

Toxicity of Crude Extract of Cyanobacteria for Embryos and Larvae of Carp (*Cyprinus Carpio* L.)

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Abstract

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Cyanobacteria and their toxic products represent a serious problem in many waters. The aim of this study was to find out how crude extract of cyanobacteria can influence the embryonal and larval development of carp on the base of embryo-larval toxicity test.

Crude extract of cyanobacteria containing the known amount of microcystins LR, YR and RR (130, 13 and 1.3 $\mu\text{g}\cdot\text{L}^{-1}$, i.e. high, medium and low concentration of the extract), was administered to carp eggs. The experiments were finished after 8 and 30 days (short- and long-term exposure). Evaluation of the tests was based on the OECD guideline for testing chemicals, direction 210 from 1992. The extract with high concentration caused 93% ($p < 0.01$) embryonal mortality, prolonged hatching, increased numbers of malformed and dead larvae ($p < 0.01$) and a decrease in average total length ($p < 0.01$). Yolk sac dropsy and abnormal behaviour were observed. The extract with medium concentration caused an increase in dead larvae after the short-term exposure ($p < 0.05$) and an increase in malformed ($p < 0.05$) and dead ($p < 0.01$) larvae after the long-term exposure. The extract with low concentration caused an increase in dead larvae only after the long-term exposure ($p < 0.05$). In general, we can conclude that the extract with high concentration results in acute toxicity for embryos of the carp. The influence of the extract with medium and low concentrations were manifested after the long-term exposure.

Microcystin, mortality, malformations, abnormal behaviour, fish, embryo-larval toxicity test

Cyanotoxins produced by cyanobacteria pose an environmental problem and influence the health status of both human and aquatic organisms. Many studies described health damage or even intoxication in animals and humans (Falconer 1989; Carmichael 1992). Cyanotoxins cause an impairment of the immune system, torpidity and overall weakness, vomiting and digestive problems, respiratory and allergic diseases, liver damage and other health problems. They also play an important role in the cancerogenesis (Bell and Codd 1994). The most common cyanotoxins are hepatotoxins. They involve acute toxicoses. Death can occur anywhere between 10 min to a few days after intoxication depending on a number factors (Ressom et al. 1994). There are a number of different hepatotoxins produced by species and strains within the genera *Anabaena*, *Cylindrospermopsis*, *Microcystis*, *Nodularia*, *Oscillatoria*, *Nostoc*, *Aphanizomenon*, *Gloetrichia* and *Coelosphaerium* (Ressom et al. 1994). There are 28 microcystins known presently. *Microcystins* are cyclic heptapeptides. They have hepatotoxic effect. Microcystin LR is the most common and the most often studied hepatotoxin. The mechanism of its influence is the same in humans as in fish and it is on cellular level (Eriksson 1990).

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Recently, the research into this area has been aimed at investigation of effects of cyanotoxins on early life stages of organisms. The effect of the toxic and non-toxic strains of *Microcystis aeruginosa* on larvae of *Procambarus clarkii* and their possible accumulation was observed by Vasconelos et al. (2001). Oidtmann et al. (2001) found higher mortality of juvenile crayfish in the lake with microcystins present. The effect of microcystin LR and YR (pure toxins at concentrations of 1, 10, 100, 500 and 2000 $\mu\text{g}\cdot\text{L}^{-1}$) was studied also in early life stages of the frog *Xenopus laevis* (Fischer and Dietrich 2000). In conclusion, the authors state that transchorial/transdermal absorption of microcystins in *Xenopus laevis* is minimal or absent and their results indicate that early life-stages of amphibians (up to five days of development) are unlikely to be affected by cyanobacterial blooms producing microcystins LR and RR.

