Toxicon 70 (2013) 98–106

Highlights

ACT *C. raciborskii* elicited significant feeding inhibition and lethality to crustacean neonates.
ACT *C. raciborskii* exerted significant retardation of *Danio rerio* embryogenesis.
ACT *C. raciborskii* do not synthesize cylindrospermopsins, anatoxins and saxitoxins.
Ecotoxicity risks to aquatic biota of present day maximal densities of *C. raciborskii* in Lake Balaton are moderate.

Toxicon 70 (2013) 98–106

# The ecotoxicological evaluation of *Cylindrospermopsis raciborskii* from Lake Balaton (Hungary) employing a battery of bioassays and chemical screening

András Ács[a], Attila W. Kovács[b], Janka Zsófia Csepregi[c], Norbert Törő[d], Gyula Kiss[d] János Győri[b], Ágnes Vehovszky[b], Nora Kováts[a], Anna Farkas[b*],

[a] University of Pannonia, Department of Limnology, Egyetem Str. 10, H-8200 Veszprém, Hungary.

[b] Balaton Limnological Institute, Centre for Ecological Research, Hungarian Academy of Sciences, Klebelsberg K. u. 3., POBox 35., H-8237 Tihany, Hungary.

[c] Faculty of Biology and Geology, Babeş-Bolyai University, Cluj Napoca, Romania

[d] MTA-PE Air Chemistry Research Group, Egyetem Str. 10, H-8200, Hungary

[*]Corresponding author. Balaton Limnological Institute, Centre for Ecological Research, Hungarian Academy of Sciences, Klebelsberg K. u. 3., POBox 35., H-8237 Tihany. Tel.: 0036 87 448 244; e-mail: farkas.anna@okologia.mta.hu

Abstract

Ecotoxicity of four *C. raciborskii* strains (ACT 9502, ACT 9503, ACT 9504, ACT 9505) isolated from Lake Balaton (Hungary) was evaluated in four aquatic bioassays including the *Thamnocephalus platyurus* acute lethality test; *Daphnia magna* acute immobilization assay; *Daphnia magna* feeding inhibition assay and *Danio rerio* embryo developmental toxicity assay, assisted by chemical screening for known toxins by HPLC-MS. For reference, we analyzed in parallel the toxin content and toxic effects of two previously characterized toxin-producing strains: the Australian cylindrospermopsin producer AQS *C. raciborskii* and the anatoxins producer *Oscillatoria* sp. PCC 6506. Bioassays were used to evaluate the overall toxicity of the hydrophilic bioactive metabolites pool synthesized by the selected cyanobacteria.

Chemical screening has proven that the ACT *C. raciborskii* extracts investigated did not contained cylindrospermopsins and anatoxins. The relative toxicity of the ACT *C. raciborskii* aqueous extracts observed in each bioassay was comparable to the effects recorded for the anatoxins producer PCC 6506 strain while toxicity values ($EC_{50}$/$LC_{50}$) calculated for the AQS extract were in general one order of magnitude lower.

Concerning sublethal effects of ACT *C. raciborskii* extracts to the *D. rerio* embryogenesis, the general morphological abnormality observed was a significant retardation of development.

Overall, our results suggest that *C. raciborskii* populating Lake Balaton produce metabolites with significant bioactive potencies. Therefore, continued investigation of these unknown compounds is required.

Keywords: *Cylindrospermopsis raciborskii*, Lake Balaton, ecotoxicity, crustaceans, fish embryogenesis

## 1. Introduction

The freshwater cyanobacterium *Cylindrospermopsis raciborskii* (Wołoszyńska) Seenayya and Subba Raju (*Nostocales, Cyanopyceae*) is a successful competitor of the phytoplankton community, occurring in almost every continent (Briand et al., 2004; Padisák, 1997). Although the species is of tropical to subtropical origin, it proved to be highly adaptive and invaded new habitats even in temperate climates (Padisák, 1997; Bernard et al., 2003; Fastner et al., 2003; Saker et al., 2003; Hamilton et al., 2005).

In Hungary, the first reports on *C. raciborskii* occurrence were documented in the 1970s. In Lake Balaton this species was first observed in 1978 and has gradually become the dominant blue-green algae species blooming several times since 1982, with maximal biomasses of aprox. 30.000 – 35.000 µg w.w. $L^{-1}$ (equiv. to aprox. 160 µg Chl *a* $L^{-1}$) observed in 1994 (Présing et al., 1996).

In general, the presence of *C. raciborskii* in freshwaters is of particular concern due to the ability of certain strains to produce various toxins, from potent hepatotoxins (CYNs: cylindrospermopsins), to neurotoxins (PSPs: paralytic shellfish poisons), (Saker et al. 2003; Fastner et al., 2007; Berry et al. 2009). Although for the European *C. raciborskii* isolates investigated so far the ability of synthesizing CYNs and PSPs has never been documented, many of them proved to be toxic in the mouse bioassay (Bernard et al., 2003; Fastner et al., 2003; Saker et al., 2003; Hamilton et al., 2005).

Similarly to other *C. raciborskii* strains isolated from European water bodies, preliminary toxicological studies have indicated variable patterns of toxicity of the *C. raciborskii* isolates from Lake Balaton as well. The first investigated isolate (NIVA-CYA 225) proved to exert protracted type symptoms in mice (Underdal et al., 1999). Another isolate (ACT 9502) showed toxic effects also on invertebrate and vertebrate models (Hiripi et al., 1998). Electrophysiological assays suggested the production of anatoxin-a like metabolites, as revealed by the cholinergic blocking effects recorded on mollusc neuronal models (Kiss et al., 2002; Vehovszky et al., 2009). In turn, other toxicological investigations failed to confirm toxicity for the ACT 9502 isolate by the mouse bioassay (Bernard et al., 2003). A recent study outlined also the induction of specific morphological alterations in CHO-K1 (*Chinese Hamster Ovary*) cells upon exposure to crude extracts of four *C. raciborskii* isolates (ACT 9502, ACT 9503, ACT 9504, ACT 9505) (Antal et al. 2011).

Owing to the regular appearance of *C. raciborskii* in summer with biomasses up to 4.000 – 5.000 µg w.w. $L^{-1}$ even nowadays (L. Vörös, unpublished data) in Lake Balaton and increasing evidence about the toxicity potential of certain ACT *C. raciborskii* isolates, we have considered important to assess also their risks to the aquatic biota.

Accordingly, the objectives of this study were to: to analytically screen the pool of hydrophilic compounds produced by the ACT *C. raciborskii* for known cyanotoxins that could theoretically be expected to be synthesized according to their phylogeny and toxicity patterns described, and to evaluate the possible ecotoxicological effects of the hydrophilic metabolite matrices synthesized by the ACT *C. raciborskii* strains with a battery of four aquatic bioassays (*Thamnocephalus platyurus* acute lethality test; *Daphnia magna* acute immobilization assay; *Daphnia magna* feeding inhibition assay; *Danio rerio* embryo developmental toxicity assay).

