

## Effect of different cyanobacterial biomasses and their fractions with variable microcystin content on embryonal development of carp (*Cyprinus carpio* L.)

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### Abstract

While numerous studies focused on the effects of microcystins, the role of other components of complex cyanobacterial water blooms in toxicity is poorly understood. In this study we have evaluated effects of various fractions of cyanobacterial biomass with different composition and microcystin content on embryolar development of carp (*Cyprinus carpio*). The following samples (fractions) of four natural water blooms were prepared and tested: complex cyanobacterial biomass, crude aqueous extract of biomass, cellular pellet remaining from aqueous extract, permeate (i.e. microcystin-free fraction prepared during C-18 solid-phase extraction; SPE), and eluate (i.e. fraction prepared by SPE containing mostly microcystins). Complex biomass and the crude aqueous extract (regardless of microcystin content and/or microcystin variants present) in the sample were the most toxic. On the other hand, eluate fractions of all samples containing microcystins in concentrations 8–255  $\mu\text{g L}^{-1}$  induced no or only weak toxic effects. Exposures of fish to permeate fractions (with removed microcystins) of two samples dominated by *Aphanizomenon* sp. and *Planktothrix* sp. resulted in significant mortality, while other two samples dominated by *Microcystis* spp. induced minor effects. We have also observed significant inhibition of glutathione S-transferases (GST) at most fractions of the *Aphanizomenon* sp. and *Planktothrix* sp. dominated samples. Our data indicate that cyanobacterial water blooms as well complex biomass extracts induce significant embryolar toxicity in common carp. However, these effects were independent of microcystin content, and the most pronounced effects were observed with the non-*Microcystis* dominated samples. Therefore, a critical examination of microcystin role in overall ecotoxicology of complex cyanobacterial blooms is needed. © 2007 Elsevier B.V. All rights reserved.

**Keywords:** Cyanobacterial biomass; Embryonal development; Common carp; Microcystins; Embryotoxicity; Glutathione S-transferase

### 1. Introduction

Cyanotoxins were intensively studied during last decades and numerous mechanisms of toxicity were identified and include for example hepatotoxicity, neurotoxicity or contact acute toxicity (Chorus et al., 2000). Numerous individual cyanotoxins were identified such as lipopolysaccharides, neurotoxic alkaloids or the cyclic peptides. Among the latter group, microcystins (MCs), received considerable attention of scientists worldwide,

and more than 70 variants of MCs were identified so far (Dietrich and Hoeger, 2005). However, under natural conditions, it is usually complicated to attribute the mortality of aquatic organisms such as fish to the effects of isolated individual cyanotoxins. For example, it has been hypothesized that one of the major reasons of deaths of aquatic organisms during degradation of cyanobacterial blooms were low concentrations of dissolved oxygen (resulting from the enhanced bacterial metabolism on the decaying biomass) rather than effects of cyanotoxins (Pechar, 2000; Snyder et al., 2002).

Laboratory experiments with aquatic organisms exposed to toxic cyanobacteria (or dissolved toxins) help to evaluate the potential toxic effect of complex water blooms (Sivonen and

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Jones, 1999). Early-life stage development plays a crucial role in the ontogenesis, and embryos/larvae can therefore be successfully used as models for studies of cyanobacterial toxicity in fish. Previous reports have shown minor toxic effects of purified microcystins and aqueous cyanobacterial extracts on embryolarval development of zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*) or chub (*Leuciscus cephalus*) (Oberemm et al., 1997, 1999). On the other hand, significant toxicities were shown for mud loach *Misgurnus mizolepis* (Liu et al., 2002) and common carp *Cyprinus carpio* (Palíková et al., 2003). It has also been shown that fish eggs are well protected against the toxic effects of pure microcystins but microinjection applications of microcystin LR caused mortality in medaka (*Oryzias latipes*) (Jacquet et al., 2004) and zebra fish (Wang et al., 2005).