Oberemm et al. (1997) studied the effect of microcystin LR (pure toxin at concentrations of 0.5, 5 and 50 $\mu\text{g}\cdot\text{L}^{-1}$) and crude extract of cyanobacteria on embryos and larvae of zebrafish (*Danio rerio*). No effects were observed during the embryonic development. High mortality and malformations were observed after exposure to various cyanobacterial crude extracts. Oberemm et al. (1999) studied the effects of microcystin LR, RR and YR (pure toxins at concentrations of 0.5, 5 and 50 $\mu\text{g}\cdot\text{L}^{-1}$), saxitoxin (pure toxin at concentrations of 10, 50, 100 and 500 $\mu\text{g}\cdot\text{L}^{-1}$), anatoxin-a (pure toxin at concentrations of 40, 200 and 400 $\mu\text{g}\cdot\text{L}^{-1}$) and crude extract of cyanobacteria (the highest concentrations of total microcystins were 40 and 45 $\mu\text{g}\cdot\text{L}^{-1}$) on the development of fish (*Danio rerio*, *Oncorhynchus mykiss*, *Rutilus rutilus*, *Abramis brama*, *Leuciscus cephalus*, *Leuciscus delineaatus* and *Cobitis taenia*) and amphibians. No acute toxic effects were observed after exposure to pure microcystins. Only in *Oncorhynchus mykiss* earlier hatching occurred.

Dissolved (extracellular) concentrations of microcystins vary from 0.1 to 1800 $\mu\text{g}\cdot\text{L}^{-1}$ of natural waters. A broad overview of extracellular microcystins samples around the world is given in Sivonen and Jones (1999). The level of dissolved microcystins in the Czech Republic was measured in drinking water reservoirs 0-45 $\mu\text{g}\cdot\text{L}^{-1}$, in recreational reservoirs 0-180 $\mu\text{g}\cdot\text{L}^{-1}$ and fish pond - with *Microcystis ichtyoblabe* dominance 225 $\mu\text{g}\cdot\text{L}^{-1}$, (Bláha and Maršálek 2001). The concentrations of microcystins in cyanobacterial biomass from Czech water bodies vary from 0 to 4450 $\mu\text{g}\cdot\text{g}^{-1}$ dry weight (Maršálek et al. 2001).

The results of embryonal (short term) and embryo-larval (long term) tests in the carp (*Cyprinus carpio* L.) exposed to crude extract of cyanobacteria containing the known amount of microcystins are presented in this study. Concentrations of microcystins were chosen to compare the results with literature and to be compared with the level of dissolved microcystins in natural waters.

Materials and Methods

Carp eggs and preparation of crude extract of cyanobacteria

The carp eggs were obtained by artificial reproduction at the fishery in Oslavany (Czech Republic). Fertilised and unsticked carp eggs were divided into four groups till 8th hours from fertilisation, each containing two hundred eggs. The eggs were incubated in glass vials contained 0.5 L and 1 L (after 8th day) of water. The water was changed every 8 hours including the crude extract of cyanobacteria to keep the concentration of crude extract of cyanobacteria. The conditions in baths were following: water temperature 21.5–22.5 °C, dissolved oxygen 65–113%, i.e. 5.5–10.1 $\text{mg}\cdot\text{L}^{-1}$ and pH was 7.9–8.9. Hatching was 96 and 93% and survival was 92.5 and 86.5% in control groups.

The larvae were fed by commercial food Artemia PREMIUM since the 5th day. Feeding was performed 20–30 min before every water changing.

Tests were performed with the crude cell extract obtained from field samples of water bloom (Brno reservoir, Czech Republic). Sample content the planktonic species *M. aeruginosa* (85%), *Microcystis ichtyoblabe* (5%) and *Aphanizomenon flos-aquae* (3%). The sample was collected from surface water bloom (0 to 0.3 m depth) and concentrated by plankton net 22 μm . The sample was stored frozen at -20 °C. The concentration of microcystins was determined by HPLC according to the method described by Lawton et al. (1994). Total microcystin concentration (MC) was 1 129 $\mu\text{g}\cdot\text{g}^{-1}$ dry weight in the biomass. To obtain the crude extract, the material was ultrasonicated for

