daily for 28 days with *Microcystis* cell extracts (group 3,4) or microcystins (10,11). Data are expressed as mean ± SD, N=7.
**Statistically significant are groups 10,11 from the controls 1+2.
(**B**) The concentration of anti-KLH antibody (ng/mL) 21 days after immunization with KLH (200 µg/rat). Figure shows stimulation of immune system in the group fed with food with MCs (group 11) . *Significantly higher then control group 3 (ANOVA + LSD test)

tion point out insufficient detoxification system. There was substantial variability both within and between experimental groups in studied biomarkers levels of antioxidant GSH and lipid peroxidation (Figure 2). Although some statistical significances were recorded (e.g. elevated LPO levels in groups 7,8 compared to some other groups with low LPO 3–6). No systematic trend in biomarker responses were observed (see Figure 2B), although some authors observed increases in hepatic lipid peroxidation (Jayaraj *et al.* 2006; Rao *et al.* 2005) and generation on reactive oxygen species in liver of mice (Weng *et al.* 2007). Interestingly, there

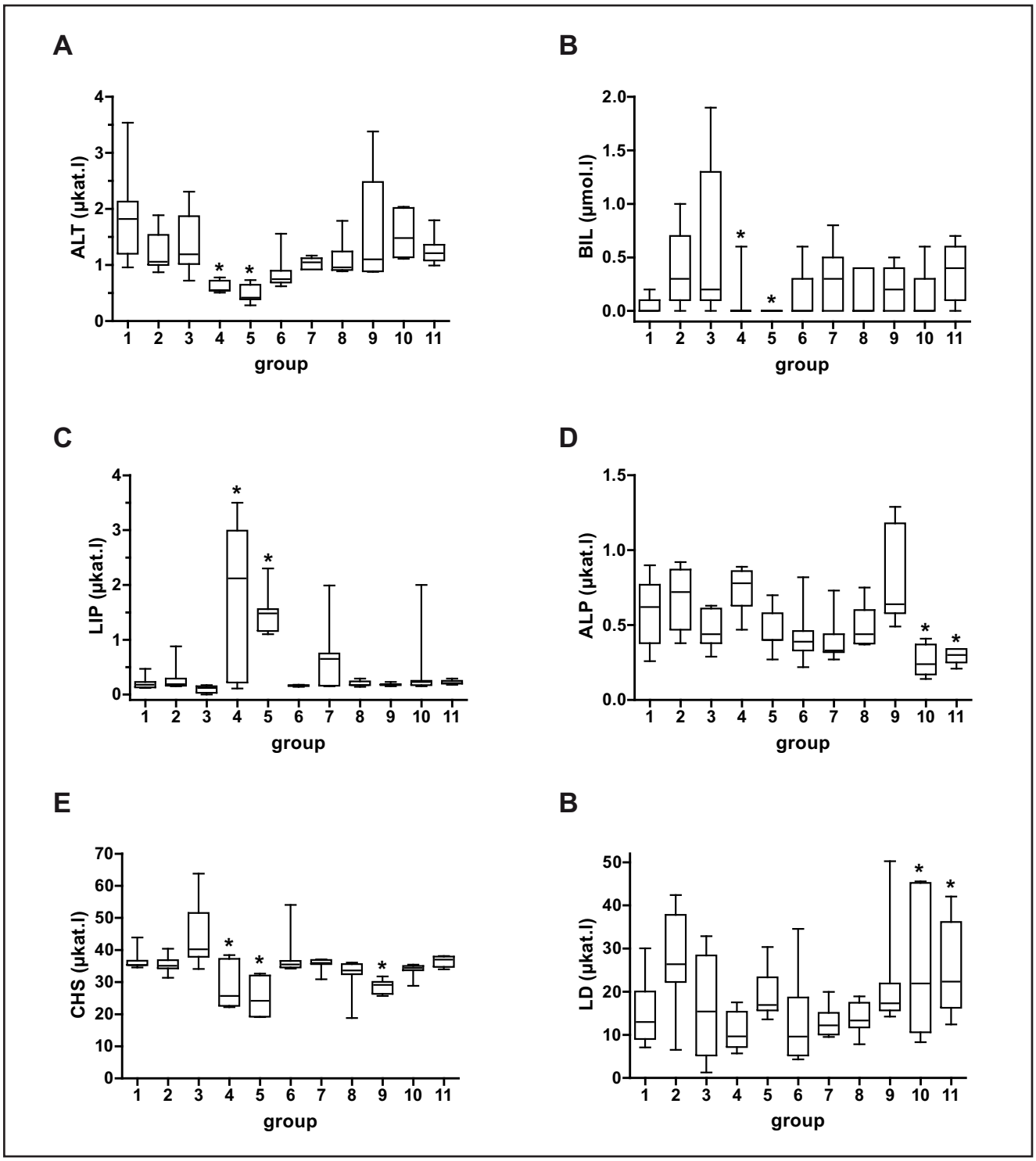

Fig. 4. Results of selected biochemical parameters of the blood that were significantly altered. Effect of cyanobacterial biomass (4,5) and MCs (10,11) on levels of (**A**) alanine amino transferase, (**B**) bilirubin, (**C**) lipases, (**D**) alkaline phosphatase, (**E**) cholinesterase and (**F**) lactate dehydrogenase. *Significantly different from control groups (LSD test or Dunn's Multiple Comparison Test, n = 7)