Besides traditional toxicological endpoints, biochemical markers are often used to investigate sublethal toxic effects, and they may predict severe chronic toxicity. Cyanobacterial metabolites have been shown to induce oxidative stress in various organisms, and activity of detoxification enzyme glutathione *S*-transferase activity (GST) has successfully been used to monitor adverse effects of cyanobacterial blooms (Wiegand et al., 1999; Pietsch et al., 2001; Best et al., 2002; Li et al., 2003; van der Oost et al., 2003; Bláha et al., 2004).

In the present study we have investigated embryolarval toxicity of cyanobacteria to common carp *C. carpio* assessing both traditional toxicity endpoints such as mortality, timing of the hatching, and the development of eye pigments, and the activities of GST. The major aims of the study were: (i) to evaluate the variability in the fish responses to different cyanobacterial blooms (four natural samples dominated by *Microcystis* spp., *Aphanizomenon* sp., and *Planktothrix* sp.); (ii) to study the role of microcystins in ecotoxicity of complex cyanobacterial samples by testing various fractions of biomass prepared by microcystin-targeted solid-phase extractions (SPE).

## 2. Materials and methods

### 2.1. Cyanobacterial samples

Four different natural cyanobacterial water blooms have been collected with plankton net and stored frozen at  $-18^{\circ}\text{C}$ . Two samples were dominated by *Microcystis* spp., and other two samples by *Aphanizomenon flos-aquae* or *Planktothrix aghardii*.

Origin of the water blooms and the details on their composition are listed in Table 1.

### 2.2. Preparation of the fractions

After thawing, biomass concentration of each sample has been adjusted with distilled water to 6 g of dry weight per litre of volume. Complex fresh cyanobacterial biomass was homogenized by sonication on a magnetic stirrer using the ultrasonic probe Bandelin Sonoplus HD2070 (15 min, cycle 0.9, 100% power). The homogenate was used as FRACTION A (complex homogenized biomass) for toxicity assessment or centrifuged (15 min,  $2800 \times g$ ). The resulting pellet and supernatant were separated, diluted with distilled water to starting volume before centrifugation and stored frozen as FRACTION B (pellet, cell debris containing fragments of cell walls including lipopolysaccharides, LPS) or FRACTION C (supernatant, crude aqueous extract), respectively. A portion of the supernatant was further extracted on solid phase using ODS SepPak 35 cm<sup>3</sup> 10 g (waters) cartridges preconditioned with methanol and equilibrated with distilled water. Permeates from the cartridge were collected (FRACTION D; permeate, polar fraction devoid of microcystins). Methanol eluate from the cartridge containing microcystins, other peptides and more hydrophobic compounds was evaporated under vacuum and residue was dissolved in distilled water to reach the volume of the sample before solid phase extraction (FRACTION E). The experimental setup is presented in Fig. 1.

### 2.3. HPLC analyses of microcystins

Aliquots of each biomass and fraction were used for HPLC-DAD analyses of microcystins. We have used Agilent 1100 Series HPLC system with Supelcosil ABZ+ Plus 150 mm  $\times$  4.6 mm 5  $\mu\text{m}$  column (Supelco) and mobile phase consisting of (A) 0.1% (v/v) TFA, (B) acetonitrile with 0.1% (v/v) TFA (gradient elution: 20–59% B in 0–30 min), flow rate 1 mL/min, temperature  $30^{\circ}\text{C}$ , injection volume 25  $\mu\text{L}$ . UV-spectra between 200 and 300 nm were collected and chromatograms evaluated at 238 nm. Microcystins were identified by retention times and UV-spectra compared with analytical standards of microcystin-LF, -LR, -LW, -RR and -YR. Peaks with significant spectral similarity but different retention times were considered unidentified microcystins. An external calibration with standards was used for quantitation. The sum of micro-