For reference, we have analyzed in parallel the toxin content and effects of two well-known toxic cyanobacterial strains: the Australian cylindrospermopsin (CYN) producer AQS *C. raciborskii* strain (Saker and Eaglesham, 1999; Saker and Neilan, 2001) and the anatoxins (anatoxin-a – AN, homoanatoxin-a – HMAN) and CYN producer *Oscillatoria* sp. PCC 6506 (Aráoz et al., 2005, Mazmouz et al. 2010).

## 2. Materials and methods

### 2.1. Cyanobacterial strains

We have evaluated the acute toxicity of four *C. raciborskii* strains ACT 9502, ACT 9503, ACT 9504 and ACT 9505, obtained from the Algal Cultures of Tihany (ACT). These strains were isolated from Lake Balaton between 1994-1995 by the classical bacteriological streaking method according to Rippka (1988). The AQS *C. raciborskii* strain was kindly provided by Dr. M.L. Saker (Emaar Properties PJSC, Dubai, United Arab Emirates), whereas the *Oscillatoria* sp. PCC 6506 strain was purchased from the Pasteur Culture Collection of Cyanobacteria (France).

### 2.2. Cyanobacteria culturing and crude extract preparation

Mass cultures (semi continuous) of each strain were grown at 22°C in BG11 medium (Rippka et al., 1979) without nitrate, illuminated under a 16:8 h L:D cycle with fluorescent white light (cool white F33, 36 W, Tungsram) at the irradiance of 60 µmol $m^{-2}$ $s^{-1}$ measured by US-SQS/L, WALZ spherical quantum micro sensor connected to Li-Cor 1400 data logger on the surface of the growth vessels and bubbled with sterile air at 500 – 800 mL $min^{-1}$. Cells were harvested in late exponential growth phase by filtration on Whatman GF/C filter paper, lyophilized and stored at -20 °C until use.

Cell-free crude extracts were prepared in MilliQ water at 5 mg dry weight $mL^{-1}$ concentration. Cell suspensions were submitted to four cycles of freeze-thawing, and checked under light microscope for complete cell lyses. If necessary, certain suspensions were additionally subjected to ultrasonic treatment (10 sec in 3 cycles, 50% duty cycle, 6 output control, Ultrasonic homogenizer COLE PARMER Series 4710 with head adapter CV17 model) on ice. The broken cell suspensions were centrifuged in two cycles (12,000g, 10 min, 4°C), the supernatants were sterilized by filtration on 0.22 µm syringeless filter devices (Mini-Uniprep™, Whatman Inc., Florham Park, NJ, USA) and stored at -20 °C until use.

2.3. Cyanotoxin analyses

Detection and quantification of cyanotoxins in aqueous extracts were performed by liquid chromatography-quadrupole ion trap mass spectrometry using an Agilent 1100 model HPLC linked to an Agilent Trap VL ion-trap mass spectrometer, according to Meriluoto and Spoof (2008). The chromatographic separation was achieved on a Merck Purospher STAR C18 column (55 mm X 4 mm I.D., 3 mm particle size) maintained at 40 °C. A binary gradient elution was used employing a mobile phase of 0.05% trifluoroacetic acid in water and 0.05% trifluoroacetic acid in acetonitrile (1-70% over 20 min) at a constant flow rate of 1 mL $min^{-1}$. The diode array detector scanned within 200–800 nm with main detection wavelength at 227 nm for typical anatoxins spectra and at 262 nm for cylindrospermopsins. MS analysis was carried out using atmospheric pressure electrospray ionization (ESI) source in positive mode. ATX and CYN were identified upon retention times and fragmentation patterns of identified peaks: *m/z* 166 – ATX; *m/z* 416 – CYN. Quantification of ATX and CYN was performed against dilutions in water of AN-fumarate (Ascent Scientific Ltd., UK) and CYN provided by Dr. Gábor Vasas who had purified the toxin from the *Aphanizomenon ovalisporum* (Forti) strain (ILC-164), as previously described (Vasas et al., 2002). In the absence of certified standards for HMAN and deoxy-CYN the identity was checked by comparing the fragment ion spectra with those reported by Furey et al. (2003) for HMAN and by Seifert et al. (2007) for deoxy-CYN. The collision energy used for fragmentation was 22 eV for each toxin. HMAN concentrations were calculated using the AN-fumarate calibration curves. Deoxy-CYN was quantified by measuring the fragmentation product of the $[M+H]^+$ ion 400 *m/z* → 194 *m/z* as previously described by Li et al. (2001).

2.4. Ecotoxicity tests

First range finding tests were conducted to define the range of concentrations that evolved 0% and 100% inhibition/mortality in test subjects. Accordingly, for *T. platyurus* and *D. magna* the ACT and PCC 6506 extracts were tested at concentrations within the 0.005 – 2 and 0.005 - 5 mg biomass dw $mL^{-1}$ concentration range, while the AQS extract was tested at one order of magnitude lower concentrations. The *D. rerio* embryo toxicity assays were run within the 20 – 500 µg biomass dw $mL^{-1}$ concentration range for ACT and PCC 6506 extracts, while the AQS extract was tested within the 10 – 125 µg biomass d.w. $mL^{-1}$ range. Each extract was tested at least in 5 – 10 concentrations.

Toxicity to the anostracean crustacean *T. platyurus* was assessed according to the Standard Operational Procedure of the Thamnotoxkit F™ (1995) assay kit (purchased from MicroBioTests INC., Nazareth, Belgium). Second to III instar larvae hatched from dormant eggs were exposed in duplicate to serial dilutions (dilution factor of 2) of cyanobacterial aqueous extracts in wells of 24-well culture plates (2.5 mL/well; 10 animals/well), and incubated at 25 ºC for 24 h in the dark. In each multiwell plate four control replicates were run in standard media. The test endpoint considered was mortality.

The *D. magna* immobilization test was performed using the Daphtoxkit F™ (MicroBioTests INC., Nazareth, Belgium). Neonates (24h old) transferred in a multiwell plate system (10 mL/well; 5 animals/well) were exposed in four replicates for each of five serial dilutions of cyanobacterial extracts (dilution factor of 2) at 20 ºC, in darkness, for 24 h, after which immobility was recorded. In each multiwell plate four control replicates were run in standard media.

The short-term *D. magna* feeding inhibition assay was run in 24-well culture plates, as described by Kovács et. al (2012). Each well was filled with 10 mL of cyanobacterial extracts dilutions similar to those used in the *D. magna* immobilization experiment. Neonates were

exposed at 20 °C in darkness for periods of 1 h and 2 h. Immediately after incubation, 0.2 ml of a suspension of red polystyrene microspheres (5μm particle size) (MicroBiotest Inc., Ghent, Belgium) was added to each well. Daphnids were allowed to feed for 30 min at 20 °C in darkness. After incubation a fixative (MicroBiotest Inc., Ghent, Belgium) was added, and dead neonates were visually assessed under stereomicroscope. Measurable end-point of the test was the number of daphnids with red microspheres in their digestive tracts versus the individuals with empty digestive tracts.

