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Physiological responses of *Xenopus laevis* tadpoles exposed to cyanobacterial biomass containing microcystin-LR

Andrea Ziková[a,*], Claudia Lorenz[a], Ilka Lutz[a], Stephan Pflugmacher[b], Werner Kloas[a,c]

[a] Department of Ecophysiology and Aquaculture, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, 12587 Berlin, Germany
[b] Department of Ecological Impact Research and Ecotoxicology, Berlin Institute of Technology, Ernst-Reuter-Platz 1, 10587 Berlin, Germany
[c] Department of Endocrinology, Institute of Biology, Humboldt University Berlin, Invalidenstr. 43, 10115 Berlin, Germany

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ABSTRACT

Cyanobacteria are the primary biomass producers and some species synthesize remarkable amounts of secondary metabolites, the so-called cyanotoxins. Several reports deal with the most common cyanotoxins, microcystins (MCs), and their effects on fishes but only a few studies investigated a natural exposure to MCs and limited information is available concerning the further aquatic vertebrate class, amphibians. In the present study, *Xenopus laevis* tadpoles at stage 52 (Nieuwkoop and Faber, 1994) were exposed for 1, 3, 7, and 21 days to diets containing lyophilized cyanobacterial biomass without and with microcystin-LR (MC-LR) at concentrations of 42.8 and 187.0 μg MC-LR/g diet, respectively, to determine impacts on MC-LR bioaccumulation, development, stress, and biotransformation. The fate of MC-LR present in diet and water was determined in whole body using liquid chromatography with tandem mass spectrometry detection. Effects on development were assessed by recording mortality, weight and developmental stage. In parallel, mRNA levels of hypophyseal thyroid stimulating hormone (TSH) associated with metamorphosis and of gonadotropins, luteinizing hormone and follicle stimulating hormone, triggering sexual differentiation, were assessed. Concerning stress, corticosteroid levels and mRNA expression of heat shock protein 70 (HSP70) as stress biomarkers were examined. Furthermore, mRNA expression of biotransformation enzymes of all three phases as well as biomarkers for oxidative stress were determined.

Surprisingly, exposure to cyanobacterial biomass containing MC-LR supplied via diet as natural exposure neither resulted in measurable bioaccumulation of MC-LR nor affected dramatically development. Only minor to negligible physiological impacts on development, stress, and biotransformation mechanisms were found suggesting that *X. laevis* tadpoles seem to have some mechanisms to be able to cope quite well with diets containing lyophilized cyanobacterial biomass even with considerable amounts of MC-LR.

1. Introduction

Cyanobacterial water bloom is for decades a worldwide issue especially concerning the production of compounds, particularly secondary metabolites known as cyanotoxins. Cyanotoxins have severe effects on vertebrates which are based on their chemical structures. Cyclic peptides are mainly associated with hepatotoxicity whereas alkaloids are known to be neurotoxic and lipopolysaccharides have the potential to be irritants (Carmichael, 1992). Microcystins (MCs), the most common hepatotoxins in freshwater systems, are a family of toxins produced primarily by the species *Microcystis aeruginosa* but also by other *Microcystis* species and other genera, namely *Anabaena*, *Oscillatoria* and *Nostoc* (Dawson, 1998). In middle Europe cyanobacterial water blooms reach MC values of 3954 μg/g d.w and concentrations of MCs in water of 36.9 μg/L (Bláha et al., 2010).

MCs were thoroughly studied using several experimental organisms and cell cultures to evaluate the potential adverse effects such as liver damage resulting in haemorrhage, hepatic insufficiency (Van Apeldoorn et al., 2007), tumour promoting activity (Campos and Vasconcelos, 2010) and estrogenic effects (Oziol and Bouaïcha, 2010; Sychrová et al., 2012). Key proteins involved in MC biotransformation and excretion have been identified, demonstrating the ability of aquatic organisms to metabolize and excrete the toxin (Pflugmacher et al., 1998; Campos and Vasconcelos, 2010). Although cyanobacteria inhabit aquatic environment and the potential target organisms of their toxins are fish and amphibians most studies have been performed with non-aquatic mammals such as rodents (Zegura et al., 2011; Campos and Vasconcelos, 2010). Effects on fish have been mostly

* Corresponding author. Tel.: +49 30 64181 683; fax: +49 30 64181 663.
E-mail addresses: zikova@igb-berlin.de, Andrea.Zikova@seznam.cz (A. Ziková).

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studied using intraperitoneal application of toxins that is far from natural exposure to these metabolites (Prieto et al., 2006). However although some studies concerning natural exposure of cyanobacterial biomass in fish exist (Adamovský et al., 2007), information about impacts of cyanobacteria and their metabolites on amphibians are very scarce. Most of the amphibian studies dealing with cyanotoxins use the frog embryo teratogenesis assay *Xenopus* (FETAX) as a 96 h standardized toxicological test (Burýšková et al., 2006; Dvořáková et al., 2002) or expose *Xenopus laevis* embryos for a longer period of 120 h (Fischer and Dietrich, 2000). Thereby, the impacts of pure MC as well as toxic and nontoxic cyanobacterial biomass, cyanobacterial fractions devoid of MCs or crude aqueous extracts of naturally derived cyanobacterial biomass containing MCs have been analysed (Dvořáková et al., 2002; Burýšková et al., 2006; Fischer and Dietrich, 2000). Frog embryos in FETAX treated with radiolabeled MC-LR for 96 h revealed no MC-LR bioaccumulation suggesting that MC cannot be taken up transchorionally/transdermally and needs to be taken up orally after hatching (Fischer and Dietrich, 2000). Furthermore, no increased mortality, malformations or growth inhibition could be detected in early life stages of *X. laevis* exposed to pure MC-LR and MC-RR. Similarly, no effects were observed in marsh frogs (*Rana ridibunda*) treated in embryonic stages with pure MC-LR and MC-YR (Oberemm et al., 1999). Exposure of free swimming and feeding tadpoles of *Bufo arenarum* at Gosner stage 17 (Gosner, 1960) for 10 days to pure MC-LR was reported to have no effect on development (Chernoff et al., 2002).