Table 1  
Cyanobacterial samples used in the study, and their characterization

Number	Locality	Date	Dominant cyanobacteria	Microcystin (MC) content
1	Brno reservoir	1 October 1999	<i>Microcystis aeruginosa</i> (98%), <i>Microcystis wesenbergii</i> (2%)	2600 $\mu\text{g/g}$ DW (MC-LR 48%; MC-YR 9%; MC-RR 7%; MC-LW 1%; unidentified MCs 35%)
2	Fraumühlh	29 July 1996	<i>M. wesenbergii</i> (85%), <i>Microcystis</i> sp. (15%)	140 $\mu\text{g/g}$ DW (MC-RR 100%)
3	Skalka	14 August 1997	<i>Aphanizomenon flos-aquae</i> (70%), <i>Microcystis viridis</i> (30%)	715 $\mu\text{g/g}$ DW (MC-LR 80%; MC-YR 20%)
4	Dubice	8 September 2004	<i>Planktothrix aghardii</i> (100%)	2600 $\mu\text{g/g}$ DW (unidentified MCs)

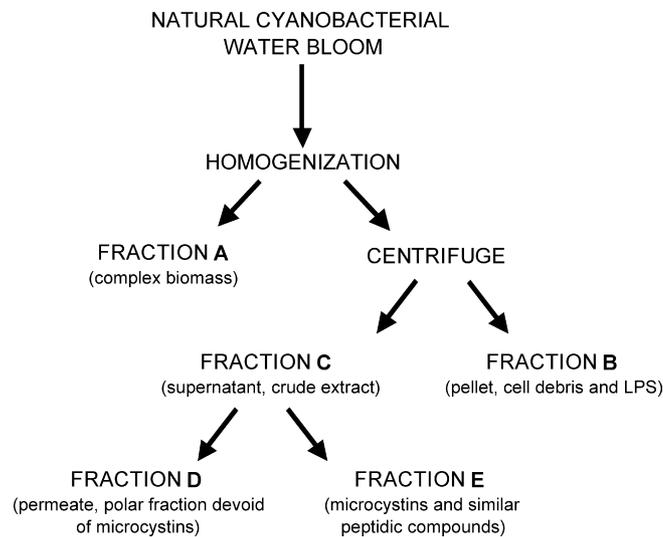


Fig. 1. Experimental design of the pre-treatment and fractionation of cyanobacterial samples.

cystins in individual prepared fractions is shown in Table 2 (coefficient of variance for repeated analyses was below 5%).

#### 2.4. Carp eggs

Fertilized carp eggs were obtained by artificial spawning from fishpond cultivation in Pohorelice Ltd. (Czech Republic). Production lines of carp (female PL, male M72) were used for spawning. The fish eggs were exposed within 8 h of fertilization.

#### 2.5. Toxicity testing—experimental design and conditions

All studied fractions were tested in two independent experiments. Individual fractions were applied at concentrations corresponding to 120, 40, 10 and 3 mg biomass dry weight  $L^{-1}$  in two replicates. Some of the lower concentrations of Skalka and Dubice samples (sample nos. 3 and 4) were not tested. Four replicates of the control group (using the dilution water) were run with each exposure. Each exposure group contained 15 carp eggs and was incubated in glass vials with 15 mL of the diluting water prepared according to the standard procedure ČSN EN ISO 7346. The exposures were semi-static, and the solutions were exchanged every 12 h, total duration of the exposure was

Table 2

Concentrations of microcystins (sum of identified MC variants) in individual fractions (equivalents corresponding to the highest tested concentration of biomass samples 120 mg dry weight  $L^{-1}$ ; coefficient of variance for HPLC analyses was below 5%)

	Biomass number (sum of MCs $\mu g L^{-1}$ )			
	1	2	3	4
Biomass	307.2	8.4	85.8	274.8
Pellet	26.9	<LOD <sup>a</sup>	18.9	13.3
Crude extract	264.2	3.3	59.5	209.4
Permeate	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>
Eluate	243.3	8.4	69.8	254.5

<sup>a</sup> LOD (limit of detection) = 2  $\mu g L^{-1}$ .

