



Endocrine, teratogenic and neurotoxic effects of cyanobacteria detected by cellular *in vitro* and zebrafish embryos assays



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HIGHLIGHTS

- Retinoid-like activity newly identified in two cyanobacterial species.
- Estrogenic and retinoid-like activity can occur simultaneously in cyanobacteria.
- Teratogenicity of cyanobacteria in zebrafish likely associated with retinoids.
- Mixture toxicity probably masked estrogenicity in transgenic fish.
- Cyanobacteria affected the locomotion of zebrafish embryos.

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ABSTRACT

Cyanobacteria contain various types of bioactive compounds, which could cause adverse effects on organisms. They are released into surface waters during cyanobacterial blooms, but there is little information on their potential relevance for effects *in vivo*. In this study presence of bioactive compounds was characterized in cyanobacteria *Microcystis aeruginosa* (Chroococcales), *Planktothrix agardhii* (Oscillatoriales) and *Aphanizomenon gracile* (Nostocales) with selected *in vitro* assays. The *in vivo* relevance of detected bioactivities was analysed using transgenic zebrafish embryos tg(*cyp19a1b*-GFP). Teratogenic potency was assessed by analysis of developmental disorders and effects on functions of the neuromuscular system by video tracking of locomotion. Estrogenicity *in vitro* corresponded to 0.95–54.6 ng estradiol equivalent (g dry weight (dw))⁻¹. In zebrafish embryos, estrogenic effects could not be detected potentially because they were masked by high toxicity. There was no detectable (anti)androgenic/glucocorticoid activity in any sample. Retinoid-like activity was determined at 1–1.3 µg all-trans-retinoic acid equivalent (g dw)⁻¹. Corresponding to the retinoid-like activity *A. gracile* extract also caused teratogenic effects in zebrafish embryos. Furthermore, exposure to biomass extracts at 0.3 g dw L⁻¹ caused increase of body length in embryos. There were minor effects on locomotion caused by 0.3 g dw L⁻¹ *M. aeruginosa* and *P. agardhii* extracts. The traditionally measured cyanotoxins microcystins did not seem to play significant role in observed effects. This indicates importance of other cyanobacterial compounds at least towards some species or their developmental phases. More attention should be paid to activity of retinoids, estrogens and other bioactive substances in phytoplankton using *in vitro* and *in vivo* bioassays.

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1. Introduction

Blooms of cyanobacteria have become a serious problem in surface waters throughout the world. Their occurrence is associated with poor water quality, accumulation of biomass and low content of oxygen in water (Wiegand and Pflugmacher, 2005). Further-

more, cyanobacteria produce a wide spectrum of substances, some of which can cause various adverse effects on organisms (Kuiper-Goodman et al., 1999). Cyanobacterial toxins are categorised into five functional groups: hepatotoxins, neurotoxins, cytotoxins, dermatotoxins and irritant toxins (Wiegand and Pflugmacher, 2005). The hepatotoxic microcystins have been investigated in the greatest detail (Bláha et al., 2009). Great attention has also been paid to the diverse group of neurotoxins produced by cyanobacteria (Aráoz et al., 2010). Effects of complex blooms often cannot be attributed

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solely to the activity of individual cyanotoxins (Berry et al., 2009, 2007; Oberemm et al., 1997; Bláha et al., 2009). This could be due to the effect of unknown substances and/or the mutual interactions of the mixture components and environmental factors.

Recent results have indicated the ability of compounds produced by cyanobacteria to interfere with signalling of several intracellular receptors, which play important roles in physiological processes and are of relevance for potential adverse effects in vertebrates including humans (Klejdus et al., 2010; Kaya et al., 2011; Rogers et al., 2011; Wu et al., 2013, 2012). Signalling pathways, in which these receptors are engaged, play roles in hormonal regulation, reproduction and development of vertebrates (Janosek et al., 2006). Results of several studies have indicated the presence of estrogenic compounds in cyanobacteria (Klejdus et al., 2010; Stěpánková et al., 2011; Rogers et al., 2011). Furthermore, a potential interference of compounds from cyanobacterial blooms with androgen receptor signalling has been observed (Stěpánková et al., 2011). However, there is little information on potential of cyanobacterial compounds to affect signalling of other important endocrine receptors, such as glucocorticoid receptors that regulate genes controlling development, metabolism, stress and immune response (Odermatt and Gummy, 2008).