It is known in general that already feeding larval stages of amphibians are more vulnerable to contaminants compared to embryonic stages assessed by FETAX (Murphy et al., 2000). Postembryonic larval stages until completing metamorphosis are characterized as the most sensitive period to determine effects on development, sexual differentiation (Miyata et al., 1999), and physiological responses of amphibians concerning stress (Kloas et al., 1997) and biotransformation (Mouchet et al., 2006). Thus the aim of the recent study was to investigate whether natural exposure to cyanobacterial biomass containing considerable amounts of the cyanotoxin MC-LR via diet affects postembryonic larval stages of *X. laevis*. In order to investigate physiological impacts bioaccumulation of MC-LR, development including potential endocrine disruption of thyroid system triggering metamorphosis as well as of sexual differentiation, stress, and transcription of genes involved in biotransformation have been investigated.

2. Materials and methods

2.1. Animals and husbandry

Two experiments, one short-term (7 days) and one long-term exposure (21 days) were carried out using *X. laevis* tadpoles derived from the animal stock of IGB, Berlin, Germany. Spawning of adult frogs was induced by injection of human chorionic gonadotropin (Sigma, Deisenhofen, Germany) into the dorsal lymph sac (Kloas et al., 1999). Tadpoles were maintained in the tank with aerated deionised water containing 2.5 g/10 L of the commercial salt "Tropic Marin Meersalz" (Tagis, Dreieich, Germany) at a photoperiod 12 h light:12 h dark similar to OECD guideline no. 231 (2009). Prior to the start of both exposures (long-term as well as short-term), tadpoles were placed into 10 L glass aquaria according to four different diets (see below) in replicate per exposure to acclimatize for 5 days before reaching the developmental stage 52 (Nieuwkoop and Faber, 1994). Thus there were altogether 8 aquaria per short-term exposure and 8 aquaria per long-term exposure. Naturally occurring cyanobacterial biomass consisting mainly of *M. aeruginosa* was collected from the fish storage tank in fish farming Pohořelice/South Moravia (Czech Republic) using 20 μm plankton-net and later on lyophilized. Four different diets were formulated: (1) control (100% Sera Micron, Heinsberg, Germany, commercial diet), (2) M10 (90% Sera Micron + 10% lyophilized biomass of *M. aeruginosa*), (3) M50 (50% Sera Micron + 50% lyophilized biomass of *M. aeruginosa*) and (4) *Spirulina* (50% Sera Micron + 50% lyophilized biomass of *Spirulina* sp.). Experimental diets were dissolved in deionised water (1 g food/10 mL) and experimental animals were fed three times per day at a feeding ratio ranging from 24 to 30 mg/individual/day according to respective developmental stages (Opitz et al., 2005; OECD test guideline no. 231, 2009). Water medium was changed completely three times a week (Monday, Wednesday, and Friday).

2.2. Sampling

2.2.1. Short-term exposure

Sampling took place after 1, 3 and 7 days of the exposure when 15 tadpoles per aquarium (in total 30 individuals per treatment) were taken for MC-LR bioaccumulation ($n = 5$/aquarium; $n = 10$/treatment), stress ($n = 5$/aquarium; $n = 10$/treatment) and transcription of biotransformation genes ($n = 5$/aquarium; $n = 10$/treatment), meaning the animal density decreased from 45 individuals/aquarium at the beginning of the experiment to 30 after first sampling point and 15 individuals remained after second sampling point till the end of exposure at day 7.

2.2.2. Long-term exposure

20 tadpoles per aquarium were kept for 21 days without changes in animal density until the sampling point. After 21 days of exposure 20 individuals per aquarium were taken for MC-LR bioaccumulation ($n = 5$/aquarium; $n = 10$/treatment), stress ($n = 5$/aquarium; $n = 10$/treatment), transcription of biotransformation gene ($n = 5$/aquarium; $n = 10$/treatment), and sexual differentiation ($n = 5$/aquarium; $n = 10$/treatment) (in total 40 individuals/treatment) measurements.

Each tadpole was gently caught by a strainer and immediately put onto melting ice for anaesthetizing before pithing spinal cord. Afterwards the weight and total length was determined. The tadpoles used for determination of MC-LR bioaccumulation were washed in 70% methanol to avoid any contamination with MC-LR at the body surface.

2.3. MC-LR determination

MC-LR extraction of whole body was carried according to Contardo-Jara et al. (2008). Briefly, whole body samples of tadpoles were washed in 70% methanol, weighed and later homogenized in 1 mL 70% methanol using TissueLyser (Qiagen) for twice 2 min, 18 beats/s. Homogenates were centrifuged at 10,000 rpm for 5 min and supernatants were collected and completely evaporated. Extracts were re-dissolved in 2×500 μL 70% methanol and transferred to HPLC vials for MC-LR detection by liquid chromatography–with tandem mass spectrometry detection (LC–MS/MS). Water samples (8 mL) were evaporated at 30 °C, extracts were re-dissolved in 2×500 μL 70% methanol and measured using LC–MS/MS.

The used LC–MS/MS system consisted of an HPLC (Agilent 1200 Series) coupled with the MS/MS system, 3200 Q Trap from AB Sciex (Darmstadt). The microcystin-specific MS/MS conditions, ionization and fragmentation settings were optimized by direct injection of standard solutions to the MS/MS system. The chromatographic separation was achieved after pre-cleaning with a guard column Eclipse XDB-C8 (C8; 2.1 mm × 12.5 mm, 5 μm particle size) on a C18 Eclipse Plus (RP 18; 4.6 mm × 50 mm, 5 μm particle size) (Agilent, Germany). The column oven temperature was maintained at

40 °C. The flow was set to 200 μL/min within a binary composition of the mobile phase consisting of acetonitrile (A) and water (B) each charged with 0.1% trifluoroacetic acid. The gradient program began with a linear increase of solvent A from 15% to 100% during 12 min, was held there for 2 min, and returned to 15% solvent A during 0.1 min. This was followed by a linear decrease of solvent A from 15% to 5% within 5.9 min. The retention time of MC-LR was 13 min. In all cases the same injection volume of 10 μL per sample or standard was used. The measurements were performed in the positive multiple reaction monitoring (MRM) mode, with the mass-transitions of (m/z) 995.5 → 135.1 for MC-LR. The method validation was achieved over a linear rage from 5 to 250 μg/L. The detection limit for MC-LR is 0.5 ng. All data were analysed by using Analyst 1.4.2 software.