120 h. The media temperature was kept on  $23.5 \pm 0.3$  and pH was between 7.1 and 7.6 during the experiment. The 14:10 light:dark period was used. Dissolved oxygen concentrations ranged from 60% to 90% of air saturation except for a decrease at the highest tested concentrations (see Section 3). The concentration of toxic ammonium was below 0.1 mg  $L^{-1}$   $NH_3$  in all treatments. A test with the standard toxicant  $K_2Cr_2O_4$  (48 h  $LC_{50}$  of 412.31 and 439.22 mg  $L^{-1}$ ) confirmed similar sensitivities of the fish eggs in both experiments.

#### 2.6. Embryotoxicity evaluation

The following endpoints were studied during the experiments: time to the beginning and the end of embryo hatching, numbers of larvae hatching each day and the presence of eye spots 48 h after fertilization. Number of healthy embryos, filling of air bladder, number of malformed individuals and cumulative mortality were evaluated at the end of exposure. Surviving embryos were immediately frozen at  $-80^\circ C$  for enzymatic analyses.

#### 2.7. Assessment of GST activity

Three to four whole embryos were homogenized on ice in 1 mL of phosphate buffer saline (PBS, pH 7.2), supernatant was collected after centrifugation (5 min at  $2500 \times g$  at  $4^\circ C$ ) and the activity of GST was measured spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate according to the method of Habig and Pabst (1974). Specific activity (mean of three replicates) was standardized to protein content and expressed as nmol of formed product per minute per milligram protein. The protein concentration was determined according to the method of Lowry et al. (1951). The GENios microplate reader (TECAN, Switzerland) was used for measurement of absorbance.

#### 2.8. Statistical analyses

Differences among the groups were compared by analysis of variance (ANOVA) followed by Scheffe's post-test. *p*-Values less than 0.05 were considered statistically significant.

### 3. Results

Cumulative mortality of carp embryos exposed to individual fractions of cyanobacterial samples is shown in Fig. 2. Significant mortality has been observed after exposures to the highest concentrations of complex biomass (100% mortality at all tested samples) and crude aquatic extract (100% mortality at sample nos. 2–4; 50% at sample no. 1; see Fig. 2). For samples 3 and 4, lower concentrations of complex biomass as well as crude aqueous extracts and permeates also caused pronounced mortality. There was no or low mortality in all of the control groups and the groups exposed to eluate fractions.

Similar responses have also been observed for timing of hatching. Complex biomasses 1 and 2 significantly delayed hatching in carp in the highest tested concentration (also for