Recently, retinoic acid derivatives were identified by chemical analysis in cyanobacterial blooms from Tai Lake, China, and in several laboratory cultures of cyanobacteria (Wu et al., 2013, 2012). Extracts of a few cyanobacteria were shown to exhibit retinoid-like activity in a yeast reporter gene assay (Kaya et al., 2011). Retinoic acid (RA) signalling is crucial for normal vertebrate development and highly conserved among different species (Rhinn and Dollé, 2012). However, RAs are potent teratogens (Selderslaghs et al., 2009) when normal physiological concentrations are exceeded. Hence, the gross malformations reported for zebrafish embryos exposed to crude extracts of cyanobacteria *Microcystis aeruginosa*, *Anabaena flos-aquae*, *Cylindrospermopsis raciborskii* and *Aphanizomenon flos-aque* (Oberemm et al., 1997; Berry et al., 2009; Ghazali et al., 2009; Acs et al., 2013) might be related to the presence of retinoids. These malformations could not be explained by the known toxins considered in these studies, such as microcystins or cylindrospermopsin (Oberemm et al., 1997; Berry et al., 2009; Acs et al., 2013).

The objective of this study was to investigate extracts of biomass from several cyanobacterial species for the presence of bioactive compounds *in vitro* and *in vivo*, using reporter cell assays and zebrafish embryos. This approach aimed to determine the relevance of the detected *in vitro* bioactivity for *in vivo* situation. Several *in vitro* cellular reporter assays were used to examine estrogenic, retinoid-like, anti/androgenic and glucocorticoid activity. Correspondingly, estrogenic activity was also assessed by a transgenic zebrafish strain tg(*cyp19a1b*-GFP). In order to identify teratogenic effects possibly related to retinoid-like compounds the frequency of malformations was analysed. Potential interference with neuromuscular development and function was assessed in zebrafish embryos using a locomotion analysis. The selection of cyanobacterial species for testing was based on our previous results which indicated endocrine disrupting potency of biomass extracts (Stěpánková et al., 2011) and designed to represent different cyanobacterial orders. The test species included cyanobacteria *M. aeruginosa* (Chroococcales), *Planktothrix agardhii* (Oscillatoriales) and *Aphanizomenon gracile* (Nostocales).

2. Materials and methods

2.1. Preparation of cyanobacterial samples

The source and characteristics of cyanobacterial strains used in this study are given in Table 1. Cyanobacteria were cultured as

described previously (Nováková et al., 2013). Details of cultivation and preparation of samples for testing are given in Supplementary Materials (Section S1).

Ultrasound was used to extract 200 mg of lyophilized biomass with 6 mL 75% MeOH. The final extract was centrifuged and the debris re-extracted with 2×2 mL 75% MeOH. Organic compounds in samples were pre-cleaned and concentrated by solid phase extraction (SPE) using Oasis HLB and Carbograff cartridges. Eluates from both columns were pooled to obtain maximal recovery. Concentrations of microcystins were determined as previously described (Bláhová et al., 2008).

2.2. *In vitro* estrogenic, (anti-)androgenic, glucocorticoid and retinoid-like activity

Complete description of the used bioassays and testing procedures is given in Supplementary Materials (Section S2). Reporter gene assays stably transfected with luciferase gene under control of estrogen-, androgen-, glucocorticoid- and retinoid-receptor activation, respectively, were used to assess the interference of the samples with signalling of the endogenous ligands. All *in vitro* assays were performed in 96 well microplates. Cells were exposed for 24 h to cyanobacterial biomass extracts in the concentration range of 0.03125–2 g dw L⁻¹, calibration standards, blanks and solvent controls. Cytotoxicity of samples was assessed using two fluorescent indicator dyes (Schirmer et al., 1997). The activity of induced reporter luciferase was measured using luciferase substrate.