7 min. and was centrifuged for 20 min at 5 000 rpm. Re-extraction was done twice by standard water. The final concentration of hepatotoxic microcystins in the crude extract used for exposure was $15.7 \mu\text{g}\cdot\text{L}^{-1}$ ($9.6 \mu\text{g}\cdot\text{L}^{-1}$ of microcystin YR, $6.0 \mu\text{g}\cdot\text{L}^{-1}$ of microcystin LR, $0.1 \mu\text{g}\cdot\text{L}^{-1}$ of microcystin RR). The amount of biomass was $22.1 \text{ mg}\cdot\text{L}^{-1}$ of dry weight. These biomass concentrations commonly occur in the Brno reservoir, the Czech Republic.

Experimental treatments

The crude extract of cyanobacteria with known amount of microcystin LR (50, 5 and $0.5 \mu\text{g}\cdot\text{L}^{-1}$) was added to the eggs in three concentrations: the first with $0.5 \mu\text{g}\cdot\text{L}^{-1}$ of microcystin LR (low concentration of the extract) the second with $5 \mu\text{g}\cdot\text{L}^{-1}$ of microcystin LR (medium concentration of the extract), the third with $50 \mu\text{g}\cdot\text{L}^{-1}$ of microcystin LR (high concentration of the extract). The control eggs were incubated in toxic free water. The cumulative amount of microcystins was 130, 13 and $1.3 \mu\text{g}\cdot\text{L}^{-1}$, respectively. Tests were finished after 8 days (i.e. short-term exposure) and after 30 days (i.e. long-term exposure). Both groups with high concentration were finished after 8 days because the mortality of embryos was very high.

Evaluation of the tests was based on the OECD Direction 210 from 1992.

During the test we observed:

- time to the start and the end of hatching
- numbers of larvae hatching each day
- numbers of malformed larvae
- abnormal behaviour in larvae

After finishing the tests, we evaluated:

- cumulative mortality
- numbers of healthy fish at the end of the test
- average total length and body mass (the average total length was determined in 10 larvae and body mass in 20 larvae, except the group with high concentrations, where 10 larvae were measured only).

Results were statistically analysed by the Student's *t*-test and χ^2 -test using the STATplus software (Matoušková et al. 1992).

Results

Results of tests with short-term exposure

Table 1
Eggs hatching and malformations in experiment with short-term exposure

Concentration of the extract	Start of hatching (h)	End of hatching (h)	Numbers of hatched larvae for a day			Percentage of malformed larvae (%)
			3 rd day	4 th day	5 th day	
high*	96	102	0	20	4	20.83 ^A
medium*	58	102	40	142	8	2.11 ^B
low*	56	102	134	52	3	1.06 ^B
control	58	102	100	87	4.5	1.56 ^B

No significant differences were found between the values indicated by the same letters. In case of their absence in any of the examined indices, the values are not indicated. Capitals are used for indicating the significance of differences at the level of $p < 0.01$. *see experimental treatment

Hatching (Eggs hatching is presented in Table 1.)

Larvae hatched during three days. In the control group, the majority of larvae hatched during the first and second day. In the group with low concentration of the extract, it was similar, but the amount of larvae hatched in the first day was higher. In the group with medium concentration of the extract, the majority of larvae hatched in the second day. No larvae were hatched in the group with high concentration of the extract during the first day, most of them were hatched by the second day. Total numbers of hatched larvae were 192 in the control, 189 in the group with low concentration of the extract, 190 in the group with medium concentration of the extract and 24 in the group with high concentration of the extract. Malformed and dead larvae (numbers of malformed and dead larvae are presented in Tables 1 and 2.)

Three malformed larvae were found in the control group (1.56% from 192 hatched larvae), two in the group with low concentration of the extract (1.06% from 189 hatched larvae), four in the group with medium concentration of the extract (2.11% from 190 hatched larvae) and five in the group with high concentration of the extract (20.83% from 24 hatched larvae) during the experiment.

