Aquatic Toxicology 155 (2014) 283–290

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Retinoid-like activity and teratogenic effects of cyanobacterial exudates

Adam Jonas[a], Veronika Buranova[a], Stefan Scholz[b], Eva Fetter[b], Katerina Novakova[a], Jiri Kohoutek[a], Klara Hilscherova[a,*]

[a] RECETOX—Masaryk University, Faculty of Science, Brno, Czech Republic
[b] UFZ—Helmholtz Centre for Environmental Research, Department of Bioanalytical Ecotoxicology, Leipzig, Germany

ARTICLE INFO

Article history:
Received 13 February 2014
Received in revised form 26 June 2014
Accepted 27 June 2014
Available online 6 July 2014

Keywords:
Cyanobacteria
Developmental toxicity
Retinoids
Zebrafish embryo
All-trans retinoic acid

ABSTRACT

Retinoic acids and their derivatives have been recently identified by chemical analyses in cyanobacteria and algae. Given the essential role of retinoids for vertebrate development this has raised concerns about a potential risk for vertebrates exposed to retinoids during cyanobacterial blooms. Our study focuses on extracellular compounds produced by phytoplankton cells (exudates). In order to address the capacity for the production of retinoids or compounds with retinoid-like activity we compared the exudates of ten cyanobacteria and algae using *in vitro* reporter gene assay. Exudates of three cyanobacterial species showed retinoid-like activity in the range of 269–2265 ng retinoid equivalents (REQ)/L, while there was no detectable activity in exudates of the investigated algal species. The exudates of one green alga (*Desmodesmus quadricaudus*) and the two cyanobacterial species with greatest REQ levels, *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii*, were selected for testing of the potential relation of retinoid-like activity to developmental toxicity in zebrafish embryos. The exudates of both cyanobacteria 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 alga. Fish embryos were also exposed to all-*trans* retinoic acid (ATRA) in a range equivalent to the REQ concentrations detected in exudates by *in vitro* bioassays. 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. 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. Hence, the characterization of retinoid-like and teratogenic potency should be included in the assessment of the potential adverse effects caused by the release of toxic and bioactive compounds during cyanobacterial blooms.

1. Introduction

In eutrophic conditions cyanobacteria can form dense blooms, which represent an unwanted ecological state due to various negative impacts on ecosystem function and environmental and human health. For instance, increasing pH and low oxygen levels associated with cyanobacterial blooms in surface waters and a reduced light penetration in water columns impact algae and macrophytes, and also fish populations (Scheffer et al., 1997; Wiegand and Pflugmacher, 2005). Moreover, cyanobacteria produce a wide spectrum of toxic metabolites. Cyanobacteria have been implicated in causing adverse effects in humans and other vertebrates (Hitzfeld et al., 2000; Ibelings and Havens, 2008; Kuiper-Goodman et al., 1999; Lévesque et al., 2013). Toxins produced by cyanobacteria include neurotoxins, hepatotoxins, cytotoxins, dermatotoxins and irritants (Aráoz et al., 2010; Kinnear, 2010; Stewart et al., 2006; Wiegand and Pflugmacher, 2005). Furthermore, compounds causing gastrointestinal tract and respiratory distress, immunotoxicity, carcinogenicity, genotoxicity and mutagenicity (Rastogi and Sinha, 2009) have been identified. The most studied cyanobacterial toxins are the hepatotoxic and tumor promoting microcystins (Bláha et al., 2009). Some recent studies have indicated the potential of cyanobacterial metabolites to interfere with the endocrine system (Rogers et al., 2011; Stěpánková et al., 2011). Because of the simultaneous presence of various bioactive compounds in cyanobacteria it is important to investigate both particular cyanotoxins and

* Corresponding author. Tel.: +0042 549493256; fax: +0042 549492856.
E-mail address: hilscherova@recetox.muni.cz (K. Hilscherova).

http://dx.doi.org/10.1016/j.aquatox.2014.06.022
0166-445X/

the toxicity of mixtures of compounds released from cyanobacteria. Numerous studies (Berry et al., 2009; Oberemm et al., 1997; Rogers et al., 2011) have shown that the toxicity of biomass extracts often cannot be explained by the level of the known cyanotoxins and have therefore suggested that other bioactive compounds contribute to the toxicity. Published studies investigating cyanobacterial metabolite mixtures have mostly focused on extracts of biomass (Berry et al., 2009; Rogers et al., 2011). However, limited information is available for cyanobacterial exudates, *i.e.* mixtures of extracellular compounds excreted during common physiological processes (Nováková et al., 2013). For instance, a recent study indicated high mortality in zebrafish embryos exposed to exudates of the cyanobacterium *Fischerella ambigua*, and this toxicity could not be explained by the level of known active compounds which were tested simultaneously (ambigol A, ambigol C, 2,4-dichlorobenzoic acid, and tjipanazole D) (Wright et al., 2006).

Recently, several retinoid compounds have been chemically identified in both biomass and exudates of some phytoplankton species (Wu et al., 2013, 2012). The same compounds were detected in water samples obtained from a eutrophic lake with cyanobacteria blooms (Taihu Lake, China) suggesting that these retinoids were probably produced by cyanobacteria. Retinoid-like activity was also detected in biomasses of seven cyanobacterial species using an *in vitro* yeast bioassays (Kaya et al., 2011). Retinoic acid (RA) plays an important role in vertebrate development and the pathways and proteins involved in retinoic acid signalling are highly conserved in vertebrates. RA is important for hindbrain, forebrain, fin and limb development and it is required to establish body axis symmetry (Rhinn and Dollé, 2012). Furthermore, germ layer formation, cardiogenesis, pancreas, eye and lung development are regulated by RA (Kam et al., 2012). Excessive amounts of retinoids, as well as their deficiency, cause teratogenicity (Collins and Mao, 1999).