2.3. *In vivo* experiments with zebrafish embryos

Experiments with zebrafish embryos were performed at the UFZ Leipzig using the transgenic zebrafish strain tg(*cyp19a1b*-GFP) (Tong et al., 2009; Brion et al., 2012). The strain was kindly provided by O. Kah, University of Rennes and was crossed to the in-house wild-type strain UFZ-OBI prior to use. Details on zebrafish culture and embryo production as well as on exposure experiments are included in Supplementary Materials (Section S3.1–S3.2). Zebrafish embryos at the stage of 24hpf were exposed to extracts of biomass prepared in methanol. Extracts were added to the test vessels and methanol was allowed to evaporate. Standard test medium (ISO, 2008) was added to the exposure dishes immediately after methanol evaporation. Exposure media were mixed by gentle agitation, briefly ultrasonicated and mixed again. The exposure was conducted for 96 h at 26 ± 1 °C and a photoperiod 12 h light: 12 h dark. Exposure media were replaced after 48 h. Dissolved oxygen (Fibox 3 trace, PreSens, Germany) and pH were recorded at the beginning and end of each exposure interval.

Due to limited availability of biomass, an initial screening experiment for reduction of oxygen levels, mortality and malformations was conducted. The screening concentrations were selected based on previous experience and literature data, which have indicated lower oxygen content at greater biomass concentrations (Burýšková et al., 2006). Based on this screening appropriate concentrations for further detailed assessment were defined. For screening, fish embryos were exposed in 6 mL glass vials containing 2 mL exposure medium. Biomass concentrations of 0.3, 1, 3 and 10 g dw L⁻¹ were tested for each species. As a positive control embryos were exposed to 1 nM ethinylestradiol (EE2). The vials were incubated on a shaker to promote oxygen exchange. The percentage of dead and malformed embryos was assessed daily. The induction of GFP reporter fluorescence was measured at the end of exposure at 120hpf.

Based on the results of the screening test detailed test was carried out in three independent replicated experiments conducted on different days. Twenty embryos were exposed per replicate and

Table 1List of cyanobacterial strains used in this study, their origin, cyanotoxin content and total retinoid (REQ in ng ATRA (g dw)⁻¹) and estrogenic equivalents (EEQ in ng E2 (g dw)⁻¹).

Order Species	Source ^a	Place of origin		MCs concentration (μg g ⁻¹) ^b			Relative equivalent <i>in vitro</i>	
		Country	Water body	MC-RR	MC-YR	MC-LR	ng ATRA (g dw) ⁻¹	ng E2 (g dw) ⁻¹
Oscillatoriales								
<i>Planktothrix agardhii</i>	CCALA 159	Germany	Lake Plussee	n.d.	22.9	7.8	1163	54.6
Nostocales								
<i>Aphanizomenon gracile</i>	RCX06 ^c	Ireland	Lake Lough Neagh	n.d.	n.d.	n.d.	1322	1.2
Chroococcales								
<i>Microcystis aeruginosa</i>	PCC 7806	Netherlands	Reservoir Braakman	n.d.	n.d.	325	1092	0.95

^a Culture collection ID for laboratory cultured strains: CCALA – Culture Collection of Autotrophic Organisms, Institute of Botany, Academy of Sciences of the Czech Republic; RCX – RECETOX Culture Collection of Cyanobacteria and Algae; PCC – Pasteur Culture Collection of Cyanobacteria.

^b Method limit of detection was 5 μg (g dw)⁻¹; MCs – microcystins; n.d. – not detected (below limit of detection).

^c This species originates from CCALA (strain 008), but has been long-term cultivated at RECETOX.

test concentration in exposure dishes in 20 mL exposure medium. Each independent experiment included three negative controls (test medium) and three positive controls (1 nM EE2). In the detailed test, hatching, teratogenicity and mortality were assessed daily. Locomotion, length and estrogenic effects were evaluated at the end of the exposure.

Details on the methods for evaluating these parameters as well as on conducted data analyses are included in [Supplementary Materials \(Section S3.3–S4\)](#).

3. Results

A. gracile did not contain detectable levels of microcystins. Microcystin-LR was detected in *M. aeruginosa*, microcystin-LR and -YR in *P. agardhii* (Table 1).

The *in vitro* assay revealed concentration-dependent retinoid receptor mediated activity in the biomass extracts of all tested cyanobacteria (Fig. 1A). Similar potencies were detected for all three species (Table 1). The LOEC for retinoid-like activity was in the range of 0.25–0.5 g dw L⁻¹ for the three cyanobacterial species.