Seven larvae died during the experiment in the control group (3.64% from 192 hatched larvae), ten in the group with low concentration of the extract (5.29% from 189 hatched larvae), 16 in the group with medium concentration of the extract (8.42% from 190 hatched larvae) and 11 in the group with high concentration of the extract (45.8% from 24 hatched larvae).

Cumulative mortality (cumulative mortality is presented in Table 2.)

In the control 185 larvae survived. In the group with medium concentration of the extract and in the group with low concentration of the extract 174 and 179 larvae survived. In the group with high concentration of the extract only 14 larvae survived.

Average total length and body mass of surviving larvae (average total length and body mass of surviving larvae) are presented in Table 2.

Table 2
Fry measurements and survival in experiment with short-term exposure

Concentration of the extract	Cumulative mortality (%)	Average total length (mm \pm SD)	Average total body mass (mg \pm SD)	Percentage of dead larvae (%)
high*	93.0 ^A	5.15 \pm 0.53 ^A	0.82	45.80 ^A
medium*	13.0 ^B	6.60 \pm 0.39 ^B	0.83	8.42 ^{B,a}
low*	10.5 ^B	6.80 \pm 0.35 ^B	0.84	5.29 ^{B,ab}
control	7.5 ^B	6.58 \pm 0.44 ^B	1.07	3.64 ^{B,b}

No significant differences were found between the values indicated by the same letters. In case of their total absence in any of the examined indices, the values are not indicated. Capitals and small letters are used for indicating the significance of differences at the level of $p < 0.01$ and $p < 0.05$, respectively.

*see experimental treatment

Abnormal behaviour of larvae

In hatched larvae of the group with high concentration of the extract we observed yolk sac dropsy and abnormal behaviour (loss of reflexes, decreased reaction to external stimuli, decreased food intake).

Examples of malformed larvae from the groups with high concentration of the extract are presented in Plate xy Fig. 1.

Results of tests with long-term exposure

Hatching (egg hatching is presented in Table 3.)

Larvae hatched during three days. In the control group, the majority of larvae hatched in the second day. In the groups with medium and low concentrations of the extract it was similar, the majority of larvae hatched in the first day. Total numbers of hatched larvae were 186 in the control, 188 in the group with low concentration of the extract and 186 in the group with medium concentration of the extract.

Malformed and dead larvae (numbers of malformed and dead larvae are presented in Tables 3 and 4.)

In the control group 4 malformed larvae were found (2.15% from 186 hatched larvae), 7 in the group with low concentration of the extract (3.72% from 188 hatched larvae) and 10 in the group with medium concentration of the extract (5.38% from 186 hatched larvae) during the experiment.

Table 3
Egg hatching and malformations in experiment with long-term exposure

Concentration of the extract	Start of hatching (h)	End of hatching (h)	Numbers of hatching larvae for a day			Percentage of malformed larvae (%)
			3 rd day	4 th day	5 th day	
medium*	58	102	160	25	1	5.38 ^a
low*	56	102	123	64	1	3.72 ^{a,b}
control	58	102	63	122	1	2.15 ^b

No significant differences were found between the values indicated by the same letters. In case of their total absence in any of the examined indices, the values are not indicated. Small letters are used for indicating the significance of differences at the level of $p < 0.05$.

*see experimental treatment

Table 4
Fry measurements and survival in experiment with long-term exposure

Concentration of the extract	Cumulative mortality (%)	Average total length (mm \pm SD)	Average total body mass (mg \pm SD)	Percentage of dead larvae (%)
medium*	26.0	14.83 \pm 2.07	36.03 \pm 15.61	20.43 ^{A,a}
low*	16.5	14.30 \pm 1.51	30.57 \pm 11.32	11.17 ^{AB,b}
control	13.5	14.55 \pm 1.96	30.87 \pm 12.47	6.98 ^B

No significant differences were found between the values indicated by the same letters. In case of their total absence in any of the examined indices, the values are not indicated. Capitals and small letters are used for indicating the significance of differences at the level of $p < 0.01$ and $p < 0.05$, respectively.