The *D. rerio* embryo toxicity test basically followed the draft OECD protocol "fish embryo toxicity (FET) test (OECD, 2006). The zebrafish (*D. rerio*) facility established at the "Toxi-Coop" Toxicological Research Center Zrt. (Hungary) provided the embryos used in this study as per the method prescribed by the OECD (2006) draft. Embryos (4- to 32-cell stage) were collected within 1 h post-fertilization (hpf), and exposed by direct immersion in wells of 6-well polypropylene plates (20 embryos per well) containing cyanobacterial extract dilutions prepared in fish breeding medium. For each plate an individual control well was included. Toxicity assays were run in duplicate, and each test was further triplicated. Approximately 90% of the exposure media was renewed daily (as presence of easily degradable metabolites could be expected). A test was considered valid if more than 70% of the controls (evaluated individually for each plate) successfully hatched and showed neither sub lethal nor lethal effects. Scoring of effects was done upon the $LC_{50}$ for cumulative mortality at 48 hpf and $EC_{50}$ for hatch rate at 96 hpf. Morphological alterations of embryos were evaluated at 24, 48, 72 and 96 hpf by means of a light microscope according to the general morphology score system (Kimmel et al., 1995; OECD, 2006). The characteristics evaluated included the presence of fully developed somites, tail detachment, heartbeat, circulation, appropriate pigmentation, spontaneous movement, development retardation, edemas, malformation and hatching. Morphological alterations were considered as fingerprint endpoints, if the concentration-response relationship was statistically significant.

### 2.5. Statistics

In all tests individual exposure concentrations were finally assessed in a minimum of 6 replicates for the *D. rerio* embryo developmental assay, or in 8 – 12 replicates for the crustacean assays (within 2 to 3 individual experiments). Statistical analyses were carried out using analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Statistical significance was accepted at $p \leq 0.05$ for all assays. Mean effective/lethality ($EC_{50}$/$LC_{50}$) concentrations and corresponding 95% confidence intervals ($CI_{95}$) were determined by non-linear curve-fitting mainly according to a four parameter logistic model or, in the case of *D. rerio* 96h $EC_{50}$ hatching inhibition to a first order exponential decay model. Threshold concentrations were expressed in terms of mg dry weight of cells extracted per mL. Differences in threshold concentrations were considered significant when 95% confidence intervals did not overlap (Wheeler et al., 2006). Statistics and graphical plotting were performed using the OriginPro software package (8.6.0, OriginLab Corporation, USA).

## 3. Results

### 3.1. Cyanotoxins analysis

In the aqueous extracts of ACT *C. raciborskii* strains tested, neither anatoxins nor cylindrospermopsins were detected. Analysis of the AQS *C. raciborskii* extract confirmed the presence of both CYN and deoxy-CYN. Based on the chromatographic separation method used extracted ion chromatograms of the AQS extract featured retention times for CYN of 1.8 (consistent with the CYN standard used) and for deoxy-CYN of 2.8 min (Fig. 1A). LC-$MS^2$

fragmentation performed on the precursor ion $[M + H]^+$ of *m/z* 416.2 (CYN) produced a mass spectrum (Fig. 1 A2) containing all the characteristic fragments with similar relative intensities as those recorded for the CYN standard (336.2, 318.2, 274.1, 194.1, 176.1). Fragmentation of the precursor ion $[M + H]^+$ of *m/z* 400.2 produced a mass spectrum containing all the characteristic deoxy-CYN fragments (320.1, 302.1, 274.1, 194 and 176) (Fig. 1 A1). The concentration of CYN and deoxy-CYN in the AQS *C. raciborskii* strain was estimated to be 8.31 and 0.52 mg $g^{-1}$ dry weight cells.

In the PCC 6506 extract we detected AN and HMAN only (Fig. 1B). The characteristic peaks for anatoxins in this extract appeared in the extracted ion chromatogram at the retention time of 3.0 min for AN (consistent with the AN standard used) and 4.2 min for HMAN (Fig. 1B). The identity of both anatoxins was justified by the $MS^2$ fragmentation of the two precursor ions $[M + H]^+$ of *m/z* 166 (AN) and *m/z* 180 (HMAN). Fragmentation of anatoxin-a of the sample extract gave major ions at *m/z* 149.0, 130.9, 107.0 that shows good agreement with the $MS^2$ mass spectrum of AN standard (Fig. 1 B1), while fragmentation of HMAN resulted a mass spectrum (Fig. 1 B2) containing the major fragment ions at *m/z* 163.0, 145.0, 135.1 with similar relative intensities as reported by Furey et al. (2003). The concentration of AN and HMAN of the PCC 6506 strain was estimated as 0.051- and 1.203 mg $g^{-1}$ dry weight cells.

3.2. Acute toxicity

In the crustacean lethality/immobilization assays mortality in controls was in all experiments below 5%, therefore the requirement of assay procedures for a valid test was always met.

Sigmoidal responses of % inhibition/lethality versus concentration were obtained for all cyanobacterial extracts within each bioassay. The estimated $EC_{50}/LC_{50}$ values with corresponding 95% confidence limits generated in ($CI_{95}$) the *T. platyurus* and *D. magna* bioassays are presented in Table 1.

In the *T. platyurus* 24h lethality assay, the $LC_{50}$ thresholds for the ACT *C. raciborskii* extracts varied within the 0.031 – 0.084 mg d.w. $mL^{-1}$ range with the ACT 9502 and ACT 9504 extract exerting significantly higher inhibition. Of the cyanobacteria strains used as positive references the PCC 6506 extract yielded an $LC_{50}$ value of 0.045 mg d.w. $mL^{-1}$, while the AQS extract exhibited the highest inhibition with an $LC_{50}$ threshold of 0.0026 mg d.w. $mL^{-1}$.

Similar differences in the toxicity potential of extracts were recorded also in the *Daphnia magna* 24 h immobilization tests. Irrespective of exposure duration, $EC_{50}$ feeding rate endpoint estimates of *D. magna* revealed fairly good positive correlation with the 24h-$LC_{50}$ data determined in the 24h *D. magna* immobilization tests. For ACT *C. raciborskii* extracts the 1h-$EC_{50}$s (1.06-2.4 mg d.w. $mL^{-1}$) and 2h-$EC_{50}$s (0.16-0.35 mg d.w. $mL^{-1}$) spanned a relatively narrow around 2-fold range with $CI_{95}$ overlapping that suggest similar toxicity potential for these isolates.

Of the positive references, the PCC 6506 extract exerted significantly higher inhibition only in the 1h exposure regime (by a factor of 1.6-3.5) compared to ACT extracts, while for the AQS *C. raciborskii* extract the 1h-$EC_{50}$ and 2h-$EC_{50}$ were 0.04 and 0.02 mg d.w. $mL^{-1}$ respectively. In the feeding experiments, for each cyanobacterial extract a strong influence of exposure duration was observed. Considering the ratios between 1h-$EC_{50}$/2 h-$EC_{50}$ highest influence of exposure time was observed for the ACT strains (5.8 – 6.9 fold increase in inhibition), followed by the PCC 6506 extract (4.5 fold), while the weakest time effect was observed for the AQS extract (2 fold).