Estrogenic potency was detected in all samples (Fig. 1B and Table 1). The greatest estrogenicity was observed for *P. agardhii*. Approximately 50-fold lower concentrations of estrogen equivalents were detected for *M. aeruginosa* and *A. gracile*. The LOECs for the detection of an estrogenic response ranged from 0.03 g dw L⁻¹ (*P. agardhii*) to 1 g dw L⁻¹ in case of *M. aeruginosa* and *A. gracile*. No androgenic, glucocorticoid or antiandrogenic activity was detected in any of the tested biomass extracts up to the highest tested concentration (2 g dw L⁻¹).

The initial screening test revealed 100% mortality in embryos exposed to extracts of *P. agardhii* or *M. aeruginosa* at 3 or 10 g dw L⁻¹ or to 10 g dw L⁻¹ of the extract of *A. gracile*. Since mortality was observed during the first 24 h after initiation of exposure, when oxygen levels in these treatments dropped to 5–31%, it might have been associated with hypoxia. However, the mortality rate of 20% at concentrations of 1 g dw L⁻¹ of *M. aeruginosa* occurred at oxygen levels greater than 80% saturation, which indicates that components in the biomass caused reduced survival. No teratogenic effects were observed in embryos exposed to the least concentration (0.3 g dw L⁻¹) of all samples and in 1 g dw L⁻¹ *P. agardhii*. Deformities of the tip of the tail were observed in zebrafish embryos exposed to all other extracts at 1 and 3 g dw L⁻¹. Extracts of *A. gracile* at 3 g dw L⁻¹ also caused edema in hearts of all embryos (Table S1).

A potential estrogenic activity was indicated in the screening experiment by greater GFP fluorescence in comparison to controls (>1) for the extracts of *A. gracile*, *P. agardhii* and *M. aeruginosa* at 0.3 g dw L⁻¹ and *A. gracile* at 1 g dw L⁻¹. At the highest sublethal test concentrations, however, i.e. *A. gracile* at 3 g dw L⁻¹ and *M.*

aeruginosa at 1 g dw L⁻¹, significantly lower GFP fluorescence was observed indicating a potential toxic interference with GFP protein synthesis (Table S1).

Given the mortality and decrease in oxygen content and the fact that the screening test indicated the greatest estrogenicity at concentration 0.3 g dw L⁻¹, the maximum test concentration in the subsequent detailed testing was limited to a biomass concentration of 0.3 g dw L⁻¹ for *P. agardhii* and *M. aeruginosa* and to 0.3–3 g dw L⁻¹ for *A. gracile*.

In the detailed testing dissolved oxygen levels were greater than 90% saturation and pH was between 7 and 8 in all test

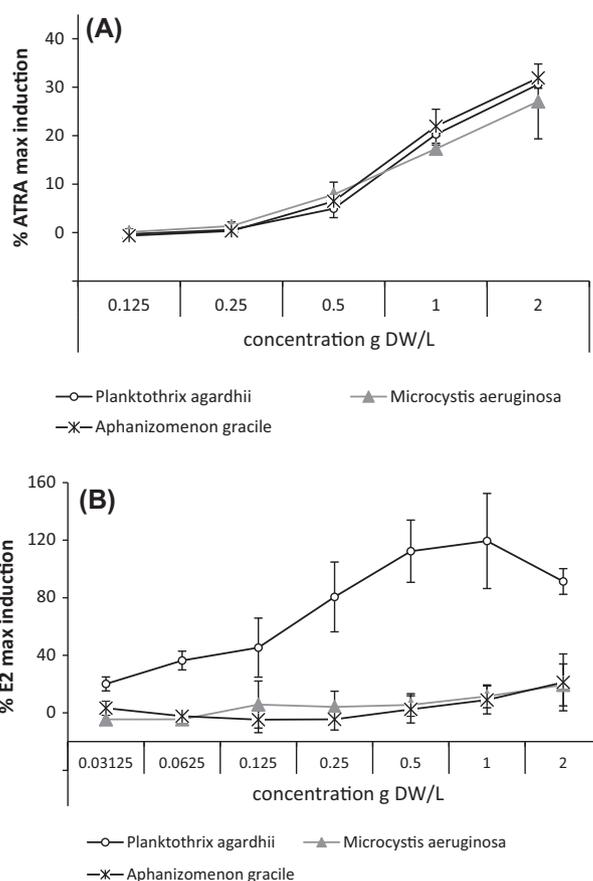


Fig. 1. Concentration–response curves of (A) the retinoid-like activity (expressed as % of maximal RAR-mediated induction caused by standard all-trans retinoic acid – ATRAm_{ax}, 500 nM) in P19/A15 cell line (B) the estrogenic activity (expressed as % of maximal ER-mediated induction caused by standard estradiol – E2m_{ax}, 500 pM) in MVLN cell line after 24 h exposure to extracts from cyanobacterial and algal biomass.