*see experimental treatment

Thirteen larvae died during the experiment in the control group (6.98% from 186 hatched larvae), 21 in the group with low concentration of the extract (11.17% from 188 hatched larvae) and 38 in the group with medium concentration of the extract (20.43 % from 186 hatched larvae).

Cumulative mortality (cumulative mortality is presented in the Table 4.)

In the control group 173 larvae survived. In the group with low concentration of the extract 167 larvae survived and in the group with medium concentration of the extract were 148 surviving larvae.

Average total length and body mass of surviving larvae (no significant differences in the average total length and body mass were found, see Table 4).

Discussion

The results indicate that embryonic development is greatly influenced by the highest concentration of crude extract of cyanobacteria (50 $\mu\text{g}\cdot\text{L}^{-1}$ of microcystin LR). Very high cumulative mortality (93.3%) at this concentration made the 30-day test impossible to continue. Hatching of this group was also influenced and started 38 h later than in control group. The number of hatched larvae was very low. Hatched larvae were characterized by yolk sac dropsy, high mortality, increased numbers of malformed individuals, no reaction to external stimuli followed by inability to feed. This concentration of the extract influenced even the body weight of the fry and the total body length, which were significantly lower.

Lower concentrations of crude extract of cyanobacteria (5 and 0.5 $\mu\text{g}\cdot\text{L}^{-1}$ of microcystin LR) during the short-term exposure resulted only in a moderate rise in cumulative mortality and no other considerable changes. On the other hand, embryos started hatching 2 hours

earlier than controls in the extract of cyanobacteria containing the low toxin concentration ($0.5 \mu\text{g}\cdot\text{L}^{-1}$ of microcystin LR).

Longer action of cyanobacteria extracts had effects even at lower concentrations, in particular, in the group exposed to the medium concentration of the extract. Cumulative mortality was higher and the number of malformed individuals was increased. Neither the total length nor the body weight of larvae was significantly altered.

We can say that the highest used concentration of crude extract of cyanobacteria ($50 \mu\text{g}\cdot\text{L}^{-1}$ of microcystin LR) influenced the development of the carp fry through acute toxicity and was not suitable for the determination of chronic toxicity. To our knowledge, there are no papers on acute embryo-larval toxicity of crude extract of cyanobacteria available. Oberemm et al. (1997) used pure microcystin LR in doses of 0.5 , 5 and $50 \mu\text{g}\cdot\text{L}^{-1}$ and no acute toxicity was found. Higher mortality, retarded larval growth and decreased survival (at 5 and $50 \mu\text{g}\cdot\text{L}^{-1}$ microcystin LR only) were observed at the end of larval period (after termination of the exposure and transfer of larvae into microcystin LR free water). High mortality and malformations were observed after exposure to various cyanobacterial crude extracts. Oberemm et al. (1999) described more pronounced effects of cyanobacterial biomass than of pure toxins after embryonic exposure. Malformations combined with high mortalities and adverse effects on outer egg structures were observed concomitantly in all species after exposure to various aqueous crude extracts of cyanobacteria. Total concentrations of microcystins they used (30 , 40 and $45 \mu\text{g}\cdot\text{L}^{-1}$), however, were lower than the ones we used in our experiment.

The used high concentration of the extract corresponds with the level of dissolved microcystins in recreational reservoirs, medium and low concentrations of the extract with the level of dissolved microcystins in drinking water reservoirs (Maršálek and Bláha 2001). The highest level of dissolved microcystins is usually present in July and September in the Czech Republic. But when the weather is warm, a high level of dissolved microcystins can be found in June as well. Then it could negatively influence the embryo-larval development of various fish species in natural waters.

Toxicita extraktu sinic pro embrya a larvy kapra obecného (*Cyprinus carpio* L.)

Cyanotoxiny produkované sinicemi vážně ovlivňují zdravotní stav organismů. Cílem studie bylo zjistit účinek extraktu sinic na embryo-larvární vývoj kapra obecného na základě embryo-larvárních testů toxicity.

