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# Retinoid-like compounds produced by phytoplankton affect embryonic development of Xenopus laevis

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ABSTRACT

Teratogenic effects, which were remarkably similar to those induced by retinoic acids, have been seen in wild frogs indicating possible source of retinoids in the environment. Recent studies indicate that some cyanobacterial species can contain teratogenic retinoic acids (RAs) and their analogues. Retinoids are known to regulate important processes such as differentiation, development, and embryogenesis. The study investigated the effects of exudates (extracellular compounds) of two cyanobacteria species with retinoic-like activity and one algae species on embryonic development of amphibians. The retinoid-like activity determined by *in vitro* reporter gene assay reached 528 ng retinoid equivalents (REQ)/L and 1000 ng REQ/L in exudates of *Cylindrospermopsis raciborskii* and *Microcystis aeruginosa*, respectively, while algal exudates showed no detectable activity. Total mean of retinoid-like copounds into exudate was 35.6 ng ATRA/mil.cells for *M.aeruginosa* and 6.71 ng ATRA/mil.cells for *C.raciborskii*, respectively. Toxicity tests with amphibian embryos up to 96 h of development were carried out according to the standard guide for the Frog Embryo Teratogenesis Assay Xenopus. Lowest observed effect concentrations (LOEC) of malformations (2.5–2.6 µg/L REQ) were two times lower than LOEC for ATRA (5 µg/L). The exudates of both cyanobacteria were indeed provoking diverse teratogenic effects (*e.g.* tail, gut and eyes deformation) and interference with growth in frogs embryos, while such effects were not observed for the algae. *Xenopus* embryos were also exposed to all-*trans* retinoic acid (ATRA) in concentration range (1 – 40 µg/L) equivalent to the REQs detected in cyanobacterial exudates. ATRA (10 µg/L) caused similar teratogenic phenotypes at corresponding REQs as cyanobacterial exudates. The study confirms the ability of some species of cyanobacteria to produce retinoids naturally and excrete them directly into the environment at concentrations which might have adverse influence on the development of amphibians.

## 1. Introduction

Along with continuous anthropogenic water eutrophication, the appearance of massive water blooms has become a worldwide problem in the last decades. Blooms dominated by cyanobacteria (photosynthesizing gram-negative bacteria), growing excessively in aquatic reservoirs have adverse impact on the quality of water and life of many aquatic and terrestrial organisms including humans. They can cause serious environmental and health problems in many surface waters (Wiegand and Pflugmacher, 2005). It is well documented that cyanobacteria produce wide range of biologically active compounds, but only few of them have been toxicologically characterized in detail. Cyanobacterial toxins (cyanotoxins) can be categorized according to their effect as neurotoxins, hepatotoxins, cytotoxins, dermatotoxins, genotoxins and irritant toxins and they were also shown to play an important role in carcinogenesis (Svrcek and Smith, 2004). Hepatotoxic microcystins (MC) belong among the most studied toxins. In many cases toxin produced by cyanobacteria occur in sources of drinking water and in recreational lakes and reservoirs. Microcystins contaminating drinking water sources have been directly linked to frequent incidence of primary liver cancer in China (Chen et al., 2009; Ueno et al., 1996). Although some purified cyanobacterial toxins have been investigated extensively (such as microcystins or cylindrospermopsins), mechanisms of toxicity and effects of other compounds produced by cyanobacteria are poorly understood or unknown (Falconer, 2007).

Remarkable similarities in malformations (all of the separate limb malformations) observed in wild frogs and laboratory frogs exposed to retinoids lead to hypothesis of presence of retinoid active compounds in the environment (Gardiner and Hoppe, 1999). It was concluded that although it is not fully possible to exclude that malformations in aquatic

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wild frogs have more reasons, the majority of observed malformations is highly likely caused by retinoid exposure (Gardiner et al., 2003). Another study argued that there is no evidence that the retinoids are reason of these malformations in wild frog population because such retinoids should be detectable in water, sediments, algae or vegetation (Stocum, 2000). These possible retinoids in water bodies were recommended to be investigated and their possible impact on drinking water supplies evaluated, because driving forces disrupting development in animals are probable candidates to impact human and other vertebrate development alike (Gardiner et al., 2003; Gardiner and Hoppe, 1999). However, there is not much information on the presence and sources of retinoids in the environment.

Retinoids are a class of compounds including vitamin A (retinol, ROH) and its natural analogues such as retinal (RAL) and retinoic acids (RAs), in addition to its synthetic derivatives. As one of the most potent known animal teratogens, RAs are generally thought of as vertebrate-specific hormones and can be transformed *in vivo* from RAL, ROH, retinyl esters (REs) and carotenoids, which are collectively called as RA precursors. It has been extensively proven that an excess of some RA precursors would produce adverse effects to humans and animals, but both excessive amount of retinoids as well as their deficiency cause teratogenicity (Collins and Mao, 1999). Retinoids are known to regulate important processes such as differentiation, development, and embryogenesis. Some effects, such as malformations in frogs or changes in metabolism of birds, could be related to disruption of the retinoid signaling pathway (Novák et al., 2007). Many pathways and proteins involved in retinoic acid (RA) signaling during development are highly conserved in vertebrate species. RA is important for hindbrain, forebrain, fin and limb development as well as responsible for body axis symmetry (Rhinn and Dolle, 2012), it regulates germ layer formation, cardiogenesis and pancreas, eye and lung development (Kin et al., 2012). The all-*trans* retinoic acid (ATRA) is the main active form of retinoids connected to gene transcription during early embryonic development (Kin et al., 2012).