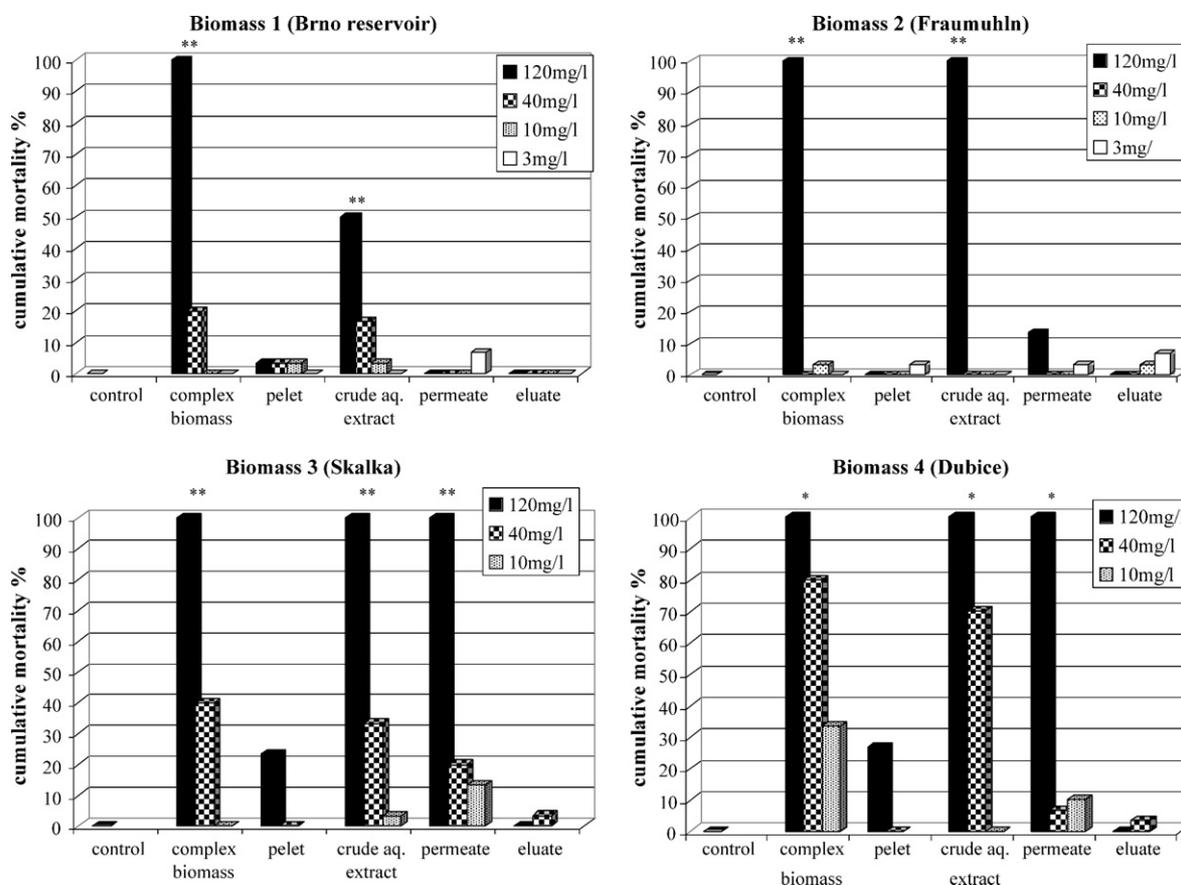


Fig. 2. Cumulative mortality of carp embryos after 120 h exposure to four different samples of cyanobacterial water blooms and its fractions. Significant differences from the control are labeled by asterisks (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ).

crude extract from biomass 2). For samples 3 and 4, hatching was delayed after exposures to the highest concentrations of complex biomass, crude extract and permeate fractions.

Missing eye pigmentation at 48 h post fertilization and incomplete filling of air bladder after 120 h was observed at some individual carps exposed to higher concentrations of some fractions from all biomasses (Table 3). The filling of air bladder was not evaluated for those variants that resulted in 100% mortality.

Although exposure media have been exchanged every 12 h, we have observed significant decrease in dissolved oxygen concentration (below 60% saturation) at 72–96 h at the highest concentrations of complex biomass (all samples), in crude aque-

ous extracts (sample nos. 2–4), and also in permeate (nos. 3 and 4).

Activity of GST in the surviving embryos was determined at the end of exposure (120 h; Fig. 3). Biomasses 1 and 2 caused variable modulations of enzyme activities (either increase or decrease) but these were not significantly different from control groups. Exposures to all fractions from biomasses 3 and 4 (with the exception of permeate) caused significant decrease of GST activity when compared to control (Fig. 3).

#### 4. Discussion

We have investigated embryolarval toxicity in carp after exposures to various cyanobacterial samples and their fractions differing by composition and the content of microcystins. The highest toxicity was observed with the complex biomass and the crude extract fractions of all types of biomass regardless of the cyanobacterial dominance or the amount and/or type of microcystin. Similar lack of correlation between the toxicity and the microcystin content in cyanobacterial samples has also been observed in our previous studies with early life stages of carp (Palíková et al., 2003). In the previous study, we have observed significantly increased cumulative mortality and delayed hatching of embryos even at lower concentrations of microcystin in cyanobacterial extract (5 and 0.5  $\mu\text{g L}^{-1}$ ) after longer exposures (30 days).