Table 2
Toxicity, teratogenic and estrogenic effects and effects on locomotion and length observed during the detailed experiments on zebrafish embryos after exposure to biomass extracts. Shading emphasizes significant effects.

Time	Endpoint	control	<i>Aphanizomenon gracile</i>			PA ^a	MA ^a
		Concentration (g dwL ⁻¹)	0.3	1	3	0.3	0.3
		REQ ^b ngL ⁻¹	397	1322	3966	349	328
48hpf	mortality ^c	0	0	0	21.7 ±38	0	0
	tail tip deformation	0	0	8.33 ±6*	68.3 ±33*	0	0
	yolk deformation	0	0	1.67 ±3	28.3 ±26	0	0
	heart edema	0	0	0	11.7 ±20	0	0
72hpf	hatched	1 ±2	7 ±12	17 ±29	45 ±17*	0	3 ±6
	mortality	0	0	0	30 ±48	0	0
	tail tip deformation	0	0	83.3 ±29*	70 ±48*	0	0
	heart edema	0	0	5 ±5	5 ±9	0	0
96hpf	spine deformation	0	0	33.3 ±57	0	0	0
	hatched	33 ±43	63 ±40*	75 ±35*	62 ±43*	62 ±40*	82 ±24*
	mortality	0	0	0	70 ±40*	0	0
	tail tip deformation	0	0	100 *	38.3 ±54	0	0
120hpf	heart edema	0	0	1.67 ±3	38.3 ±54	0	0
	spine deformation	0	0	33.3 ±58	0	0	0
	mouth deformation	0	0	33.3 ±58	0	0	0
	hatched	76 ±21	100	97 ±3	-	100	100
120hpf	mortality	0	0	0	88.3 ±13*	0	0
	tail tip deformation	0	0	100 *	11.7 ±13	0	0
	heart edema	0	0	3.33 ±3	11.7 ±13	0	0
	spine deformation	0	0	100 *	0	0	0
120hpf	mouth deformation	0	0	36.7 ±55	0	0	0
	hatched	90 ±10	100	100	-	100	100
	Estrogenicity ^d	1.01 ±0.3	1.06 ±0.1	0.61 ±0.3	-	0.96 ±0.2	1.22 ±0.5
	Locomotion ^e						
	mean distance	0.8 ±0.2	3.3 ±1.6	2.7 ±1.4	-	5.5 ±1.8*	4.1 ±1.3*
	OA	1	0.60 ±0.13	0.62 ±0.22	-	0.44 ±0.04*	0.52 ±0.07*
	Length (µm) ^f	3755 ±21	3952 ±93*	3842 ±94	3165 -	3943 ±77*	3958 ±53*

^aPA is *Planktothrix agardhii* and MA *Microcystis aeruginosa*.

^bREQ = equivalent concentration of ATRA in exposure media.

^cFrequency of effects (in %) for mortality and malformations are shown as an average of three replicate experiments (60 embryos each) with standard deviation.

^dInduction of fluorescence in transgenic cyp19a1b-GFP zebrafish embryos as an indicator of *in vivo* estrogenicity shown as means and standard deviation.

^eLocomotion expressed as mean moved distance and overlapping area (OA, see material and methods for details) with corresponding standard deviation.

^fLength of embryos (µm) shown as an average length and standard deviation of three independent experiments.

*Statistically significant difference from control ($p < 0.05$) – not assessed due to mortality.

concentrations and controls. Hatching rates were particularly affected by all extracts and test concentrations at 72hpf due to an earlier hatching of exposed embryos. The effect on hatching at 48hpf appeared to be dependent on the concentration of biomass as indicated by exposure to different concentrations of *A. gracile* extracts (Table 2).

Teratogenic effects were only observed in zebrafish embryos exposed to extract of *A. gracile* at ≥ 1 g dw L⁻¹. Specifically, deformities of the tip of the tail and spine were observed (Table 2). Edema of the heart and trunk, small head and yolk retention, were only noted at greater concentrations (3 g dw L⁻¹) that already caused mortality. Details of types of malformations along with the time of their occurrence are shown in Table 2.