Pokusným skupinám jsme přidávali do vody extrakt sinic se známými koncentracemi microcystinů (130 , 13 a $1.3 \mu\text{g}\cdot\text{L}^{-1}$, tj. vysoká, střední a nízká koncentrace extraktu). Testy byly ukončeny po osmi a třiceti dech (tj. krátká a dlouhá expozice). Vyhodnocení testů jsme prováděli podle směrnice OECD 210 z roku 1992.

Vysoká koncentrace extraktu způsobila 93% ($p < 0.01$) embryonální mortalitu, prolongované kulení, zvýšený počet malformovaných a mrtvých larev ($p < 0.01$) a snížení celkové délky larev ($p < 0.01$). Zaznamenali jsme vodnatelnost žloutkového váčku a odchylky chování. Střední koncentrace extraktu a krátká aplikace způsobila vzestup mrtvých larev ($p < 0.05$), dlouhá expozice vzestup mrtvých ($p < 0.05$) i malformovaných ($p < 0.01$) larev. Nízká koncentrace extraktu způsobila vzestup počtu mrtvých larev ($p < 0.05$) pouze při dlouhé expozici. Zjistili jsme, že nejvyšší použitá koncentrace extraktu sinic působí na vývoj plůdku kapra akutně toxicky a není vhodná již pro zjišťování chronické toxicity. Účinek nižších koncentrací toxinů se projevil až po delší expozici.

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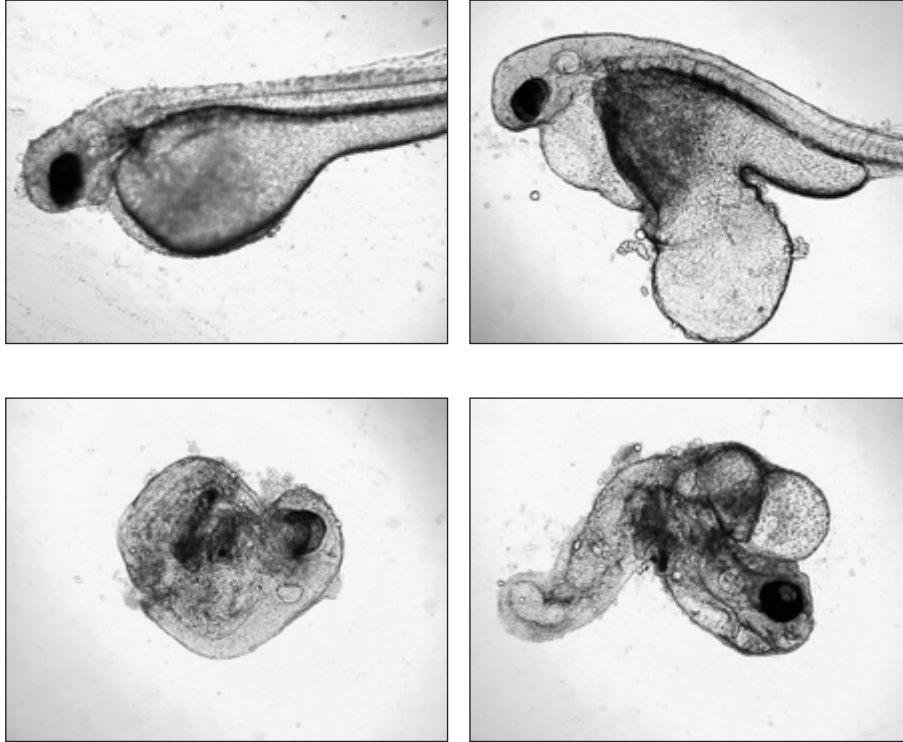


Fig. 1: Examples of malformed larvae with non-resorbed and deformed yolk sack or deformed body from the group with high concentration of extract of cyanobacteria after finishing of the short-term exposure.