Table 3

Effects of cyanobacterial biomasses and their fractions on eye pigmentation and air bladder filling in carp embryos (120 h exposures)

Biomass	Total absence of eye pigmentation (after 48 h)	Air bladder filling (after 120 h)
1	A, C (120, 40)	A (40) 55%, C (120) 5%
2	A, C, D (120)	D (120) 18%
3	A, B, D (120)	A, B, C, D (120, 40) 0–10%
4	A, B, C, D (120)	A, C (40) 0%

Fraction (A) complex biomass, (B) pellet, (C) crude aqueous extract, (D) permeate; no effects were observed with the eluate—fraction (E). Numbers in parentheses are the dry weight concentrations of biomass ( $\text{mg L}^{-1}$ ). Percentage indicates how many of the surviving embryos had an air filled bladder.

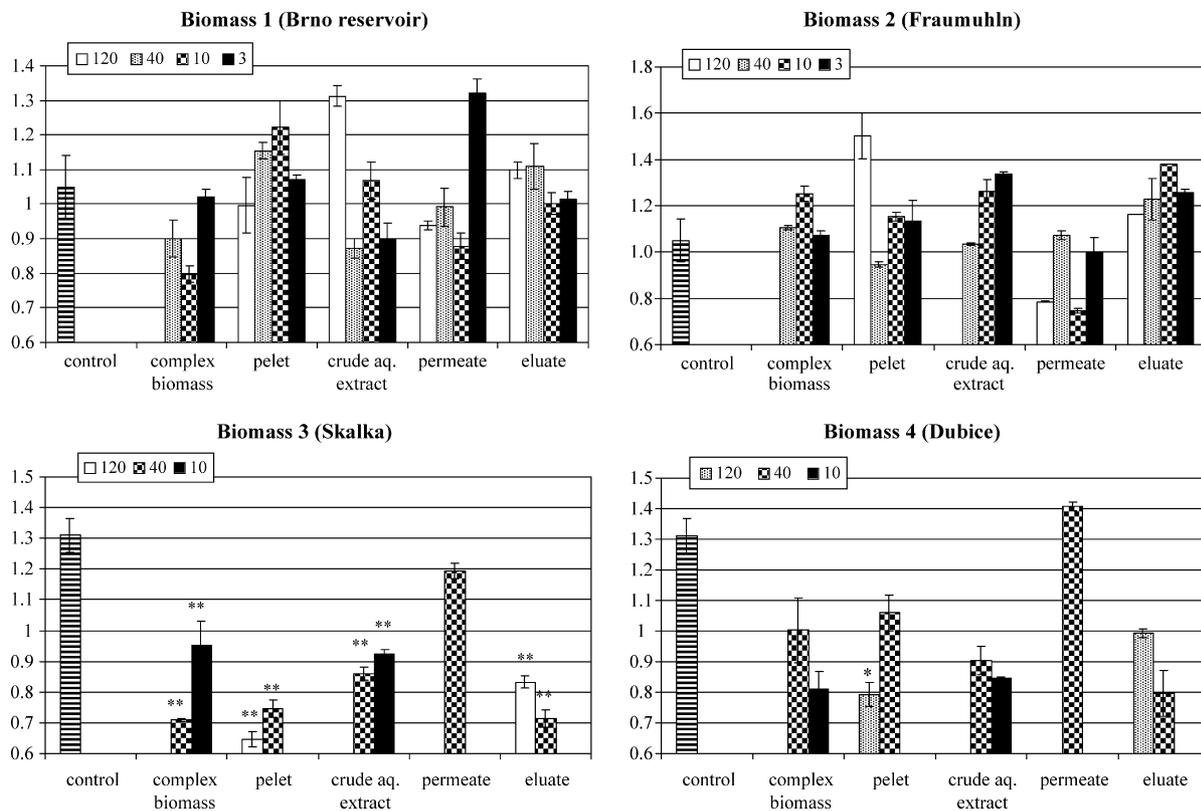


Fig. 3. Glutathione *S*-transferase (GST) activity (nmol GST/min mg protein) in carp embryos exposed for 120 h to individual fractions of cyanobacterial biomasses at indicated concentrations (3, 10, 40 and 120 mg L<sup>-1</sup>). Differences statistically significant from the control are labeled by asterisks (\*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ). Bars represent means  $\pm$  standard error of three replicates (three to four individual embryos per sample).