2.4. Developmental assessment

Mortality was recorded daily during short and long-term exposure. At the different samplings, each tadpole was weighed and developmental stage was assessed according to Nieuwkoop and Faber (1994). In addition, sex was determined gross morphologically using a binocular.

2.5. Corticosteroid determination

Extraction of the stress hormones, corticosterone and aldosterone, took place in glass vials. The whole experimental animal was homogenized in 1 mL distilled water and three times 6 mL of diethylether were added. After each addition sample was shaken on a horizontal shaker for 1 h and then placed for 30 min at −80 °C for phase separation. Steroid containing liquid organic phase was poured into a new empty glass vial and left over night to evaporate. Evaporated samples were redissolved in 1 mL of 5% ethanol and processed according to manufacturer's protocol for commercial kits of enzyme linked immunoassays to determine the stress hormones aldosterone and corticosterone, respectively (IBL International, Hamburg, Germany).

2.6. mRNA expression

mRNA expression of hormones concerning development associated with the (thyroid system thyroid stimulating hormone – TSHβ) and sexual differentiation (luteinizing hormone – LHβ and follicle stimulating hormone – FSHβ) were assessed in part of the brain containing diencephalon and pituitary, whereas parameters associated stress (heat shock protein 70 – HSP70) and biotransformation (cytochrome P450 – Cyp1A1; glutathione-S-transferases – muGST, piGST; multidrug resistance protein – MDR) and oxidative stress (catalase – CAT; superoxide dismutase – SOD) were determined in liver by quantitative RT-PCR.

2.6.1. RNA isolation and reverse transcription into cDNA

Total RNA isolation from brain and liver tissue was performed using Trizol reagent according to the manufacturer's instructions (Invitrogen, Germany). RNA concentrations and purity were determined by UV-absorbance measurements at 260 and 280 nm using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Germany). cDNA was reversely transcribed from 1 μg of DNase (AmpGrade, Invitrogen) treated total RNA as described by Urbatzka et al. (2010).

2.6.2. Quantitative PCR

All qPCR reactions were carried out using real-time PCR cycler (MX3005P, Stratagene) according to Opitz et al. (2009). Elongation factor 1α (EF-1α) was used as housekeeping gene and mRNA expression of target genes was normalized to the corresponding level of EF-1α mRNA. Reverse transcription reactions performed without reverse transcriptase served as negative controls to check the absence of genomic contamination. qPCR reactions performed with RNase free water (Qiagen) replacing the cDNA template served as additional negative controls to check for the specificity of target cDNA amplification. qPCR data were analysed using MxPro software (Stratagene). Gene-specific primers are listed in (Table 1).

Table 1
Gene specific primers used for RT-PCR assays.

Gene name	Accession number	Primer (5′–3′)
hsp70	BI939172	CCCAGCGACAGGCTAC AGTGTTCACCAGCGGTTAGA
SOD	X51518	GTGTGCTGGCGGGAAG CCGGGGCTCCGTGATT
TSHβ	L07618	AGAGTGCGCTTACTGCCTTG GGTAGGAAAAGAGCGGGTTC
FSHβ	AB175888	TGCTCGTTCTGTGTTGGAAGATG CCTGTTTGATGAGTGGATGCTTTG
LHβ	AF360397	CACTGACGCTTCTGGGGTTCTAC GATTGGGCAGTCGTCTTTCTCT
EF-1α	M25504	TGCCATTGTTGACATGATCCC TACTATTAAACTCTGATGGCC
CAT	BC054964	ACGCTGAGGGGGCAAAGAAGAAA CAGAGCGTGCAGCCAATGAGTA
Cyp1A1	NM_001097072	CTCATGTATCTTGTGGCCCATC GCTTCAGTATAGGGCAGTTGAG
muGST	BC054171	GTTGGCTCATTCTATCCGTCTCC ATCCCAGCTTCTCTTTTTCATTCA
piGST	AJ489617	GACTGGAGCCAATGATGAGGAAC ATAGGCAGTGAGCAAAGGGTAGG
MDR	U17608	CTGGGCAAAGGTATTGTCTG TGACCACCAAAGGCAAAC

Abbreviations: *hsp70*, heat shock protein; *SOD*, superoxide dismutase; *TSHβ*, thyroid-stimulating hormone; *FSHβ*, follicle-stimulating hormone; *LHβ*, luteinizing hormone; *EF-1α*, elongation factor 1α; *CAT*, catalase; *Cyp1A1*, cytochrome P450; *muGST*, mu glutathione-S-transferase; *piGST*, pi glutathione-S-transferase; MDR, multidrug resistance protein.

2.7. Statistical analyses

Data were analysed for normal distribution by Kolmogorov–Smirnov and significances were pre-checked by Kruskal–Wallis or one way analysis of variance ANOVA (passed if $P < 0.05$) using SigmaStat 2.0 software (Jandel Scientific, San Rafael, USA). Accordingly pairwise comparisons among all treatment groups at each sampling time were carried out by nonparametric Dunn's test or by parametric posthoc Tukey's test. Results are presented as the mean ± SD or median with 5, 25, 75 and 95 percentile values (developmental stages).

3. Results

Chemical parameters of water environment were maintained during whole exposure time within both experiments as follows, O_2: 73.7 ± 4.5%; 6.42 ± 0.39 mg/L; temperature: 22.1 ± 0.1 °C; pH: 6.8 ± 0.2; conductivity: 505 ± 13 μS/cm.