In the *D. rerio* embryo toxicity assay the cumulative mortality and incidences of morphological malformations in controls at 96 hpf was in all experiments below 30%.

Therefore the requirement of the draft OECD protocol "fish embryo toxicity" (FET) test (OECD, 2006) for a valid test was always met and the observed effects could be attributed to the bioactive effects exerted by cyanobacterial extracts. Irrespective of algal species, cyanobacterial extracts have caused the mortality of embryos almost exclusively in the first 24 h of exposure along with significant dose-dependent development retardation. In few cases, some embryos which revealed development retardation within the first 24 h exposure could catch up this delay in embryogenesis until 96 hpf. The mean $LC_{50}$ for mortality at 48 hpf and $EC_{50}$s for hatch rate at 96 hpf with corresponding 95% CI are presented in Fig. 2. Overall, the 48h-$LC_{50}$s and 96h-$EC_{50}$s for *D. rerio* spanned relatively narrow 2.1-fold and 3.4-fold ranges with $CI_{95}$s in general overlapping (48h $LC_{50}$: 0.05 – 0.11 mg d.w. $mL^{-1}$; 96h $EC_{50}$: 0.02 – 0.08 mg d.w. $mL^{-1}$) (Fig 2.). However, both the cumulative lethality thresholds (48h-$LC_{50}$) and the effective concentrations influencing the hatching success (96h-$EC_{50}$), indicated higher toxicity to *D. rerio* embryos for the AQS *C. raciborskii* extract.

Fig. 2.

No significant morphological alterations were observed for embryos in the control groups during experiments (Fig. 3 A1, A2, A3). The most prevalent sublethal developmental effect observed upon exposure to ACT *C. raciborskii*- and the PCC 6506 extracts was the particular inhibition of embryogenesis at the end of the gastrula period (Fig. 3 B1, C1, E1) and this effect was relevant even below the $LC_{50}$ threshold concentrations. Of particular importance was the significant delay in the formation of somites for the ACT 9502 and ACT 9504 strains (Table 2.). However, affected embryos continued embryogenesis (Fig. 3 B2, C2, E2) but were significantly slowed compared to the normally developing ones and the majority succeeded to hatch by the 6 dpf time point (data not shown). Embryos incubated in the AQS extract were typically halted in development in the segmentation period (Fig. 3 D1) while embryos succeeding to pass this stage consistently developed severe pericardial and peritoneal edemas that persisted even post hatching (Fig. 3 D2, D3; Table 2.).

Fig. 3.

By 4 dpf embryos affected upon exposure to the ACT *C. raciborskii* and the PCC 6506 *Oscillatoria* extracts reached the complete embryogenesis and appeared similar to normally developing embryos (Fig. 3 inserts b, c), however the rate of embryos finally hatched was always significantly lower (by 30 – 40%) than the percent of embryos that have apparently normally developed throughout the 96 h exposure. Embryos exposed to extracts of AQS *C. raciborskii*, showing developmental malformation at 1-2 dpf survived the 96 h exposure period (Fig. 3 insert d), but none of the apparently affected embryos succeeded to hatch, moreover, even of the normally developing embryos approximately 15- 20% failed to hatch by the end of 96 h exposure (Table 2.).

Table 2.

## 4. Discussion

Long-term analysis of the biomass and community structure of the phytoplankton in Lake Balaton has revealed that *C. raciborskii* is one of the most prevalent cyanobacteria during the months of July to October (Hajnal and Padisák, 2008), systematically reaching cell densities of 25,000 – 31,000 cells $mL^{-1}$ (L. Vörös and J. Padisák, unpublished data). This level is rather close to the threshold of 20,000 cells $mL^{-1}$ set by the WHO as provisional guideline value for relative low probability of adverse health effects in recreational waters at which short-term adverse health outcomes, e.g. skin irritation and/or gastrointestinal illness might occur in less than 30% of the individuals exposed (WHO, 2003). Thus, evaluation of the toxicity potential of this cyanobacteria species is justified. Previous toxicological

investigations resulted in highly variable potencies in terms of lethality to mice: Underdal et al. (1999) reported protracted type effects following i.p. exposure of BOM:MNRI F mice (14 – 17 g) to 2900 – 3500 mg $kg^{-1}$ cyanobacterial cell suspensions (NIVA-CYA 225). Hiripi et al. (1998) recorded a 24 h $LD_{50}$ threshold of 230 mg $kg^{-1}$ on BalbC male mice (24 – 27 g) for lysed cell suspensions (ACT 9502), while Bernard et al. (2003), found the ACT 9502 extract (free of cellular debris) to be non-toxic against Male Swiss Albino mice (IOPS OFI strain) weighing 20 – 40 g at nominal doses ranging between 500 – 1000 mg $kg^{-1}$. We assume that the apparent controversies in assay results are most probably related to differences in assay methodology as: 1. the type of extract preparation prior injection (lysed cell suspension v.s. extracts devoid cellular debries); 2. the variation in weight and most probably also the condition of test organisms, as already noted by Humpage et al. (2012). Bioassays performed on molluscan neuronal models suggested the production of anatoxin-a like metabolites for several ACT *C. raciborskii* isolates, as revealed by the cholinergic blocking effects recorded by Kiss et al. (2002) and Vehovszky et al. (2009, 2012), but this fact was not analytically justified in these investigations. Moreover, recent cellular assays have outlined for the aqueous extracts of ACT *C. raciborskii* significant cytotoxicity as well as the ability to alter the actin filamental structure of CHO-K1 cells by the reduction of stress fibers and their shift to the perinuclear region (Antal et al., 2011). In this later study we also chemically screened the ACT *C. raciborskii* strains for the presence of CYN by HPLC/MS and all these algal materials proved to be free of this toxin.

Consequently, in this study we aimed at clarifying whether the ACT *C. raciborskii* are capable to synthesize anatoxins and, to evaluate the risks of ecotoxicological impact of this cyanobacteria genera.

Based on the results of chemical screening of the ACT *C. raciborskii* cyanobacterial materials performed so far, none of the toxins (cylindrospermopsins, anatoxins or saxitoxins) that could theoretically be expected to be synthesized according to their phylogeny have been ever detected. ACT *C. raciborskii* were previously checked for saxitoxins by fluorimetric analysis according to Gerdts et al. (2002) (data not shown). The reliability of our analytical screening was justified by the fairly good cyanotoxin quantifications in the extracts of cyanobacteria strains used as positive references. In the aqueous extract of the AQS strain in addition to CYN we have also detected also the deoxy- variant of this toxin. To our knowledge this is the first time when deoxy-CYN is reported in relation with the AQS *C. raciborskii* strain. Anatoxins were quantified in the PCC 6506 scum, as previously reported by Aráoz et al., (2005) and Mann et al. (2012). Accordingly, the neurological alterations previously described are caused by yet unidentified bioactive compounds synthesized by the ACT *C. raciborskii* strains.