In general, in the present experiments we have observed effects that were highly sample-specific. Generally higher toxicities were observed in sample nos. 3 and 4 dominated by *Aphanizomenon* and *Planktothrix*, while lower toxicities were associated with *Microcystis* dominated samples. Further, we did not observe correlations between the microcystin content in the samples and their toxicities. According to the latest findings (Preussel et al., 2006), *A. flos-aquae* may produce cylindrospermopsin, a potent alkaloid hepatotoxin with additional effects on kidneys, heart, thymus, spleen and intestine (Hawkins et al., 1997; Falconer et al., 1998). We cannot also exclude possible presence of cyanobacterial neurotoxic metabolites (such as anatoxin-a, anatoxin-a (S) or saxitoxins) in the extracts of *Anabaena*, *Aphanizomenon* and *Planktothrix* dominated water blooms (Chorus and Bartram, 1999). Further, it has been shown that some lipids with negative effects to fish embryo larval development are produced by *A. flos-aquae* (Papendorf et al., 1997). Minor role of microcystin in overall toxicity of complex cyanobacterial samples is also supported by our observations of 100% mortality in biomass no. 2 containing low microcystin concentrations and high mortality of the permeate fractions of sample nos. 3 and 4 (no detectable microcystins in these fractions). Additionally, tested eluate fractions (containing mostly microcystins) had no effects on fish mortality at any of the studied biomass samples.

After fractionation of the crude aqueous extract into permeate (without MCs) and eluate (with MCs) there was general

decrease in overall toxicity (no toxicity at eluate and permeate of the biomass samples 1 and 2; lower toxicities at nos. 3 and 4). Although the effect of SPE extraction cannot fully be excluded (such as degradation of some chemicals, weak efficiency of eluting solvent, etc.), our observations could also be explained by possible synergistic interactions between microcystins and other unknown substances originally present in the complex extract. This hypothesis has also previously been suggested by Oberemm et al. (1997, 1999), who observed more significant effects of cyanobacterial extract on embryolarval development of zebrafish compared to purified microcystin-LR. Similarly, higher toxicity of cyanobacterial extract compared to purified microcystins was observed with *Salmo trutta* (Best et al., 2001). Correspondingly, in the study with brine shrimp (*Artemia salina*) (Feuillade et al., 1996), high toxic effects were observed in the crude extract of cyanobacteria *Planktothrix rubescens* that contained no detectable microcystins. There is also a series of studies that attributed higher toxicity to other unknown compounds in permeate rather than to microcystins (Jungmann et al., 1991; Jungmann, 1992, 1995; Jungmann and Benndorf, 1994).

In a previous study, Liu et al. (2002) reported increase in mortality of mud loach exposed to microcystin-LR. Teratogenic effects of microcystins were observed in fish eggs (EC<sub>50</sub> 244.6  $\mu\text{g L}^{-1}$ ) as well as with hatched larvae (LC<sub>50</sub> 164.3  $\mu\text{g L}^{-1}$ ). These findings do not fully correspond to our observations with carp (no toxic effects up to 250  $\mu\text{g L}^{-1}$ ), and could eventually be explained by either variable experi-

mental designs in both studies or different sensitivity of fish species.

In our study, highest concentration of the crude biomass samples affected also the start of egg hatching. Our previous investigation (Palíková et al., 2003) revealed delayed hatching of carp even at lower concentrations of microcystins in the complex cyanobacterial extracts. On the other hand, Oberemm et al. (1999) reported earlier hatching in rainbow trout at lower microcystin concentrations but delays when higher microcystin concentrations were used. Other experiments with microcystin microinjection into the fish eggs suggested that the egg shell is not easily permeable barrier for microcystins, and that fish embryos are relatively protected from microcystin toxicity (Jacquet et al., 2004; Wang et al., 2005; Delerme et al., 2005).