is no statistical difference in the levels of GSH among groups (1–11), although it was described depletion of GSH (Gupta *et al.* 2003; Rao *et al.* 2005) as well as higher synthesis of GSH (Gehringer *et al.* 2004) (Jayaraj *et al.* 2006) after exposure of MCs in mammals.

In most of the assessed hematological parameters such as white blood cell count (WBC), red blood cell count (RCB), hemoglobin (HBG), hematocrit (HCT), platelet count (PLT) and mean cell volume (MCV), no significant differences among groups were observed. Interestingly, there was a statistical significant decline in MCH (mean corpuscular hemoglobin – Figure 3A) and corpuscular hemoglobin concentration (MCHC) at groups 10+11 in comparison with controls (1+2). Lower cell-bound hemoglobin but no changes in RBC and total HGB, might indicate possible lyses of red blood cells. Similar trends of HCM after exposure of MCs were observed in several studies with fish (Gupta & Guha 2006). Theoretically, lower MCH could be due to production of early forms of red blood cells with lower level of hemoglobin. This argument is supported with statistically lower levels of MCHC in exposed groups (10+11). Taken together, hematological parameters point out negative impact on blood composition of rats after oral exposure with MCs.

Determination the levels of anti-KLH antibody in blood after stimulation with KLH bring information about adverse effects on the functioning of the immune system. The analysis shows statistical difference in concentration of anti-KLH antibody between control group 3 and 11 (MCs exposure) using ANOVA and LSD test (Figure 3B). The results demonstrated that treatment with MCs evoked robust antibody response on antigen KLH and showed significant stimulation of immune