3.1. MC-LR bioaccumulation/detection

MC-LR determination by LC–MS/MS revealed that only the diets M10 and M50 contained considerable amounts of MC-LR with

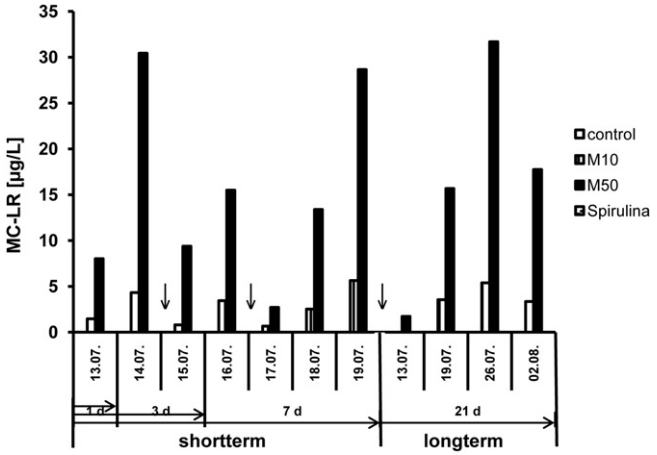

Fig. 1. Microcystin-LR (MC-LR) concentrations in aquaria rearing *Xenopus laevis* tadpoles fed with four diet variants (control, M10, M50 and *Spirulina*) during short- and long-term exposure. Values for long-term exposure at days 19.07, 26.07 and 02.08 correspond to 3 days exposure without water change. Arrows indicate water changes. MC-LR concentration in control and *Spirulina* variants was not detectable.

42.8 μg/g and 187.0 μg/g diet, respectively. MC-LR was below the detection limit (n.d.) of 0.5 ng/sample, both in control and in *Spirulina* diet.

Surprisingly, MC-LR in whole body of any tadpole fed with MC-LR containing diets was n.d. Concentrations of MC-LR in water varied according to feeding and water changes. The concentrations were lowest shortly after water exchange and increased with time by feeding before next change. Measured concentrations of MC-LR ranged in short-term experiment from 0.7 to 5.6 μg/L and from 2.7 to 30.4 μg/L for M10 and M50, respectively. In long-term experiment MC-LR concentrations were from n.d. to 5.4 μg/L and from 1.7 to 31.7 μg/L for M10 and M50, respectively (Fig. 1).

3.2. Development

3.2.1. Mortality, weight and developmental stages

During the whole study, either in short-term or in long-term exposure no mortality was recorded for any of the 4 diets.

No significant changes were observed concerning development between control and treatments within short-term as well as long-term exposures. In all treatment groups, developmental stages increased in time dependent manner in all treatment groups (Fig. 2A), starting with the developmental stage 52 in the beginning, reaching stage 54 after short-term and stage 58 after long-term exposure.

Total body weight of the tadpoles after 7 days of exposure reached 0.7 ± 0.2 g for control, M10 and *Spirulina* groups and 0.6 ± 0.2 g in the M50 experimental group. At the end of long-term exposure after 21 days the body weight of the tadpoles was significantly lower in the M50 group (1.2 ± 0.3 g) compared to control (1.4 ± 0.2 g) ($P < 0.01$). Animals fed with *Spirulina* diet had slightly higher weight (1.5 ± 0.2 g) than controls being very close to M10 (1.4 ± 0.3 g) but the difference was not statistically significant (Fig. 2B).

3.2.2. TSHβ mRNA expression

Thyroid stimulating hormone (TSHβ) mRNA as an indicator for potential endocrine disruption of the thyroid system did not significantly differ between control and any treatment after 21 days

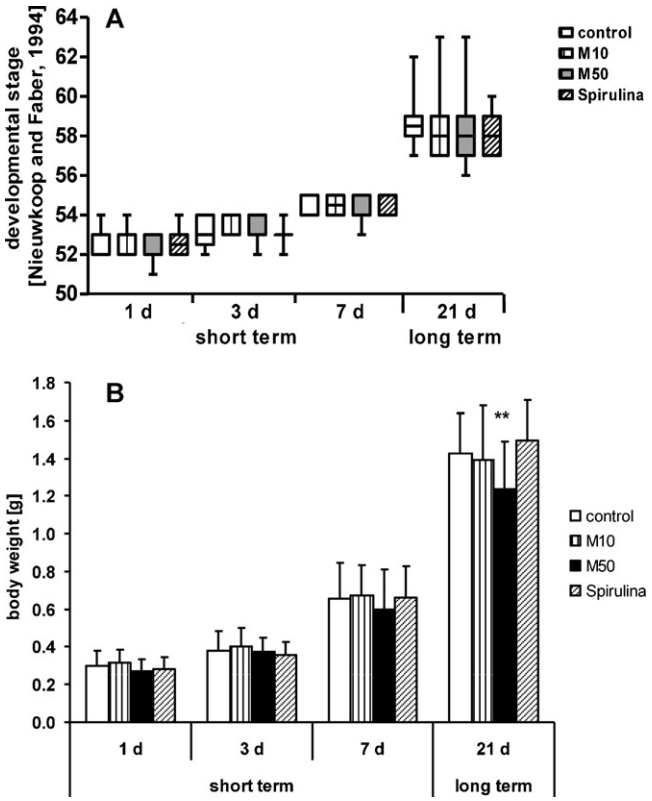

Fig. 2. Developmental stages according to Nieuwkoop and Faber (1994) (A) and growth rates (B) of tadpoles fed with control, M10, M50 and *Spirulina* during short and long-term exposure. Results are shown as median and 5, 25, 75 and 95 percentile values (A) and mean values + SD (B) ($n = 10$ for short-term and $n = 40$ for long-term exposure). Significant differences compared to control are marked by asterisks (**$P < 0.01$).

(long-term exposure) which confirmed the similarities concerning developmental stages.

3.2.3. Sex ratio and mRNA expression of FSHβ, LHβ

After 21 days of exposure to experimental diets, neither gross morphological determination of gonads using binocular, nor mRNA expression of FSHβ showed any difference. The only significant difference ($P < 0.05$) was recorded concerning higher LHβ mRNA in the M10 compared to control group (Fig. 3). Sex ratios (female/male) were as follows: 12/8 (control), 9/11 (M10), 8/12 (M50) and 13/7 (*Spirulina*).

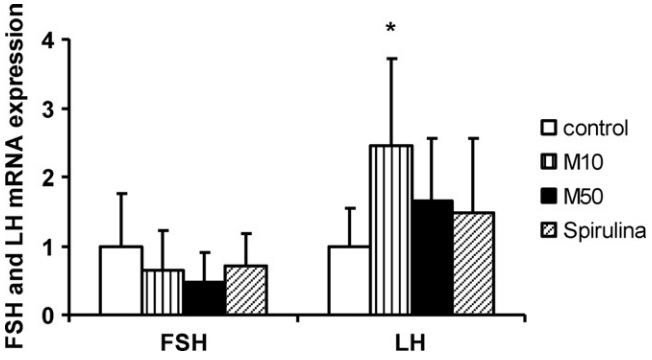

Fig. 3. mRNA expression of follicle stimulating hormone (FSH) and luteinizing hormone (LH) after 21 days of feeding with four diet variants (control, M10, M50 and *Spirulina*). Data represent mean values + SD ($n = 10$) and significant differences compared to control are marked by asterisks (*$P < 0.05$).