Embryos measured at 5dpf were significantly ($p \leq 0.05$; by about 5%) longer when exposed to extracts of *P. agardhii*, *M. aeruginosa* and *A. gracile* at 0.3 g dw L⁻¹ (Table 2, Fig. S1). Lengths of embryos exposed to 1 g dw L⁻¹ of *A. gracile* extract were not significantly different from controls. Surviving embryos exposed to

3 g dw L⁻¹ of *A. gracile* extract (8 out of 60) were on average 16% shorter than in controls, but this difference was not statistically significant due to greater mortality at this concentration.

The weak estrogenic effects initially observed in the screening test could not be confirmed in the detailed test (Table 2). Mean GFP levels relative to control from the three independent experiments ranged from 0.6 to 1.2, but the differences compared to control were not statistically significant.

Statistically significant differences in locomotion compared to control were observed for 0.3 g dw L⁻¹ extracts of *M. aeruginosa* and *P. agardhii* (Table 2). Effects were observed only in the second light phase of the behavioural assays, when an increase in the moved distance was noted for exposed embryos.

4. Discussion

Receptor transactivation studies have demonstrated the occurrence of bioactive compounds in cyanobacteria. However, there is a

lack of information on relevance of the *in vitro* potencies for *in vivo* situations. Therefore, in the present study *in vitro* bioactivities of cyanobacteria were compared to effects in a whole-organism model.

The species selected for this study are common in the environment and can dominate aquatic habitat in case of cyanobacterial blooms (Stěpánková et al., 2011; Wu et al., 2012). The lower concentrations used in this study (0.3 g dw L^{-1}) can be considered environmentally relevant during cyanobacterial blooms with densities over $1000000 \text{ cells mL}^{-1}$, which occur frequently in the water bodies. Our field studies document concentrations of biomass up to 3 g dw L^{-1} (approximately $70000000 \text{ cells (mL)}^{-1}$) in lakes with massive cyanobacterial water bloom dominated by *M. aeruginosa* (unpublished data).

The mortality results showed the importance of measuring concentrations of dissolved oxygen in testing of cyanobacterial biomass extracts because of the biological oxygen demand (BOD) they exerted. In the initial screening test oxygen deficiency in some samples might have contributed to mortality, which is indicated by association of oxygen content of less than 35% saturation with 100% mortality (Table S1). A minimum oxygen concentration of 80% is suggested by fish embryo testing guidelines (OECD, 2013) and supported by experimental observations (Küster and Altenburger, 2008). Difficulties in maintaining sufficient concentration of dissolved oxygen and appropriate pH was indicated previously as a possible limitation in biotests with cyanobacteria samples (Burýšková et al., 2006). However, the mortality caused by insufficient oxygen due to the BOD of cyanobacterial blooms represents an environmentally relevant effect (Kuiper-Goodman et al., 1999). Therefore, oxygen depletion should be measured and considered in order to assess the environmental relevance of toxicants versus oxygen insufficiency.

Despite that the tested species belong to distant orders (Oscillatoriales, Nostocales, Chroococcales) of both unicellular (*M. aeruginosa*) and filamentous (*A. gracile*, *P. agardhii*) cyanobacteria they contained similar concentrations of retinoid-like compounds. Among these species previous information on total retinoid-like activity had been available only for a different strain of *M. aeruginosa* analyzed by *in vitro* yeast RA activity assay (Kaya et al., 2011). There is a similarity in observed total retinoid-like potency with the previous study (2500 and $1092 \text{ ng ATRA (g dw)}^{-1}$) despite the different origin and different bioassays used for the assessment. Also chemical analyses documented the presence of a few analogues of retinoic acids in *M. aeruginosa* strains isolated from two Chinese lakes (Wu et al., 2012). Our study is the first to report the presence of compounds with retinoid-like activity also for the cyanobacterial species *P. agardhii* and *A. gracile*.