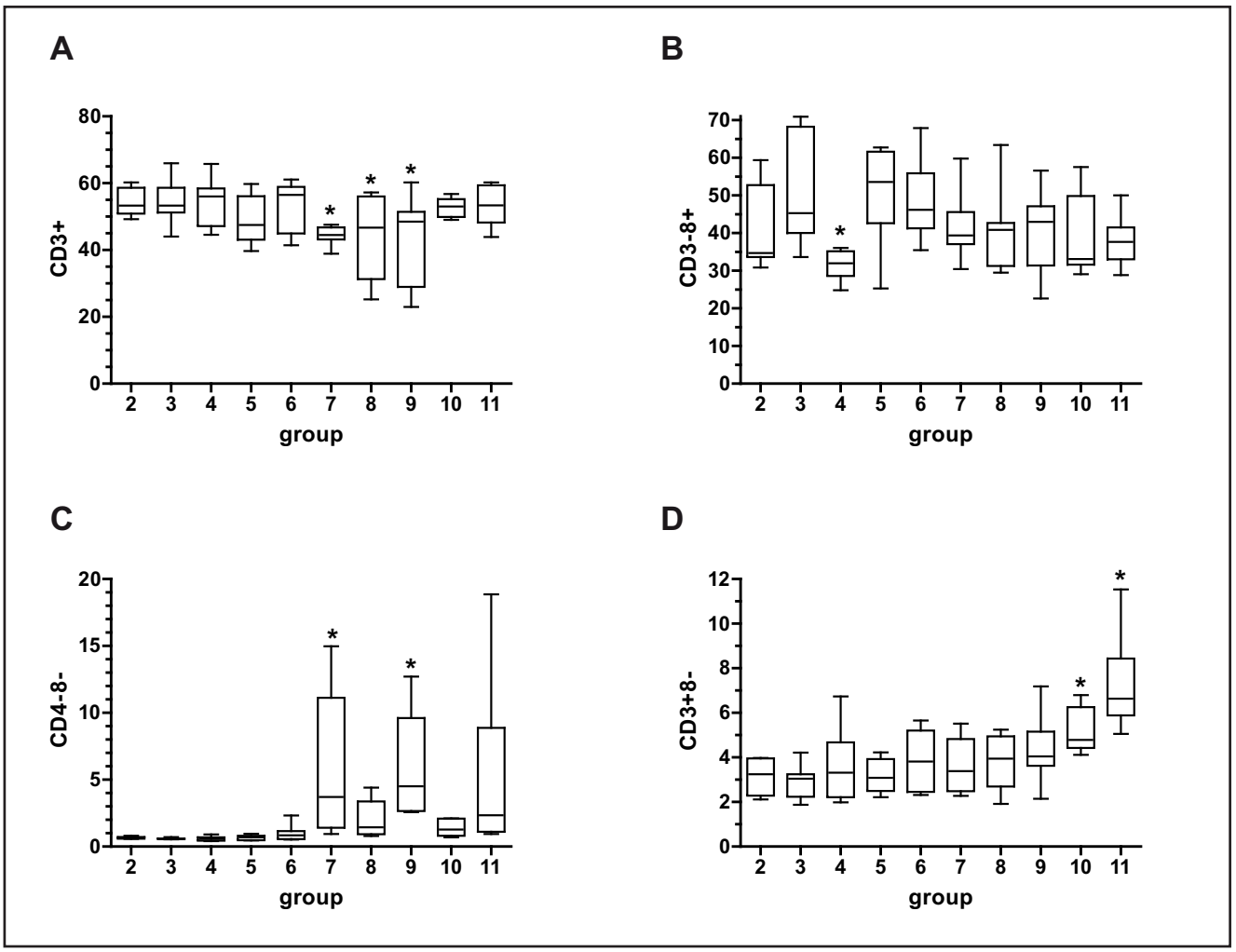

Fig. 5. Distribution of selected lymphocyte markers in 11 groups of rats exposed for 28 days to various food contaminated by cyanobacteria. Results of CD3+ distribution (**A**) and CD3–8+ lymphocytes (**B**) show variability among groups with problematic interpretation of statistical differences. Panel (**C**) - elevated CD4–8– values in groups 7 and 8 (KLH-challenged groups fed with nontoxic cyanobacteria and algae). Panel (**D**) - elevated CD3+8– lymphocytes in groups exposed to isolated microcystins in food.
*(**A**) Groups no. 7,8,9 had statistically lower values than groups 2,3,4 (, ANOVA, LSD $p<0.05$).
(**B**) Group 4 had significantly lower values of CD3–8+ then group 3,5,6.
(**C**) Groups 7,9 had significantly higher values in comparison to corresponding "non-KLH" groups (7 vs. 6; 9 vs. 8).
(**D**) Significantly increased CD3+8– lymphocytes values in groups 10+11 in comparison with the majority of the experimental variants.

system. The modest modulation of immune system was also observed in other experimental variants treated with KLH (groups 5, 7, 9), but without statistical significance (Figure 3B). Information about changes of immune system after exposure of MCs are rare, but several studies indicate that MCs can modulate immune system of mammals, significantly induce changes in the WBC number (Yuan *et al.* 2010) and deregulate lymphocytes (Lankoff *et al.* 2004; Yea *et al.* 2001).

We determined the set of biochemical blood parameters to observe the markers of health status and effect on liver such as levels of albumin (ALB), alkaline phosphatase (ALP), alanine amino transferase (ALT), aspartate aminotransferase (AST), bilirubin (BIL), amylase (AMS), cholesterol (CHOL), creatinkinase (CK), total serum protein (TP), glucose (GLU), creatine (CREA), uric acid (KM), lactate (LACT), lactate dehydrogenase (LD), phosphorus (P), triglycerides (TRIG), ferrum (Fe), urea (UREA), lipases (LIP), cholinesterase (CHS), acid phosphatase (ACP), sodium (Na) and chlorine (Cl). For majority of the parameters were observed no significant differences for exposed group with *Microcystis* (4,5) or pure MCs (10,11) (ALB, AST, CK, LACT, AMS, TP, GLU, KM, TRIG, UREA and ACP). In comparison with control groups, there were statistically significant increases in group 4 and 5 (animals treated with *Microcystis*) in levels of CHOL, CREA, P and LIP but interestingly, some parameters were statistically decreased (ALT, BIL, CHS). Experimental group treated with pure MCs in diet (group 10+11) show different pattern in examined biochemical parameters. Statistically increased were parameters CHOL, Fe, LD, Na, Cl but only ALP was statistically decreased. Statistically significant effects of microcystin or cyanobacterial biomass on biochemical markers including hepatic enzymes show Figure 4.