Toxicity data ($EC_{50}$ and $LC_{50}$ for each endpoint) reflected very similar bioactivity potential between ACT *C. raciborskii* strains as revealed by the relatively narrow range that effective concentrations thresholds have spanned, and by corresponding confidence intervals overlapping within particular bioassays. Moreover, these threshold data suggest that the toxicity potential of the ACT *C. raciborskii* extracts is comparable to that of the anatoxins producer PCC 6506 *Oscillatoria* sp. isolate for these particular test organisms. All assays revealed highest toxicity for the cylindrospermopsins producer AQS *C. raciborskii* strain.

Comparing our ecotoxicity data with $EC_{50}$/$LC_{50}$ data reported in the literature for cyanobacterial aqueous extracts determined by the *T. platyurus* lethality assay ($LC_{50}$: 0.11 – 0.35 mg d.w. $mL^{-1}$) and by immobilization assays with *Daphnia* ($EC_{50}$: 1.1 – 6.4 mg d.w. $mL^{-1}$) (Marsalek and Blaha, 2004) the relative toxicities of the ACT *C. raciborskii* strains to crustaceans has to be considered as significant. The relatively low differences between the toxicities exerted on the *D. rerio* embryo development by the ACT *C. raciborskii* and the AQS extracts (approx. 2 fold) supports the findings published by Berry et al. (2009) that is

CYN does not permeate the chorion of embryos, and toxic effects observed are related to unidentified cyanobacterial metabolites. Similarly, Wang et al. (2006), have reported membrane impermeability also for microcystin-LR i.e. impaired delivery of the water-soluble toxin into the cytoplasm of *D. rerio* embryos. Our results are in line with previous findings suggesting that toxic effects recorded for complex cyanobacterial matrices in many routinely used bioassays cannot always be directly related to the production of some prevalent cyanobacterial toxins only (microcystins, cylindrospermopsins, anatoxins etc.). These effects may rather be caused by additional, less investigated toxic metabolites synthesized by cyanobacteria, or by metabolites that even if non-toxic themselves, can modify the effects of the priority toxins (Buryskova et al., 2006; Falconer, 2007; Oberemm et al., 1997; Pietsch et al., 2001).

Results of feeding inhibition experiments with *D. magna* basically outlined the same trend of toxicity potential as the lethality/immobilization assays, and moreover, revealed a distinct time-dependent difference in the toxicity potency of the ACT *C. raciborskii* and PCC 6506 *Oscillatoria* sp. as compared to the AQS *C. raciborskii*. Upon exposure to the AQS extract daphnids have practically stopped to graze as soon as they came in contact with the test material, while upon exposure to the other five extracts similar inhibition rate was achieved in a twice longer time span. The $EC_{50}$ thresholds and corresponding confidence intervals revealed significantly higher inhibiting efficiency for the PCC 6506 *Oscillatoria* sp. compared to the ACT *C. raciborskii* extracts, which might suggest a better predictive ability of anatoxins prevalence for the *D. magna* feeding inhibition assay compared to the classical crustacean based lethality/immobilization assays.

Apart from mortality rate, for the ACT *C. raciborskii* and PCC 6506 *Oscillatoria* sp. extracts the general morphological abnormality of the *D. rerio* embryo development was the relevant delay in the segmentation period in the first 24h exposure. For embryos exposed to the AQS *C. raciborskii* extract the prevalent toxicity pattern was the formation of yolk sac and cardiac edemas that persisted even post hatching.

The apparent poor sensitivity of the assay models used in this study most presumably relies on the fact that grazers and fish are adapted, in an evolutionary sense, to the presence of bioactive metabolites synthesized by cyanobacteria (Chorus and Bartram, 1999; Wilson et al., 2006). However, these bioassays are still useful tools in evaluating the potential hazards of cyanobacterial materials owing to their relative simplicity, low cost and fairly good predictive abilities and allow management decisions to be taken.

Acknowledgments

This work was granted by the Hungarian Scientific Research Fund (OTKA grant) K63451 and by a grant from the Balaton Project of the Office of the Prime Minister of Hungary (MEH) and EuLakes project (implemented through the CENTRAL EUROPE Programme co-financed by the ERDF). We thank "Toxi-Coop" Toxicological Research Center Zrt. who provided support by GOP-1.3.1-09/-2009-0010. Special thanks to Dr. Martin L. Saker for providing AQS *C. raciborskii* strain, and we thank Gábor Vasas, for the provision of purified cylindrospermopsin.

References

Antal, O., Karisztl-Gácsi, M., Farkas, A, Kovács, A., Ács, A., Törő, N., Kiss, Gy., Saker, M. L., Győri, J., Bánfalvi, G., Vehovszky, Á., 2011. Screening the toxic potential of Cylindrospermopsis raciborskii strains isolated from Lake Balaton, Hungary. Toxicon 57, 831–840.
Aráoz, R., Nghiem, H., Roppka, R., Palibroda, N., de Marsac, N.T., Herdman, M., 2005. Neurotoxins in axenic oscillatorian cyanobacteria: coexistence of anatoxin-a and homoanatoxin-a determined by ligand-binding assay and GC/MS. Microbiology 151, 1263–1273.
Bernard, C., Harvey, M., Briand, J.F., Biré, R., Krys, S., Fontaine, J.J., 2003. Toxicological comparison of diverse Cylindrospermopsis raciborskii strains: evidence of liver damage caused by a French C. raciborskii strain. Environ. Toxicol. 18, 176–186.
Berry, J.P., Gibbs, P.D.L., Schmale, M.C., Saker, M.L., 2009. Toxicity of cylindrospermopsin, and other apparent metabolites from *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*, to the zebrafish (*Danio rerio*) embryo. Toxicon 53, 289–299.
Briand, J.F., Leboulanger, C., Humbert, J.F., Bernard, C., Dufour, P., 2004. Cylindrospermopsis Raciborskii (cyanobacteria) invasion at mid-latitudes: selection, wide physiological tolerance, or global warming? J. Phycol. 40, 231–238.
Buryskova B, Hilscherova K, Babica P., Vrskova D, Marsalek B., Blaha, L. 2006. Toxicity of complex cyanobacterial samples and their fractions in Xenopus laevis embryos and the role of microcystins. Aquat. Toxicol. 80, 346-354.
Chorus, I., J. Bartram., 1999. Toxic Cyanobacteria in Water: a Guide to Public Health Significance, Monitoring and Management. Monitoring and Management. Für WHO durch E & FN Spon /Chapman & Hall, London, 416.
Falconer, I.R., 2007. Cyanobacterial toxins present in *Microcystis aeruginosa* extracts – More than microcystins!. Toxicon 50, 585-588.
Fastner, J., Heinze, R., Humpage, A.R., Mischke, U., Eaglesham, G.K., Chorus, I., 2003. Cylindrospermopsin occurrence in two German lakes and preliminary assessment of toxicity and toxin production of Cylindrospermopsis raciborskii (Cyanobacteria) isolates. Toxicon 42, 313–321.
Fastner, J., Rücker, J., Stüken, A., Preussel, K., Nixdorf, B., Chorus, I., Köhler, A., Wiedner, C., 2007. Occurrence of the cyanobacterial toxin cylindrospermopsin in northeast Germany. Environ. Toxicol. 22, 26–32.
Furey, A., Crowley, J., Shuilleabhain, A.N., Skulberg, O.M., James, K.J., 2003. The first identification of the rare cyanobacterial toxin, homoanatoxin-a, in Ireland. Toxicon 41, 297–303.
Gerdts G., Hummert C., Donner G., Luckas B., Schütt, C., 2002. A fast fluorimetric assay (FFA) for the detection of saxitoxin in natural phytoplankton samples. Mar. Ecol. Prog. Ser. 230: 29-34.
Hajnal, É., Padisák, J., 2008. Analysis of long-term ecological status of Lake Balaton based on the ALMOBAL phytoplankton database. Hydrobiologia 599, 227–237.
Hamilton, P.B., Ley, L.M., Dean, S., Pick, F.R., 2005. The occurrence of the cyanobacterium *Cylindrospermopsis raciborskii* in Constance Lake: an exotic cyanoprokaryote new to Canada. Phycologia 44(1), 17–25.
Hiripi, L., Nagy, L., Kalmár, T., Kovács, A., Vörös, L., 1998. Insect (Locusta migratoria migratorioides) test monitoring the toxicity of cyanobacteria. Neurotoxicology 19, 605–608.
Humpage, A., Falconer, I., Bernard, C., Froscio, S., Fabbro, L., 2012. Toxicity of the cyanobacterium *Limnotrix* AC0243 to male Balb/c mice. Water Res. 46, 1576–1583.
Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of Embryonic Development of the Zebrafish. Dev. Dynamics 203, 253–310.