Potentially linked to the retinoid compounds and activities reported in phytoplankton species, teratogenic effects in zebrafish embryos were found at greater concentrations of *A. gracile* extracts. Effects included tail tip, spine, mouth and yolk deformation and heart edema (Fig. 2C). These effects were also observed in exposure tests with ATRA (Jonas et al., 2014; Herrmann, 1995; Haldi et al., 2011). Concentrations of REQ detected in cyanobacteria by *in vitro* tests corresponded to concentrations of ATRA that were shown to cause teratogenicity in zebrafish embryos. Content of retinoid equivalents in the least extract concentration causing malformations ($1.3 \mu\text{g ATRA L}^{-1}$ in 1 g dw L^{-1} *A. gracile*) corresponded with the LOEC of ATRA (Jonas et al., 2014; Herrmann, 1995) for some of these malformations.

All exposures to 0.3 g dw L^{-1} cyanobacterial biomass equivalents (containing $0.3\text{--}0.4 \mu\text{g ATRA L}^{-1}$) caused a small but statistically significant increase in the length of embryos. A similar effect was observed after exposure to 0.4 and $1.3 \mu\text{g L}^{-1}$ ATRA (Jonas et al., 2014). RAs are known to increase growth hormone levels and mRNA *in vitro* in human- and fish-cells (Guibourdenche

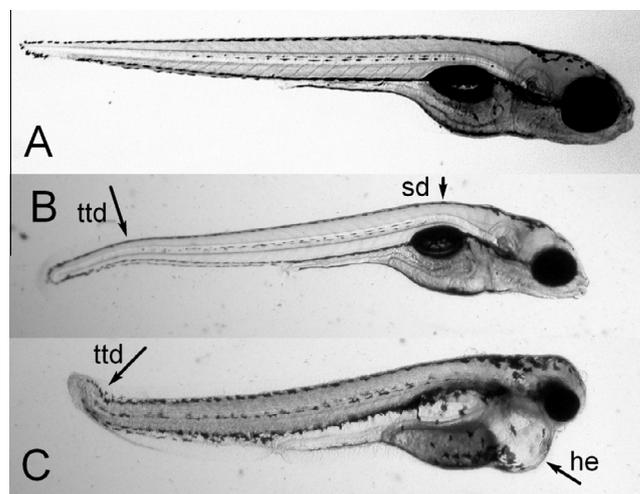


Fig. 2. Comparison of control and exposed zebrafish embryos (120hpf): control (A) compared to embryos exposed to biomass extracts of *Aphanizomenon gracile* $1 \text{ g dry weight L}^{-1}$ (B) and $3 \text{ g dry weight L}^{-1}$ (C). Arrows and abbreviations indicate heart edema (he), tail tip (ttd) and spine deformation (sd).

et al., 1997; Sternberg and Moav, 1999) and the observed greater length could be related to the stimulation of growth hormone synthesis. For greater concentrations of *A. gracile* extracts as well as for greater ATRA concentrations no increased body length was reported, which might be due to interfering toxic effects as indicated by the high rates of malformations and mortality. The lesser length indicated at 3 g dw L^{-1} of *A. gracile* corresponds to observations in zebrafish embryos exposed to greater concentrations of ATRA (Jonas et al., 2014; Herrmann, 1995).

Exposures to cyanobacterial extracts caused earlier hatching compared to controls, particularly at 72hpf, while no such effect was observed for ATRA (Jonas et al., 2014). Hence, effects on hatching are probably not associated with RA activity, but possibly with some other compounds produced by cyanobacteria.

Given the complexity of extracts of cyanobacteria other compounds than retinoids or their analogues may have contributed to the observed teratogenicity. Microcystins were found in two cyanobacteria species in this study, i.e. *P. agardhii* and *M. aeruginosa*. Microcystins probably did not play a significant role in most of the effects observed in this experiment. *In vitro* potencies of the species with greatest microcystins content (*M. aeruginosa*) were lesser (estrogenicity) or comparable (retinoid-like activity) than in species with no or lesser concentrations of microcystins. Mortality and malformations in embryos were induced by greater concentrations of the extract of *A. gracile*, which does not contain these toxins. This is consistent with previously published reports where it was argued that the weak effect of microcystins on zebrafish embryos was due to a limited uptake (Wang et al., 2005; Berry et al., 2007).

A small *in vitro* estrogenic potential was detected for extracts of *M. aeruginosa* and *A. gracile* (near limit of detection). More than 50-fold higher estrogenic potency was observed for extract of *P. agardhii*. An estrogenic activity of different types of extracts from cyanobacterial species used in this study has been reported previously (Rogers et al., 2011; Stěpánková et al., 2011). Particularly, cyanobacterial species forming massive water blooms could contribute estrogenic compounds into water bodies, which might interfere with the hormonal control of aquatic organisms.