3.3. Stress parameters

3.3.1. Corticosteroids (aldosterone, corticosterone)

After one day of exposure to experimental diets, the *Spirulina* group had a lower concentration of aldosterone compared to control whereas M10 and M50 groups reached slightly but not significantly higher concentrations than the control. In general, increases of aldosterone levels were observed in all groups after 3 days; surprisingly highest values were obtained in control (control vs. M50), $P < 0.05$ and lowest in *Spirulina* group (control vs. *Spirulina*, $P < 0.05$). At the end of long-term exposure M50 reached a significantly higher aldosterone value compared to control and the other of experimental groups ($P < 0.01$) (Fig. 4A).

Similarly to aldosterone, the concentration of corticosterone was lowest in *Spirulina* group after one day and even significantly different ($P < 0.001$) between control and *Spirulina* group (Fig. 4B). Increase in values was observed during second and third sampling point of short-term exposure where M50 was significantly lower compared to control ($P < 0.05$) after 3 days and at the end of long-term experiment the corticosterone level in M50 was only slightly but not significantly elevated (Fig. 4B).

3.3.2. HSP70 mRNA expression

One day after the beginning of the experiment only the mRNA expression of HSP70 was significantly higher in M10 compared to control group ($P < 0.05$) (Fig. 4C) but no further significance was discovered neither within short-term, nor in long-term exposure.

3.4. Biotransformation

mRNA expression of enzymes associated with biotransformation including biotransformation phase I (Cyp1A1), phase II (muGST and piGST) and phase III (MDR) and oxidative stress parameters (CAT and SOD) have been determined and revealed no significant difference among the experimental groups within both, short-term and long-term exposures except for *Spirulina* after the first day, where SOD mRNA level was significantly elevated ($P < 0.05$) in comparison with control (Fig. 5A and Table 2). There were only slight but significant elevations concerning mRNA of the gene encoding multidrug resistance protein (MDR) in M10 after 3 days and in M10 and M50 after 21 days compared to control ($P < 0.05$) (Fig. 5B).

4. Discussion

Cyanobacterial water blooms have been in many cases considered to be harmful to aquatic as well as terrestrial animals (Magalhaes et al., 2003; Frazier et al., 1998). Several studies dealing with impacts of cyanobacterial toxins on experimental animals have been reported mainly in laboratory rodents and fishes, mostly using unnatural exposure via intraperitoneal application of cyanotoxins (Guzman and Solter, 1999; Atencio et al., 2008). However, there are some cases of adverse affects of MC on human beings exposed either intravenously to MC during kidney dialysis (Hilborn et al., 2007) or via consumption of fish and shellfish that were harvested from three large Chinese lakes (Peng et al., 2010). In fish, natural exposure to MC-LR via diets seems to be less harmful in comparison with intraperitoneal application (Tencalla et al., 1994).

4.1. MC-LR bioaccumulation

In order to verify a potential impact of MC-LR contained in cyanobacterial biomass used for M10 and M50 diets which are comparable with MC concentrations of cyanobacterial water blooms assessed during monitoring of natural waters (Bláha et al., 2010), the potential bioaccumulation has to be determined. However, neither during short-term nor long-term exposures any MC-LR has been measurable in feeding stages of *X. laevis* tadpoles that might be due to a fast metabolization or excretion of MC-LR, which does not lead to higher concentrations of MC-LR within the bodies of tadpoles above the detection limit of the LC–MS/MS (0.5 ng MC-LR). According to Fischer et al. (2005), who studied the uptake of radiolabeled MC-LR via known human and rodent organic anion transporting polypeptide (OATP) expressed in *X. laevis* oocytes, demonstrated that MC-LR transport was inhibited by co-incubation with taurocholate and bromosulfophthalein. A potential explanation for detection of no MC-LR might be that no OATP is present