Kiss, T., Vehovszky, Á., Hiripi, L., Kovács, A., Vörös, L., 2002. Membrane effects of toxins isolated from a cyanobacterium, Cylindrospermopsis raciborskii, on identified molluscan neurones. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 131, 167–176.
Kovács, A., Abdel-Hameid, B.A., Ács, A., Ferincz, Á., Kováts N., 2012. Novel protocol for assessing aquatic pollution, based on the feeding inhibition of Daphnia magna. Knowl. Managt. Aquatic Ecosyst. 404, 07, DOI: 10.1051/kmae/2012001
Li, R.H., Carmichael, W.W., Brittain, S., Eaglesham, G.K., Shaw, G.R., Noparatnaraporn, A.M.N., Yongmanitchai, W., Kaya, K., Makoto, W., 2001. Detection of Cylindrospermopsin from a strain of Cylindrospermopsis raciborskii (Cyanobacteria) isolated from Thailand. Toxicon 39, 973–980.
Marsalek, B., Blaha, L., 2004. Comparison of 17 biotests for detection of cyanobacterial toxicity. Environ. Toxicol. 19(4), 310-317.
Mazmouz, R., Chapuis-Hugon, F., Mann, S., Pichon, V., Méjean, A., Ploux, O., 2010. Biosynthesis of Cylindrospermopsin and 7-Epicylindrospermopsin in *Oscillatoria* sp. Strain PCC 6506: Identification of the cyr Gene Cluster and Toxin Analysis. Appl. Environ. Microbiol., 76(15), 4943-9.
Meriluoto, J.A.O., Spoof, L.E.M., 2008. Chapter 21: Cyanotoxins: sampling, sample processing and toxin uptake. In: Hudnell, H.K. (Eds.), Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs. Adv. in Exp. Med. and Biol. 619, 483–501.
Oberemm, A., Fastner, J., Steinberg, C., E.,W. 1997. Effects of microcystin-LR and cyanobacterial crude extracts on embryo-larval development of zebrafish (*Danio rerio*) Water Res. 31,: 2918-2921.
OECD, 2006. Fish Embryo Toxicity Test. Organization for Economic Co-Operation and Development.
Padisák, J., 1997. *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: worldwide distribution and review of its ecology, Archiv für Hydrobiologie/Suppl. 107, 563–593.
Pietsch, C., Wiegand, C., Ame, M.V., Nicklisch, A., Wunderlin, D., Pflugmacher, S., 2001. The effects of cyanobacterial crude extract on different aquatic organisms: evidence for cyanobacterial toxin modulating factors. Environ. Toxicol. 16, 535-542.
Présing, M., Heródek, S., Vörös, L., Kóbor, I., 1996. Nitrogen fixation, ammonium and nitrate uptake during a bloom of *Cylindrospermopsis racyborskii* in Lake Balaton. Arch. Hydrobiol. 136, 553–562.
Rippka R., DeReuelles J., Waterbury J.B., Herdman M., Stanier R.Y., 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111, 1–61.
Rippka, R., 1988. Isolation and purification of Cyanobacteria. *In*: Cyanobacteria eds: L. Packer, A.N. Glazer, Methods in Enzymology, 167. 3-28.
Saker M.L., Eaglesham G.K., 1999. The accumulation of cylindrospermopsin from the cyanobacterium *Cylindrospermopsis raciborskii* in tissues of the Redclaw crayfish *Cherax quadricarinatus*. Toxicon 37 (7), 1055-1077.
Saker M.L., Neilan B.A., 2001. Varied diazotrophies, morphologies, and toxicity of genetically similar isolates of *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) from Northern Australia. Appl. Environ. Microbiol. 67 (4), 1839-1845.
Saker, M.L., Nogueira, I.R., Vasconcelos, V.M., 2003. Distribution and toxicity of *Cylindrospermopsis raciborskii* (Cyanobacteria) in Portuguese freshwaters. Limnetica 22, 131–138.
Seifert, M., McGregor, G., Eaglesham, G., Wickamasinghe, W., Shaw, G., 2007. First evidence for the production of cylindrospermopsin and deoxy-cylindrospermopsin by the

freshwater benthic cyanobacterium, *Lyngbya wollei* (Farlow ex Gomont) Speziale and Dyck. Harmful Algae 6, 73–80.
Thamnotoxkit F. 1995. Crustacean toxicity screening test for freshwater. Standard Operational Procedure, Creasel, Deinze, Belgium. 23 p.
Underdal, B., Nordstoga, K., Skulberg, O.M., 1999. Protracted toxic effects caused by saline extracts of *Aphanizomenon flos-aquae* (Cyanophyceae/cyanobacteria). Aquat. Toxicol. 46, 269–278.
Vasas, G., Gáspár, A., Surányi, G., Batta, G., Gyémánt, G., Hamvas, M.M., Máthé, C., Molnár, É., Borbély, G., 2002. Capillary electrophoretic assay and purification of cylindrospermopsin, a cyanobacterial toxin from *Aphanizomenon ovalisporum*, by plant test (blue-green Sinapis test). Anal. Biochem. 302, 95–103.
Vehovszky, Á., Ács, A., Kovács, W.A., Szabó, H., Győri, J., Farkas, A., 2009. Isolated strains of Cylindrospermopsis raciborskii from Lake Balaton (Hungary) produce anatoxin-a like neurotoxins. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 153, S88.
Vehovszky, Á., Kovács, A.W.,Szabó, H., Győri, J., Farkas, A., 2012. Neurotoxic effects evoked by cyanobacterial extracts suggest multiple receptors involved in electrophysiological responses of molluscan (CNS, heart) models Acta Biol. Hung., 63 (S2) 160-170.
Wang, P.J., Chien, M.S., Wu, F.J., Chou, H.N., Lee, S.J., 2006. Inhibition of embryonic development by microcystin-LR in zebrafish, *Danio Rerio*. Toxicon 45, 303-308.
Wheeler, M.W., Park, R.M., Bailer, A.J., 2006. Comparing median lethal concentration values using confidence interval overlap or ratio tests. Enciron. Toxicol. Chem. 25, 1441–1444.
WHO, 2003. Guidelines for safe recreational water environments. Volume 1, Coastal and fresh waters. WHO Library Cataloguing-in-Publication Data. ISBN 92 4 154580 1. Geneva, Switzerland: World Health Organization.
Wilson, A. E., O. Sarnelle, and A. R. Tillmanns, 2006. Effects of cyanobacterial toxicity and morphology on the population growth of freshwater zooplankton: Meta-analyses of laboratory experiments. Limnol. Oceanogr. 51(4), 1915–24.