Although estrogenicity was detected *in vitro*, no significant estrogenic effects were detected *in vivo*. Potencies of estrogenic compounds in the exposures might have been too small to cause effect in zebrafish embryo assay. The EC_{50} of 17β -estradiol for

GFP induction in transgenic fish is 130 ng L^{-1} causing approximately 10-fold induction. Since the exposure to 0.3 g dw L^{-1} biomass corresponds to concentrations of $0.3\text{--}16.4 \text{ ng EEQ L}^{-1}$, these concentrations might only cause low levels of GFP induction that could in addition be mitigated by other factors (see below). *In vivo* estrogenicity could be expected from greater concentrations of extracts, particularly from *P. agardhii* samples ($546 \text{ ng EEQ L}^{-1}$ in 10 g dw L^{-1}). However, at these concentrations mortality prevented detection of estrogenic activity. Several other reasons could also account for the lack of estrogenic effects in fish embryos. For instance, fish embryos might have greater capacity for metabolisation and deactivation of estrogenic compounds in phytoplankton biomasses. Furthermore, other compounds present in the extracts such as RA could reduce the estrogenic effects. RA is known to regulate the development of the brain (Rhinn and Dollé, 2012) and impact on differentiation of stem cells into the neural radial glial cells progenitors (Plachta et al., 2004). This could potentially affect production of aromatase B and consequently GFP, because aromatase B is mainly produced in the radial glial cell progenitors in the brain of zebrafish embryos (Tong et al., 2009).

Among the various toxins known to be produced by cyanobacteria also an array of different neurotoxins can be found (Aráoz et al., 2010). The neuroactivity or -toxicity is difficult to detect *in vitro* since a functional nervous system would be required. However, behavioural assays (i.e. analysis of movements) represent a simple and efficient tool to detect functional interference in zebrafish embryos (Selderslaghs et al., 2010). We detected a weak increase of the moved distance in zebrafish embryos exposed to biomass extracts of *M. aeruginosa* and *P. agardhii*. The observed effects might not necessarily represent a specific neurotoxic response but could also indicate sublethal effects, subtle morphological changes or avoidance reactions rather than specific interaction with e.g. neuronal receptors or ion channels. Increased locomotion of 120hpf old embryos was observed after exposure to 600 ng L^{-1} ATRA (Wang et al., 2014), which indicates possible influence of retinoids on increased locomotion in our samples. It has been also reported that exposure to 0.5 and $5 \mu\text{g L}^{-1}$ microcystin-LR resulted in an increased day-time locomotion of adult zebrafish (Baganz et al., 1998). The strains, of which the extract increased movement of zebrafish embryos, contain microcystins. Hence, non-neurotoxic compounds such as microcystins may have contributed to the response in behavioural assays. Acetylcholinesterase (AChE) inhibitor anatoxin-a was reported in several species from genera *Aphanizomenon*, *Microcystis* and *Planktothrix* (Aráoz et al., 2010). The potential presence of anatoxin in samples used in our study is unknown. Nevertheless, AChE inhibitor would rather decrease the locomotion of zebrafish embryos as was shown with AChE inhibitor diazinon (Yen et al., 2012). It will require further investigations to identify the compounds responsible for the observed effects and their mechanism of action.

In conclusion, this study demonstrates the diversity of bioactive compounds in biomass extracts. Our findings indicate that endocrine activities detected by *in vitro* assays might not be directly reflected by whole organism assays. This could, however, be provoked by various relevant mitigating factors (metabolism, down-regulation via other pathways, toxic effects, see above) not present in *in vitro* assays. We confirmed that phytoplankton species produce estrogenic, retinoid-like and/or teratogenic compounds, which could be released into the aquatic environment and affect the development of organisms living in surface waters. Fish embryos often live in littoral zones where greater accumulation of cyanobacterial water blooms can be expected (Malbrouck and Kestemont, 2006). Hence, retinoid-like effects of phytoplankton indicate the need to consider early life stages, which could be more vulnerable than adults due to sensitive developmental processes, for the estimation of the environmental impact of

cyanobacterial blooms. More attention should be paid to the activity of retinoids, estrogens and other bioactive compounds in cyanobacteria using *in vitro* and *in vivo* bioassays.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chemosphere.2014.07.074>.

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