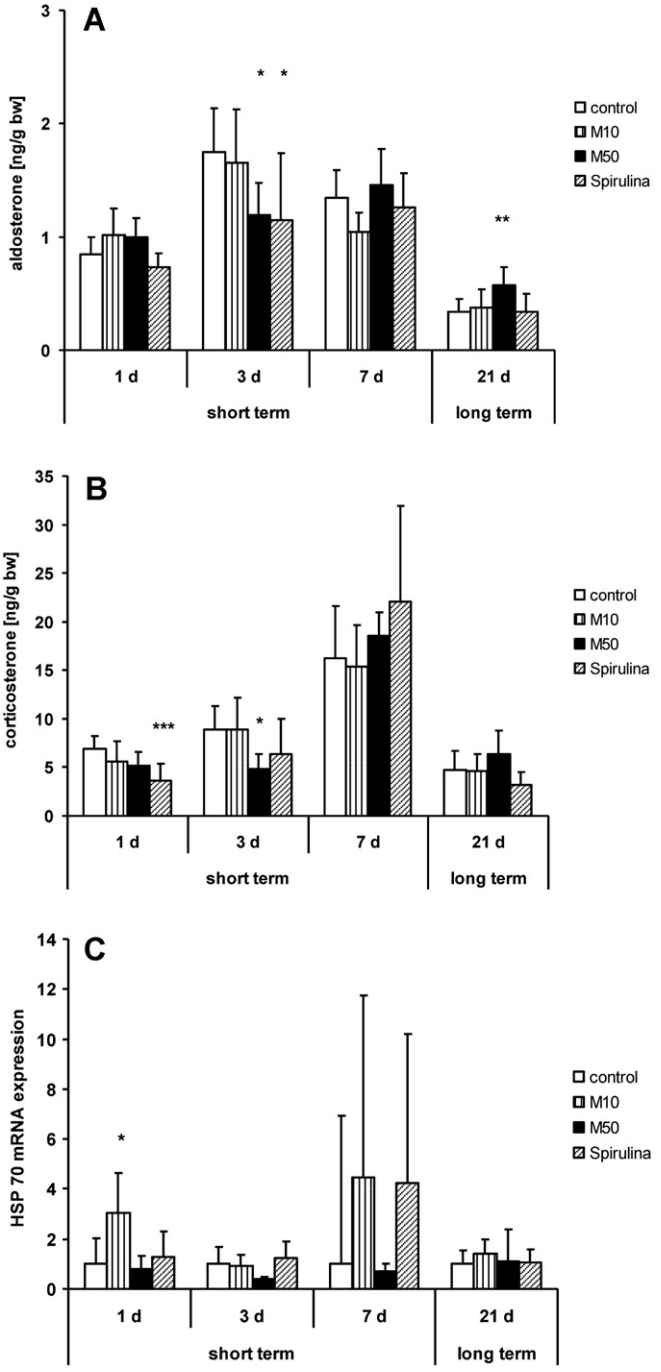

Fig. 4. Impact of four different diet variants (control, M10, M50 and *Spirulina*) on the stress parameters aldosterone (A), corticosterone (B), and heat-shock protein mRNA expression (C). Data represent mean values + SD ($n = 10$) after one, 3, 7, and 21 days of feeding and significant differences compared to control are marked by asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Table 2
mRNA expression of biotransformation biomarkers. heat shock protein 70 (HSP 70), superoxide dismutase (SOD), catalase (CAT), cytochrome P450 (CYP 1A1), mu and pi glutathione-S-transferase (muGST, piGST), multidrug resistance protein (MDR). Data are expressed by mean values ± SD ($n = 10$) and significant differences compared to control are in bold and marked by asterisk (*$P < 0.05$).

		1 d	3 d	7 d	21 d
HSP 70	Control	1.0 ± 0.6	1.0 ± 1.0	1.0 ± 0.4	1.0 ± 0.6
	M10	**3.0 ± 1.6***	0.9 ± 0.4	4.4 ± 7.3	1.4 ± 0.6
	M50	0.8 ± 0.6	0.4 ± 0.1	0.7 ± 0.3	1.1 ± 1.3
	Spirulina	1.3 ± 1.0	1.2 ± 0.7	4.3 ± 6.0	1.0 ± 0.6
SOD	Control	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.2
	M10	1.3 ± 0.1	1.0 ± 0.2	1.4 ± 0.3	1.1 ± 0.3
	M50	0.9 ± 0.3	0.8 ± 0.1	1.2 ± 0.1	1.0 ± 0.2
	Spirulina	**1.4 ± 0.2***	1.1 ± 0.3	1.4 ± 0.4	1.2 ± 0.1
CAT	Control	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.4
	M10	1.1 ± 0.1	0.7 ± 0.2	0.9 ± 0.1	0.9 ± 0.3
	M50	0.8 ± 0.2	1.0 ± 0.2	1.2 ± 0.1	1.2 ± 0.7
	Spirulina	1.1 ± 0.3	0.7 ± 0.2	0.9 ± 0.2	1.4 ± 0.6
CYP 1A1	Control	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.1
	M10	0.9 ± 0.2	0.7 ± 0.1	1.3 ± 0.5	0.8 ± 0.2
	M50	0.8 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.3
	Spirulina	1.1 ± 0.2	0.8 ± 0.3	0.7 ± 0.1	0.7 ± 0.1
muGST	Control	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2
	M10	1.0 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	0.9 ± 0.2
	M50	0.9 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	0.9 ± 0.1
	Spirulina	1.0 ± 0.1	1.2 ± 0.4	0.9 ± 0.2	1.1 ± 0.1
piGST	Control	1.0 ± 0.3	1.0 ± 0.6	1.0 ± 0.4	1.0 ± 0.5
	M10	1.0 ± 0.5	1.6 ± 1.3	2.3 ± 2.0	0.6 ± 0.5
	M50	0.8 ± 0.5	1.9 ± 1.1	1.7 ± 1.1	1.1 ± 0.5
	Spirulina	0.9 ± 0.4	0.9 ± 0.8	4.3 ± 3.2	1.1 ± 0.8
MDR	Control	1.0 ± 0.5	1.0 ± 0.2	1.0 ± 0.4	1.0 ± 0.3
	M10	1.0 ± 0.1	**1.7 ± 0.6***	1.3 ± 0.6	**2.1 ± 1.1***
	M50	0.8 ± 0.3	1.3 ± 0.4	1.3 ± 0.3	1.0 ± 0.4
	Spirulina	1.0 ± 0.5	0.9 ± 0.4	1.7 ± 0.8	1.9 ± 0.6

in *X. laevis* suggesting no active MC-LR uptake. Another hypothesis might be that OATPs in *X. laevis* tadpoles are distinct to mammalian ones possessing other physiological functions and thus the animals are unable to take up MC-LR which has to be proven by further experiments.

Most authors investigating MCs effects in *X. laevis* embryos focused mainly on the physiological response without measuring MCs concentrations in experimental embryos after application. Only the study of Fischer and Dietrich (2000) investigated the uptake of ^{3}H-MC-LR in *X. laevis* after 96 and 120 h suggesting that MC cannot be taken up transchorionally or transdermally and needs to be taken up orally after hatching to exert any effect in developing amphibians, which raises the question whether embryo-larval liver can become fully functional and susceptible for specific MC uptake and toxicity. However, the authors did not clarify whether ^{3}H measured was really due to intact ^{3}H-MC-LR, its metabolites or further degradation products including radiolysis to ^{3}H.

4.2. Development

X. laevis tadpoles are known to filter their feed composed mainly of primary producers, but the exact composition and availability of diet is dependent on many variables. According to results of Pryor (2003), larval bullfrogs (*Rana catesbeiana*) are able to feed on bloom-forming algae such as *Anabaena*. The relation between different diet sources (chlorophytes, diatoms, cyanobacteria, etc). varying in amounts of protein, carbohydrates, and lipid and anuran metamorphosis using *R. catesbeiana*, *Hyla regilla* and *Rana boylii* was reviewed by Kupferberg (1997) suggesting that the diet quality might be linked to size and time to metamorphosis.