Figure legends

Fig. 1. Extracted ion chromatograms (EIC) and ESI-$MS^2$ fragmentation pattern of toxins detected in the AQS *C. raciborskii* and PCC 6506 *Oscillatoria* sp. extracts compared to CYN and AN standards. EIC chromatogram of the AQS extract (black line) and of CYN standard (red line) (A). ESI-$MS^2$ fragmentation patterns of the $[M + H]^+$ peaks at *m/z* 400.0 (deoxy-CYN) (A1) and at *m/z* 416.0 (CYN) (A2) detected in the AQS extract. EIC chromatogram of the PCC 6506 extract (black line) and of AN standard (red line) (B). ESI-$MS^2$ fragmentation patterns of the $[M + H]^+$ peaks at *m/z* 166.0 (AN) (B1) and at *m/z* 180.0 (HMAN) (B2) detected in the PCC 6506 extract.

Fig. 2. Embryo toxicity of cyanobacterial extracts: (A) Lethal concentration of 50% ($LC_{50}$) for cumulative mortality at 48 h post fertilization (hpf) and corresponding 95% confidence interval ($CI_{95}$); (B) Effect concentration of 50% ($EC_{50}$) for hatching inhibition at 96 h post fertilization (hpf) and corresponding 95% confidence interval ($CI_{95}$). Mean of three independent experiments with duplicated test concentrations.

Fig. 3. Effects of cyanobacterial extracts on the embryonic development of *D. rerio*: Controls: A1 – spontaneously moving embryos (1 dpf), A2- pigmentation completed (2 dpf), A3 – hatched eleuthero embryos (4 dpf). Exposure to 0.125 mg $mL^{-1}$ ACT 9502 extract: B1 – 25% of embryos in the initial phase of segmentation, B2 – initiation of pigmentation at 2 dpf, B3 – 25% embryos hatched at 4dpf (insertion “b” – non-hatched apparently healthy embryo). Exposure to 0.125 mg $mL^{-1}$ ACT 9504 extract: C1 – 22% of embryos in the 90% of epiboly at 1 dpf, C2 – initiation of pigmentation at 2 dpf, C3 – 27% of embryos hatched at 4 dpf (insertion “c” – 30% of apparently healthy embryo still lacking pigmentation). Exposure to 0.075 mg $mL^{-1}$ AQS extract: D1 – 40% of embryos in the initial phase of segmentation at 1 dpf, D2 – 15% of embryos still lacking pigmentation and with severe pericardial- and yolk sac edemas at 2 dpf, D3 – hatched eleuthero embryo only by the six$^{th}$ dpf with persisting pericardial and yolk sac edemas (insertion “d” – at 4 dpf none of the exposed embryos succeeded to hatch). Exposure to 0.125 mg $mL^{-1}$ PCC 6506 extract: E1 – 25% of embryos in the 75% of epiboly at 1dpf, E2 – initiation of pigmentation at 2 dpf, E3 – 36% of embryos hatched at 4 dpf.