Recent studies indicate that some cyanobacterial species can contain teratogenic retinoic acids (RAs) and their analogues, 4-oxo-RAs (Kaya et al., 2011; Wu et al., 2012). Retinoids have been detected even outside the cyanobacterial cells of a few species, but most cyanobacteria did not produce detectable RAs into their surroundings. Moreover, most studied species contained retinal (RAL), which is considered as RA precursor and teratogenic agent (Wu et al., 2013).

Study by Kaya et al. (2011) described new RA analogue (7-hydroxy RA) isolated from laboratory cultured cyanobacteria, and also provided information about total *in vitro* retinoid-like activity of *Microcystis aeruginosa* analyzed by yeast RA activity assay. Their detected total retinoid-like potency of biomass (2500 ng REQ/g dm) was comparable as in another more recent study reporting 1092 ng REQ/g dm (Jonas et al., 2015) despite the different origin of cyanobacteria culture, different method of extraction and different bioassays used for the assessment. Study by Wu et al. (2012, 2013) described presence of retinoids in cyanobacteria and surrounding water. They have brought information on a few individual retinoids in environmental biomasses, surrounding water extracts, and laboratory cultures of cyanobacteria commonly found in aquatic environments.

The only available *in vivo* study focused on the effect of cyanobacterial exudates containing retinoids showed teratogenic effects in fish embryos (Jonas et al., 2014). The study documents that some cyanobacteria are able to produce and release retinoid-like compounds into the environment at concentrations equivalent to those causing teratogenicity in zebrafish. The exudates of both tested cyanobacteria species (*Microcystis aeruginosa, Cylindrospermopsis raciborskii*) were indeed provoking diverse teratogenic effects (*e.g.* tail, spine and mouth deformation) and interference with growth in zebrafish embryos, while such effects were not observed for the exudates of the tested alga (*Desmodesmus quadricauda*). Both the phenotypes and effective concentrations of exudates corresponded to ATRA equivalents, supporting the hypothesis that the teratogenic effects of cyanobacterial exudates are likely to be associated with retinoid-like activity.

Since the effects on amphibians potentially associated with environmental retinoids have been of major concern and a subject of intense discussion (Gardiner et al., 2003; Gardiner and Hoppe, 1999) we aimed to investigate the relevance of the retinoid-like activity that has been shown to cause effects on fish embryos on frog embryos also to compare the sensitivity and effects produced by exposure to standard toxicant ATRA and by the exudates. Thus, this study aims to characterize retinoid-like activity of the metabolites produced by phytoplankton species extracellularly into the environment (exudates), which are of high relevance for the aquatic ecosystems, and their teratogenic potential on early embryonic development of amphibians. The studied species were selected to correspond to the previous study on fish embryos (Jonas et al., 2014) to compare the effects on fish and frog embryos. They represent taxonomically and morphologically different groups of algae, coccal and filamentous cyanobacteria. The research specifically focused on the environmentally relevant species dominating water blooms in many surface waters worldwide (*Microcystis aeruginosa* and *Cylindrospermopsis raciborskii*), which both have repeatedly shown retinoid-like activity in exudates, and algae that were also investigated in our previous study of fish embryos. Frog Embryo Teratogenesis Assay Xenopus (FETAX) was used for the investigation of embryotoxicity and teratogenicity of cyanobacterial and algal exudates with differing retinoid-like activity.

## 2. Materials and methods

### 2.1. Preparation of cyanobacterial and algal exudates samples

The identification and source of investigated cyanobacterial and algal strains and the microcystin content of their exudates are listed in Table 1. All tested species were long-term cultivated in RECETOX labs in mixture of Zehnder medium (Schlösser, 1994) and Bristol Bold medium (Stein, 1975) with distilled water in the ratio 1:1:2 (v/v/v). Organisms were grown in glass flasks for 21 days at 22 ± 2 °C under continuous light (cool white fluorescent tubes, 2000 lx) and aeration with air filtered through 0.22 μm filter (Labicom, Czech Republic). Spent growth media were separated from the cyanobacterial and algal cells (biomass) by centrifugation (4675*g*, 3 min, 23 °C) after 21 days of

**Table 1**
List of phytoplankton species used in this study, their origin, microcystin, number of cells and total retinoid equivalent (REQ) of exudates determined by *in vitro* assay.

| Species | Source | Place of origin | | MCs concenrtation (ng/L) | | | REQ | Number of cells. $10^6$/mL | REQ |
|---|---|---|---|---|---|---|---|---|---|
| | | Country | Water body | MC-RR | MC-YR | MC-LR | ng ATRA/L | | ng ATRA/milion cells |
| *Microcystis aeruginosa* | PCC 7806 | Netherlands | Braakman Reservoir | 0.13 | 14.8 | 9200 | 1000 | 28.1 | 35.6 |
| *Cylindrospermopsis raciborskii* | SAG 1.97 | Hungary | Lake Balaton | < 0.125 | < 0.25 | < 0.25 | 528 | 78.6 | 6.71 |
| *Desmodesmus quadricauda* | CCALA 463 | Germany | Greifswald | < 0.05 | < 0.25 | < 0.25 | < 16 | 1.75 | |

Culture collection: PCC-The Pasteur Culture Collection of Cyanobacteria, SAG-Culture Collection of Algae at the University of Goettingen, CCALA - Culture Collection of Autotrophic Organisms
REQ- relative equivalent of ATRA ( MW 300.4*g*/mol) in exudates ( *in vitro* retinoid-like activity)