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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.

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). Furthermore, 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 Gumy, 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