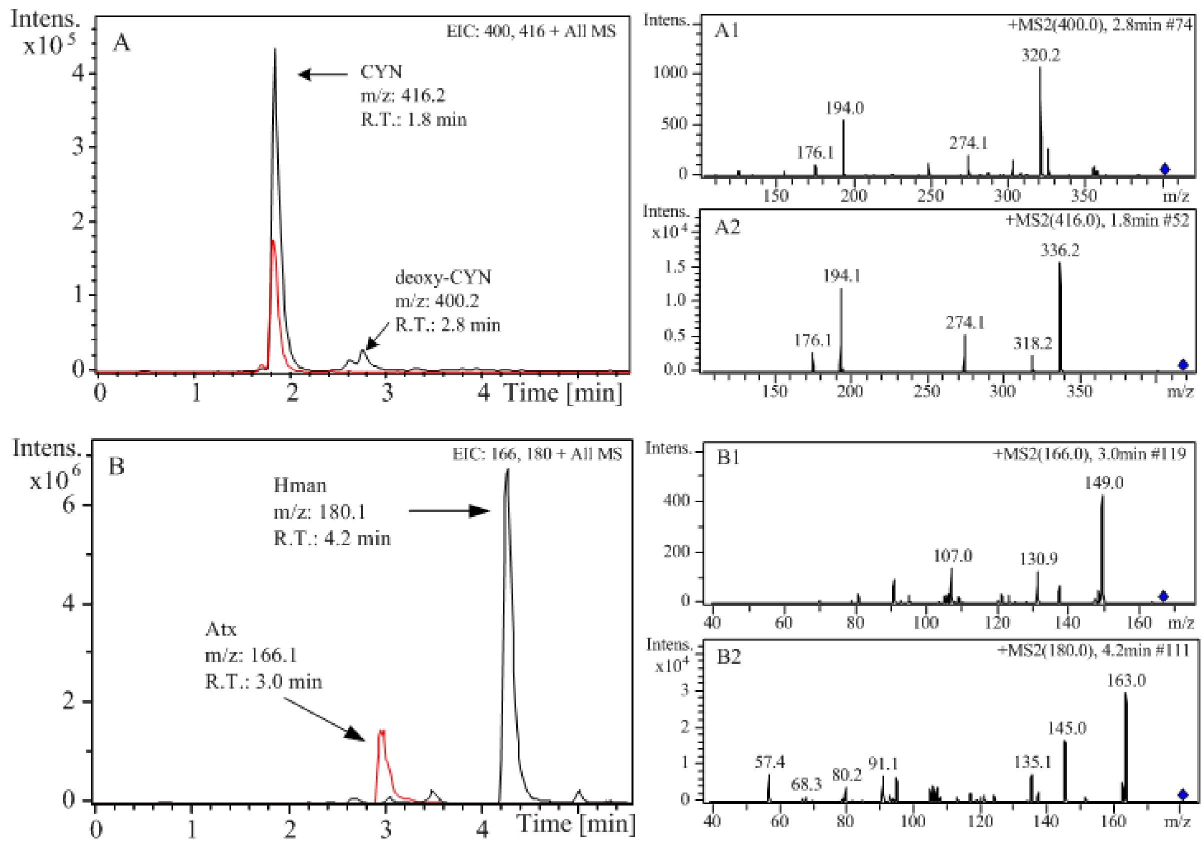


**Fig. 1.**

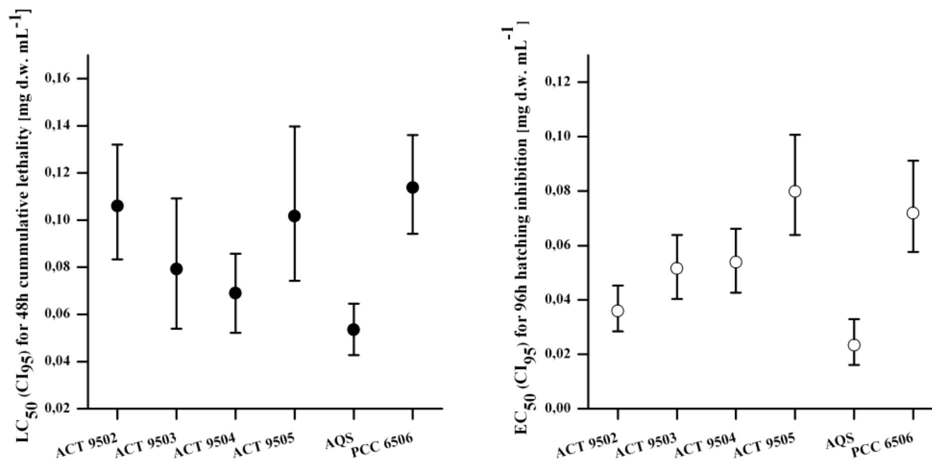


**Fig. 2.**

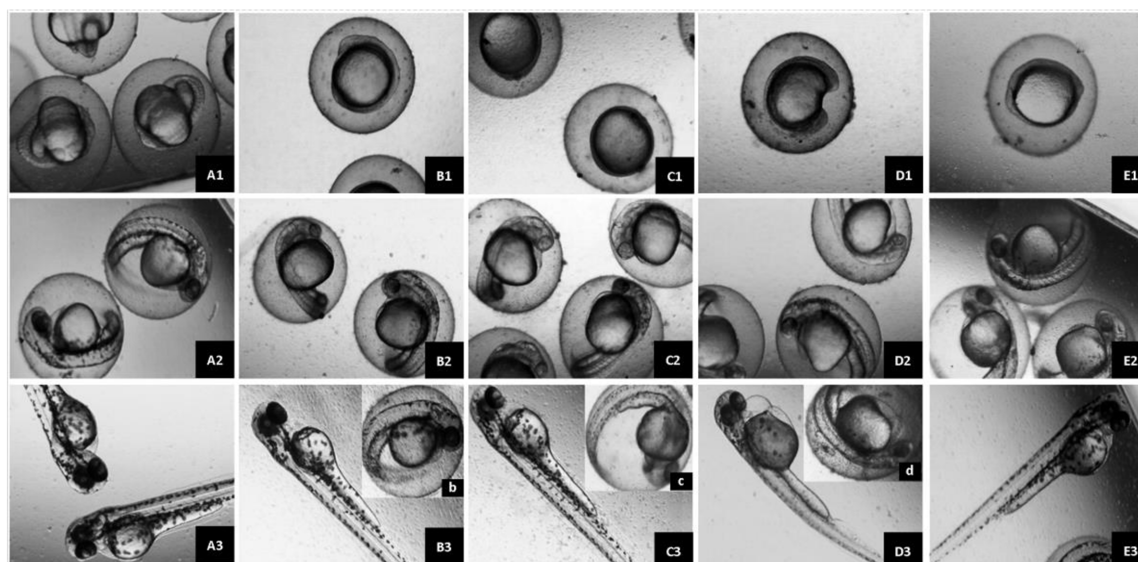


**Fig. 3.**

Table 1. Ecotoxicity data for cyanobacterial extracts on selected species and endpoints.

| Cyanobacteria | $EC_{50}$ or $LC_{50}$ (mg algal mass d.w. $mL^{-1}$) – $CI_{95}$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Thamnocephalus platyurus* 24 h immobilization | | *Daphnia magna* 24 h immobilization | | *Daphnia magna* 1 h feeding inhibition | | *Daphnia magna* 2 h feeding inhibition | |
| | $LC_{50}$ | $CI_{95}$ | $LC_{50}$ | $CI_{95}$ | $EC_{50}$ | $CI_{95}$ | $EC_{50}$ | $CI_{95}$ |
| ACT 9502 | 0,031 | 0.028-0.037 | 2.20 | 2.01-2.60 | 1.46 | 1.24-1.78 | 0.25 | 0.10-0.34 |
| ACT 9503 | 0,084 | 0.078-0.093 | 2.39 | 1.79-2.61 | 1.67 | 1.34-2.10 | 0.27 | 0.11-0.37 |
| ACT 9504 | 0,061 | 0.052-0.074 | 1.52 | 1.01-1-91 | 1.06 | 0.86-1.31 | 0.16 | 0.08-0.20 |
| ACT 9505 | 0,035 | 0.030-0.042 | 4.21 | 3.86-4.33 | 2.41 | 1.92-3.05 | 0.35 | 0.13-0.39 |
| AQS | 0,0026 | 0.002-0.0032 | 0.13 | 0.09-0.19 | 0.04 | 0.032-0.058 | 0.016 | 0.004-0.027 |
| PCC 6506 | 0,045 | 0.037-0.052 | 1.31 | 1.09-1.69 | 0.67 | 0.41-0.97 | 0.15 | 0.09-0.20 |

Table 2. Summary of developmental abnormalities observed during the embryogenesis of *D. rerio* upon exposure to cyanobacterial extracts

| Cyanobacteria | Somites | Tail detachment | Heartbeat | Circulation | Spontaneous movement | Pigmentation | Edemas | Delayed hatching |
|---|---|---|---|---|---|---|---|---|
| ACT 9502 | ++ | + | + | + | + | ++ | - | ++ |
| ACT 9503 | + | - | + | - | - | - | - | ++ |
| ACT 9504 | ++ | ++ | + | + | + | ++ | - | ++ |
| ACT 9505 | + | - | - | - | - | - | - | + |
| AQS | - | ++ | + | + | - | - | +++ | +++ |
| PCC 6506 | + | - | + | + | ++ | + | - | + |

The severity of observed alterations were classified as (-) no alterations; (+) slight alterations for statistically significant incidences of failure less than 25% observed at exposure concentrations above the $LC_{50}$ threshold; (++) moderate alterations for statistically significant incidences of failure less than 25% observed at exposure concentrations below the $LC_{50}$ threshold; (+++) severe alterations for incidences above 25% at exposure concentrations below the $LC_{50}$ threshold.