All our experimental animals regardless the experimental diet applied reached the developmental stage 58. Only the individuals fed with the M50 diet containing 50% cyanobacterial biomass with MC-LR had lower weights at the end of long-term experiment suggesting that this was rather due to present MC-LR or other specific compounds of biomass dominated by *M. aeruginosa* such as lipopolysaccharides than to changed nutrient value because the *Spirulina* treatment contained the same amount (50%) of commercial diet Sera Micron and 50% of lyophilized non-toxic cyanobacterial biomass of *Spirulina* sp. However, it seems that tadpoles put energy more likely into development than somatic growth, which was confirmed by revealing similar results for the TSH mRNA expression in all treatments. This represents the most sensitive biomarker for indication of disruption of the thyroid system triggering development. During the whole experiment no mortality was recorded at any time for any of the 4 diets indicating that the concentrations of MC-LR in cyanobacterial biomass applied via oral exposure exhibits neither acute nor chronic toxicity.

Thus our study dealt for the first time with natural cyanobacterial biomass containing cyanotoxin MC-LR and its natural application via diets in tadpoles, whereas the other studies with *X. laevis* dealt with FETAX test and/or use of pure toxin.

Burýšková et al. (2006) used five sources of cyanobacterial biomass occurring naturally during vegetative season with different dominant genera (*Microcystis*, *Aphanizomenon*, *Anabaena* and *Planktothrix*) from five localities in the Czech Republic to determine the impacts of naturally derived cyanobacterial biomass containing MCs on *X. laevis* embryos. The results show that homogenates of complex biomass and aqueous extracts were generally the most toxic fractions in terms of mortality and growth inhibition, whereas eluates containing MCs were generally less toxic.

Pure MC-LR (1) and three sources of natural cyanobacterial biomass (2) *M. aeruginosa* containing MC-LR, (3) *Microcystis wesenbergii* without MC-LR, and (4) *M. wesenbergii* plus addition of purified MC-LR were applied to *X. laevis* in FETAX testing (Dvořáková et al., 2002). Both biomasses of cyanobacterial blooms

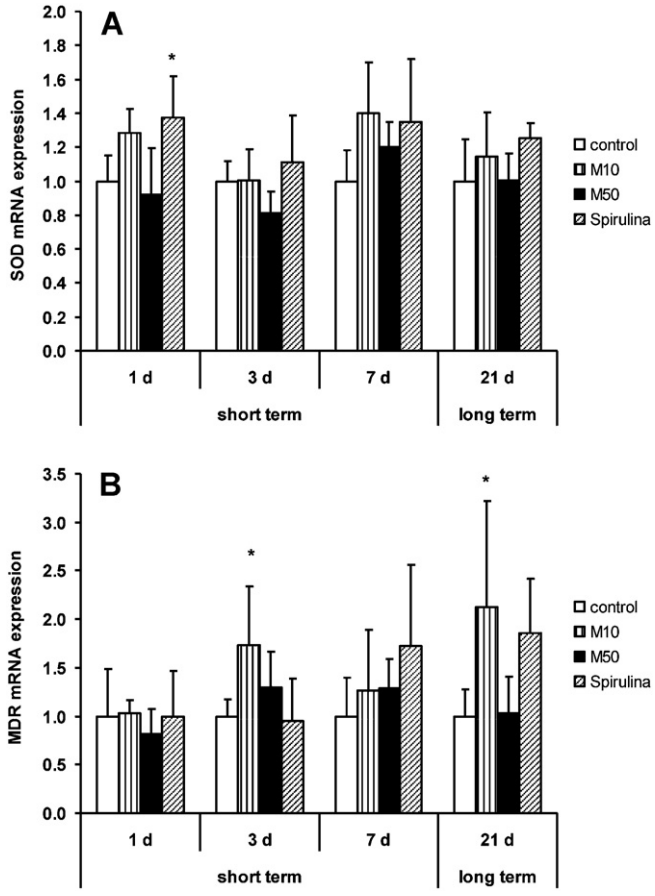

Fig. 5. mRNA expression of the biotransformation biomarkers superoxide dismutase (SOD) (A) and multidrug resistance protein (MDR) (B) after feeding tadpoles with four diets (control, M10, M50 and *Spirulina*) during short-term and long-term exposure. Results are normalized to house keeping gene and to control. Data represent mean values + SD ($n = 10$) and significant differences compared to control are marked by asterisks (*$P < 0.05$).

were embryotoxic; exposure to MC-LR containing biomass led to more than 60% malformation of the embryos, but exposure to biomass without MC-LR showed effects in a similar range indicating that other cyanobacterial compounds rather than MC-LR induce the observed adverse effects because external addition of purified MC-LR to the bloom enhanced the overall toxicity of the material only slightly.

Concerning development, no increased mortality, malformations or growth inhibition could be detected in embryos of *X. laevis* exposed to pure MC-LR and MC-RR up to 120 h (Fischer and Dietrich, 2000).

Two further studies exposed tadpoles of *B. arenarum* (Chernoff et al., 2002) and marsh frogs (*R. ridibunda*) (Oberemm et al., 1999) to pure MC-LR and MC-YR, respectively, but no effects on development concerning any abnormalities were recorded.

Until now, no study concerning estrogenic effects of MC-LR on any life stage of *X. laevis* has been published. Oziol and Bouaïcha (2010) exposed mammalian cells to cyanotoxins MC-LR and nodularin-R and declare the first evidence of estrogenic potential of these cyanotoxins. The only aquatic animal exposed to lyophilized *Microcystis* and MC-LR to investigate their endocrine disrupting effects was *Danio rerio* (Rogers et al., 2011) suggesting that *Microcystis* may be a natural source of environmental estrogens upregulating vitellogenin gene expression. Concern about effects of *Microcystis* blooms may be raised beyond those associated with toxic MC. We exposed for the first time tadpoles of *X. laevis* to cyanobacterial biomass containing cyanotoxin MC-LR without any impact on sexual differentiation as suggested by mRNA expression of LHβ and FSHβ together with sex ratio determination using a binocular. mRNA levels of LHβ and FSHβ as well as sexual development in our study displayed no differences except for LHβ mRNA between M10 and control. The observed significance seems to be rather occasional because no dose response could be observed in M50 and thus biomass containing MC-LR used in diet might not have relevant impact on sexual differentiation, which was confirmed by gross morphological sex determination demonstrating no difference concerning differences in sex ratios among the groups. However, to determine a slight potential impact on sexual differentiation, larger experimental groups are needed, which will be addressed in future experiments.