Serum enzymes of hepatic origin (ALT, AST) have shown significant decrease or no effect in all experimental variants (Figure 4A). Increase in ALT and AST is a characteristic toxic effect of microcystins and reported in a number of animal models. They are cytosolic enzymes that are released into the blood following hepatocellular damage or necrosis (Clark *et al.* 2007; Gupta *et al.* 2003; Hermansky *et al.* 1990; Rao *et al.* 2005). Decrease in ALT in group 4,5 (exposed with complex biomass of *Microcystis*) corresponds with the previous findings suggesting that subchronic MCs toxicosis decrease ALT synthesis in hepatocytes (Solter *et al.* 2000). Decrease of the levels of ALT could be also explained with the presence of other biologically active agents in complex biomass of *Microcystis*. Lactate dehydrogenase (LD) release is another commonly used marker for lethal hepatocellular injury. For example, (Clark *et al.* 2007; Rao *et al.* 2005) reported that MCs exposed i.p. or orally were able to increase enzyme activity of LD. Our results correspond well with these findings, that exposure to microcystins causes tissue damage in rats as demonstrated by significant increase lactate dehydrogenase (LD) activities and modest increase in AST activity in group 10,11 (Figure 4F). However several studies showed positive effect of MCs on ALP activities in the liver of mice and rats, our results demonstrate decrease of ALP in the blood of rats (Figure 4D) (Hooser *et al.* 1989). Modulated ALP level, enzyme involved in membrane transport, show negative impact of interaction of MCs with liver (Atencio *et al.* 2008). In our study, the increases in lipases (LIP, Figure 4C) observed in the group exposed with complex cyanobacterial biomass were likely caused by processes of inflammation. Although the most abundant peptide in cyanobacterial biomass is microcystin, cyanobacterial cells contain wide range of biological active compounds. These compounds have apparently an influence on the levels of some biochemical parameters of blood in comparison with group exposed with pure MCs (Figure 4). Generally, there are a number of studies on acute and subacute toxic effects of microcystin, but there are very few studies on comparison of effects caused by complex cyanobacterial biomass and pure MCs on extensive set of biochemical parameters of blood.

Multiple immunological markers related of the lymphocyte phenotype were analyzed by flow cytometry in exposed animals. For majority of the parameters substantial variability within groups was observed with no significant differences among exposures (CD4+, CD8+, CD4+CD8+, γδ+, γδ8+, γδ8–, CD161+, CD3+8+, CD3–8–), and less pronounced differences were observed also for other markers. For example at CD3+, groups no. 7,8,9 had statistically lower values than groups 2,3,4 (Figure 5A, ANOVA, LSD $p<0.05$), but the differences were caused by few outliers and biological interpretation was problematic (group 7 was fed with Fish meat+*Arthrospira*+KLH and groups 8–9 contained *Chlorella* algae +/– KLH challenge). The difference with poor biological interpretation was also observed at CD3–8+ (Figure 5B) where group 4 (fish meat with *Microcystis* biomass) had significantly lower values of CD3–8+ then group 5 (the same exposure as no. 4 but challenged with KLH), group 3 (control + KLH) or group 6 (meat with *Arthrospira* without KLH challenge).

For CD4–8–, elevated values were recorded in groups fed with non toxic cyanobacteria *Arthrospira* (group 7) and green alga *Chlorella* (group 9) and simultaneously challenged to KLH in both groups (Figure 5C). Interestingly, these groups had higher CD4–8– also in comparison to corresponding "non-KLH" groups (7 vs. 6, 9 vs. 8), and this may indicate some interference between exposure to non-toxic phototrophs and immunogen (KLH). The results thus demonstrate stimulation by KLH challenge in combination with non-toxic cyanobacteria and alga *Arthrospira* and *Chlorella* (CD4–8–), which corresponds to literature. Number of studies have shown immunostimulatory effects of *Spirulina* (currently *Arthrospira*) in mice (Hayashi *et al.* 1994,

1998, 2006, 2009). Also lower concentrations of cyanotoxins were shown to stimulate the immune system (Mundt *et al.* 1991, Hernandez *et al.* 2000).

From the perspective of the hypothesis of the present work, i.e. investigation of cyanobacterial toxins, the most interesting result was represented by increased CD3+8– lymphocytes in groups no. 10 and 11 (i.e. groups exposed to isolated microcystins in food, Figure 5D). CD3+8– is a subpopulation of NK T-cells, which has cytotoxic functions towards tumors and virus-infected cells. Although B- and T-lymphocyte numbers were previously studied, to our knowledge none of the studies investigated specifically CD3+8– cells. Our previous investigation (unpublished results) also suggested increase in NK cells in rats exposed to isolated toxins, and this issue will require further attention.

Apparent difference between groups exposed to isolated microcystins (groups 10+11) and similar groups fed with microcystins present in the form of cyanobacterial biomass (groups 4+5) indicate specific mixture interactions with potential protective effects of the complex green biomass.

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