One of the factors that significantly affect biotests with early life stages of aquatic organisms may be insufficient monitoring of the oxygen during the experiment. Although the exposure media were renewed every 12 h in our experiment, we have observed significant decrease in oxygen below 60% of saturation at the highest concentrations of complex biomass and aqueous extracts of all samples, and also at the permeate fractions of samples 3 and 4. According to OECD 212 (1998) methodology, toxicity results obtained under these conditions should not be considered valid when testing chemical compounds. However, in our study, toxic effects of cyanobacterial metabolites were clearly apparent. For example, significant fish mortality, other embryotoxic effects and modulations of GST activity were observed also in variants with sufficient oxygen saturation (>60%). However, we should also consider possible synergism in the effects of tested samples with the lack of oxygen, especially when testing complex samples rich with organic material such as cyanobacterial biomass. The role of oxygen was, however, overlooked in several previous studies focused on ecotoxicity of complex cyanobacterial samples.

Although cyanobacteria form a natural part of ecosystems, fish and other aquatic organisms react to their metabolites as to xenobiotics. Detoxification processes were shown to have adverse side effects such as activation of protoxicants and also elevated energy demand. Changes in activity of detoxification enzyme glutathione *S*-transferase (GST) have been used as a biomarker of chronic cyanobacterial toxicity in fish. However, the responses in GST in various exposure scenarios may be highly variable (Bláha et al., 2004). Previous study with carp hepatocytes has shown significant increase in the production of reactive oxygen species (ROS), induction of detoxication enzymes SOD, CAT, GPX, but the authors observed no significant changes in reduced glutathione (GSH) or GST (Li et al., 2003). Similarly, weak responses of GST to microcystin-LR exposure were also previously observed with early life-stages of zebrafish (Wiegand et al., 1999). On the other hand, Pietsch et al. (2001) reported significant suppression of GST in zebrafish after 24 h exposure of fish eggs to cyanobacterial extract, but significant increase of GST was observed after exposure of fish to purified MC-LR and MC-RR. Statistically significant decreases in GST activities was also observed after co-exposure of zebrafish to microcystin-LR and cyanobacterial lipopolysaccharides, LPS (Best et al., 2002).

Our experiments have shown highly sample-specific responses with a decrease of GST activity at all tested concentrations of all fractions containing microcystins of biomass 3 and 4. In agreement with Best et al. (2002), the effects were pronounced also at low concentrations of cellular pellet containing LPS. Interestingly, there were no effects on GST in microcystin-free permeates from sample nos. 3 and 4, even though the toxic effects of these fractions were documented by an increase of cumulative mortality (Figs. 2 and 3). GST activities may also be highly variable with exposure time, and, consequently, there could be a no-effect window at 120 h of our exposures. However, these investigations will require further research attention.

## 5. Conclusion

In summary, our results demonstrate significant embryotoxic effects of various samples of complex cyanobacterial biomasses and their crude aqueous extracts to carp. The toxic effects included mortality, delayed hatching, lower number of hatched embryos, suppression in embryonic development and disturbance of air bladder filling, and modulations of GST activities. However, the toxicity was highly sample-specific (the most prevalent effects occurred at samples dominated by filamentous cyanobacteria *Aphanizomenon* and *Planktothrix*), and we did not observe any significant toxicity that could directly be attributed to microcystins (weak effects of eluate fractions containing mostly microcystins, and, on the other hand, significant effects in permeate fractions of sample nos. 3 and 4 with no microcystins).

Our data thus indicate either the presence of unidentified toxic metabolites in the cyanobacterial samples, and/or possible synergistic interactions among various constituents of complex cyanobacterial biomass. Further, we suggest careful monitoring of sufficient oxygen supply during the ecotoxicological experiments with cyanobacterial samples, and to critically discriminate between the effects of cyanobacteria and toxicity related to oxygen deficiency. Although the toxicology of microcystins in mammals and humans has been well established, further research is needed to evaluate the role of these peptides in ecotoxicology of aquatic environment.

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