4.3. Stress

Adrenal corticosteroids as well as catecholamines have regulatory effects during premetamorphosis and metamorphic climax (Kloas et al., 1997). Our experiment deals for the first time with stress determination in *X. laevis* tadpoles exposed to cyanobacterial toxin MC-LR. Stress was assessed by plasma levels of the corticosteroids, corticosterone and aldosterone, and by heat shock protein 70 (HSP70) mRNA expression. The results suggest only negligible to moderate stress effects of MC-LR containing cyanobacterial biomass in M50 at the end of exposure. Plasma levels of aldosterone and corticosterone were comparable to findings by Kloas et al. (1997).

The aim of most studies previously published was to evaluate stress towards food deprivation using both pre- and prometamorphic tadpoles of spadefood toad (*Spea hammondii*) (Crespi and Denver, 2005) or juvenile *X. laevis* (Crespi et al., 2004). Both pre- and prometamorphic tadpoles of spadefood toad (*Spea hammondii*) increased whole-body corticosterone content with food deprivation, but the magnitude of the response of premetamorphic tadpoles was significantly greater. Juvenile toads did not respond to food deprivation by activating hypothalamic–pituitary–interrenal (HPI) axis, but instead pursued a strategy of energy conservation that involves a reduction in plasma corticosterone concentration (Crespi and Denver, 2005). Glucocorticoids also stimulate food intake by inhibiting corticotropin-releasing factor (CRF) while facilitating orexigenic peptides. Stress axis is activated in response to food intake but not activated during periods of food deprivation in juvenile *X. laevis* (Crespi et al., 2004).

During exposure to M50 the HSP70 mRNA level differed only after one day of exposure between control and M10. No clear pattern in continuous increase or decrease of these stress markers was detected among the experimental treatments, where tadpoles were fed by natural cyanobacterial biomass with or without cyanotoxin MC-LR.

4.4. Biotransformation

MC-LR conjugation to glutathione is mediated by soluble glutathione S-transferase in various aquatic organisms ranging from plants (*Ceratophyllum demersum*), invertebrates (*Dreissena polymorpha* and *Daphnia magna*) up to fish eggs and fish (*D. rerio*), stating that the pathway for xenobiotic metabolism expressed in all major groups of organisms is involved in the biotransformation of MC (Pflugmacher et al., 1998; Pflugmacher, 2004). Nevertheless the underlying pathways and enzymes of MC biotransformation/excretion have not been identified yet (Campos and Vasconcelos, 2010). mRNA expression of enzymes associated with biotransformation including biotransformation phase I (monooxygenase, Cyp1A1), phase II (glutathione-S-transferases, muGST and piGST) and phase III (multidrug resistance protein, MDR) and oxidative stress (catalase (CAT) and superoxidedismutase (SOD)) have

been determined using *X. laevis* tadpoles exposed to cyanobacterial biomass with and without MC-LR and resulted in no significant differences among all the experimental groups. There was only a significant elevation concerning MDR mRNA in M10 compared to control after three and 21 days. SOD mRNA level was significantly elevated in the *Spirulina* group in comparison with controls after one day. However, all these significances did not show any consistency and might therefore be due to occasionally occurring changes. Due to the small size of individual liver samples biotransformation and other enzyme activities could not be measured; all the data related to individual mRNA levels.

Biotransformation of the compounds of natural origin such as cyanotoxins in *X. laevis* tadpoles has not been elucidated yet and according to our knowledge only Burýšková et al. (2006) used different fractions, prepared from natural cyanobacterial biomass dominated by *M. aeruginosa*, and evaluated them in FETAX to assess the effects on biochemical markers of oxidative stress and biotransformation. Biomarkers were affected in variable manner but the authors did not observe any significant effect of MCs. Gillardin et al. (2009) showed that the early growth but not the development and survival of *X. laevis* are impaired when young tadpoles (stage 35/36–45) are facing contamination by a mixture of polychlorinated biphenyls (PCBs). The energy cost of the PCBs metabolization resulted in growth delay being in accordance to our results. Their results also highlighted that young tadpoles are able to express antioxidant systems, which could give them an effective protection during their development. Fort et al. (2000) evaluated toxicity of thalidomide using FETAX and demonstrating that CYP2E1 is involved in biotransformation of thalidomide. Thus tadpoles should be responsive to toxic/xenobiotic compounds concerning biotransformation but our results suggest only minor to negligible impacts of natural cyanobacterial biomass on biotransformation parameters.

5. Conclusions

In the present study a combination of various biomarkers has been applied to determine for the first time direct impacts of cyanobacterial biomass applied via diets on postembryonic stages of amphibians. Beside bioaccumulation of MC, corticosteroid determinations, classical growth and development parameters, we determined further specific biomarkers concerning sex differentiation, thyroid system and biotransformation only at the transcriptional level assessing changes in mRNA levels. It has to be mentioned that transcriptional changes or a lack of changes does not immediately mean that the similar patterns are also seen at the protein and/or enzyme activity levels. However, the overall picture provided at the organismic level concerning corticosteroids, sexual differentiation and development indicating only minor to negligible impacts by cyanobacterial biomass on tadpoles is in accordance to the findings that the mRNA levels did not change remarkably and thus we did not go further to confirm mRNA levels by proteomics and enzyme measurements. Our results, derived from short-term as well as long-term exposures showed only a slight impact of diets containing lyophilized cyanobacterial biomass with MC-LR especially in the case of M50 group resulting into lower weight, however, the overall development was not affected. It seems that tadpoles fed with natural cyanobacterial biomass dominated by *M. aeruginosa* and containing MC-LR that was lyophilized put energy rather into development than into somatic growth suggesting that the animals needed some energy for moderate to minor biotransformation or the energy content of lyophilized *M. aeruginosa* in the diet is slightly lower. In addition, no impact on sexual differentiation by *M. aeruginosa* compounds could be observed. One possible explanation might be that *X. laevis* might possess special mechanisms to cope physiologically quite well with toxic cyanobacteria occurring in the same natural habitat, enabling survival even at environmental MC concentrations being toxic for mammals.

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