High levels of retinoids might explain previous observations of diverse malformations, including several types of oedema, tail bents, undeveloped eyes or neural tube malformations in zebrafish embryos exposed to extracts of cyanobacteria *Microcystis aeruginosa*, *Anabaena flos-aquae*, *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, *Planktothrix agardhii*, and *Aphanizomenon flos-aquae* (Acs et al., 2013; Berry et al., 2009; Ghazali et al., 2009; Oberemm et al., 1999). Retinoic acids are known to cause various types of malformations in zebrafish embryos, such as yolk sac and heart edemas, brain and tail malformations, duplication of otic placodes and otoliths (Herrmann, 1995), elongated heart chambers, small intestine, absence of liver tissue (Haldi et al., 2011), and neurotoxicity (Parng et al., 2007).

The goal of this study was to determine *in vitro* retinoid-like activity of phytoplankton exudates and their effects on zebrafish embryo development and reveal the potential relation of the *in vitro* activity to *in vivo* effects. Exudates (metabolites produced and released into water by living cells) of ten phytoplankton species, including both algae and cyanobacteria were studied using *in vitro* assay for retinoid-like activity. The two most potent and one negative exudate were then tested in detail in zebrafish embryos. Fish embryos were also exposed to all-trans retinoic acid (ATRA) in a range corresponding to the retinoic acid equivalents (REQ) detected in exudates by *in vitro* bioassays. ATRA was used as a positive control due to its frequent detection in cyanobacterial extracts and exudates (Wu et al., 2012), reported highest teratogenicity among retinoids in zebrafish (Herrmann, 1995) and its use as standard ligand in *in vitro* assays for total retinoid-like activity, which is generally expressed as concentration equivalents of ATRA (Kaya et al., 2011; Novák et al., 2007). The phenotypes provoked by the exudates in zebrafish embryos and the effective concentrations of *in vitro* determined REQ were compared to those from exposure to ATRA.

2. Materials and methods

2.1. Cyanobacterial strains and culture conditions

The identification and source of investigated cyanobacterial and algal strains and the microcystin content of their exudates are listed in Table 1. All strains were cultivated in a mixture of Zehnder (Schlosser, 1994) and Bristol (modified Bold) medium (Stein, 1973) with distilled water in the ratio of 1:1:2 (v/v/v). Organisms were grown for 21 days at 22 °C ± 2 °C under continuous light (cool white fluorescent tubes, 3000 lx) and aeration with air filtered through a 0.22 μm membrane (Labicom, Czech Republic). The cultivations were started with a 20% (v/v) inoculum of a previous culture.

2.2. Exudate preparation

Spent growth media were separated from the cyanobacterial and algal cells (biomass) by centrifugation (2880 × *g*, 10 min, 25 °C) after 21 days of culture and filtered through a 0.6 μm glass fiber filter (Fisher Scientific, Czech Republic). Organic compounds present in the media (exudates) were concentrated by solid phase extraction (SPE) using an Oasis HLB column (Waters, USA) and Carbograff column (Alltech, USA) in sequence. The SPE procedure was performed according to the manufacturer's instructions for HLB and Carbograff columns. Each sample was first passed through the HLB, then through the Carbograff column. Both columns were then eluted with 100% MeOH. The eluates were concentrated using a rotary evaporator at room temperature (22 ± 1 °C). For exposure, eluates from both columns were pooled to obtain maximal recovery. A final concentration of exudates that corresponded to 2000-fold concentrated original media was reached using evaporation under a stream of inert gas (nitrogen) at room temperature and the addition of 100% methanol (Nováková et al., 2011).

2.3. Microcystin analyses

Microcystins were analysed in exudates after SPE extraction by HPLC Agilent 1100 Series coupled with a PDA detector (Agilent Technologies, Germany) using C18 Supelcosil ABZ + Plus column, 150 × 4.6 mm, 5 μm (Supelco, USA), and gradient elution with acetonitrile (Babica et al., 2006). Microcystins were identified by comparing the UV spectra and retention times with standards of microcystin-LR, -YR, -RR (MW 995, 1045, 1038 g/mol, respectively, Enzo Life Sciences, Switzerland) and quantified using calibration standards (limit of detection 0.025 μg/L).

2.4. Reporter gene assay

For the study of *in vitro* retinoid-like activity, we used the murine embryonic carcinoma cell line P19 (European Collection of Cell Culture, UK) transfected with a luciferase reporter pRAREβ2-TK-luc plasmid (P19/A15 clone) (Novák et al., 2007). The plasmid contains a reporter luciferase gene under the control of a retinoic acid-responsive element. Cells were cultured in plastic tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum Mycoplex (PAA, Austria) at 37 °C in a humidified atmosphere of 5% CO_2.

For the RAR/RXR transactivation assay, 10,000 cells per well were seeded into 96-well microplates in DMEM with gentamicin (1%) and incubated overnight under above described conditions. After 24 h, the cells were exposed to tested samples and calibration standard diluted in dimethylsulphoxide (DMSO), which was also used as a solvent control. The exudates of six cyanobacteria and four