Ecotoxicology and Environmental Safety 150 (2018) 312–319

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Intracellular and extracellular retinoid-like activity of widespread cyanobacterial species

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ARTICLE INFO

Keywords:
Cyanobacteria
Retinoids
Retinoid-like activity
Extract
Exudates
ATRA

ABSTRACT

Cyanobacterial species produce wide range of bioactive compounds. This study characterized production of retinoid-like compounds with embryotoxic and teratogenic potential by commonly occurring cyanobacterial species with tendency to form massive water blooms. The major goal was to simultaneously assess the intracellular and extracellular retinoid-like activity from several independent cultivations of one coccal (*Microcystis aeruginosa*) and four filamentous cyanobacteria (*Aphanizomenon gracile, Cylindrospermopsis raciborskii, Limnothrix redekeii,* and *Planktothrix agardhii*) and characterize the variability in its production among cultivations. The retinoid-like activity was evaluated by *in vitro* assay along with chemical analyses of nine retinoids: all-*trans* retinoic acid (ATRA), 9-*cis* retinoic acid (9cis-RA), 13cis-RA, 13cis-RA methyl ester, 5,6 epoxy-RA, 4keto-ATRA, 4keto-retinal, 4hydoxy-retinoic acid (4OH-ATRA), retinal and retinol. The production of retinoid-like compounds was recalculated per volume, per biomass dry weight and per cell to provide relevant data for risk assessment in relation to occurrence of massive water blooms in the environment. Total produced retinoid-like activity of five selected species ranged from 170 to 25,600 ng ATRA-equivalents (REQ)/g dm corresponding to 0.001–0.392 ng REQ/10^6 cyanobacterial cells. Results from chemical analyses showed that all tested extracts contained 4keto-ATRA and retinal. All-*trans* retinoic acid, 9/13cis-retinoic acid and 5,6 epoxy-retinoic acid were detected in most exudate and extract samples. The reported results of recalculated total retinoid-like activity enable potential predictions of its production by the studied species in water blooms of known cell densities relevant for risk assessment.

1. Introduction

Cyanobacteria are prokaryotic photosynthetic microorganisms that are of concern because of their ability to produce toxins and taste and odour compounds as well as disrupt drinking water treatment (Scott and Marcarelli, 2012; Zamyadi et al., 2013). Freshwater cyanobacteria and their toxins (cyanotoxins) pose a risk to human and animal health *via* contamination of water sources and aquatic communities globally (Mur et al., 1999; Paerl, 2008). Nutrient and hydrologic conditions strongly influence harmful planktonic and benthic cyanobacterial bloom dynamics in aquatic ecosystems ranging from streams and lakes to coastal ecosystems. Urbanization, agricultural and industrial development has led to increased nitrogen and phosphorus discharge, which support formation of cyanobacterial blooms in surface waters (Paerl, 2008). 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 can be classified based on biological effects into five functional groups including hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and irritant toxins (Codd et al., 2005; Sivonen and Jones, 1999). Microcystin (MC) is the most frequently detected cyanotoxin in fresh water bodies (Jang et al., 2006), and is becoming a problem to ecological health due to its adverse effects (Babica et al., 2006).

It has been indicated that some cyanobacterial species can contain teratogenic retinoic acids (RAs) and their analogues (Javůrek et al., 2015; Smutná et al., 2017; Sychrová et al., 2016; Wu et al., 2012b). Moreover, retinal (RAL) and β-carotene (considered RA precursor) were detected in *Microcystis* species cultivated in laboratory as well as in samples from Taihu lake, China (Wu et al., 2013, 2012a). 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, retinol (ROH), retinyl esters (REs) and carotenoids. Carotenoids are biological precursors of retinoids (Handelman, 2001; Linan-Cabello et al., 2002). Cyanobacteria grow by photosynthesis, and contain chlorophyll and carotenoids, whose main functions are light harvesting and photoprotection. The major carotenoids in cyanobacteria are β-

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https://doi.org/10.1016/j.ecoenv.2017.12.048
Received 17 July 2017; Received in revised form 19 December 2017; Accepted 20 December 2017
Available online 04 January 2018
0147-6513/

carotene, its hydroxyl derivatives, its keto derivatives and the carotenoid glycosides (Takaichia and Mochimaru, 2007). The carotenoid molecule is unstable. Degradation of carotenoids can produce metabolites with biological effects (*e.g.* retinoids). Free-radical attack on carotenoids is often linked with metabolites that can contribute to retinoid activity (Handelman, 2001).

Retinoids comprise a family of polyisoprenoid lipids, which include vitamin A (retinol, ROH) and its natural analogues such as retinal (RAL) and retinoic acids (RAs). These substances regulate proliferation, differentiation, apoptosis, cytokine production, gut mucosal immunity and malignant transformation of cells (Collins and Mao, 1999; Novák et al., 2008). Physiological processes are controlled by retinoic acid after it is activated by metabolism from vitamin A (retinol). Environmental retinoids, especially RAs, have become an increasing concern due to their teratogenicity and potential role in causing deformations in amphibians and fish in the field (Collins and Mao, 1999; Jonas et al., 2015, 2014; Novák et al., 2008). Retinoid signalling is primarily mediated through two classes of nuclear receptors, the retinoid acid receptor (RAR) and retinoid X receptor (RXR). The endogenous activators of RAR are all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9cis-RA). RXR is activated by 9cis-RA but not by ATRA. RAR requires heterodimerization with RXR in order to bind DNA and activate transcription of RAR target genes. An interesting and significant feature of RXR is that, in addition to heterodimerization with RAR, it is a common heterodimeric partner for ten other families of nonsteroidal nuclear receptors including the thyroid hormone and vitamin D3 receptors. Therefore, inappropriate modulation of retinoid signalling would be expected to have pleiotropic effects on development and adult physiology (Gardiner et al., 2003).

Generally, the reported concentrations of the few analysed retinoids ranged from undetected to hundreds ng/L range in cyanobacterial water blooms (Wu et al., 2012b). However, there can be a number of other not analysed metabolites able to interfere with RAR/RXR with different potency, which can interact, and thus the total retinoid-like activity determined in bioassays can be a better indicator of the potential toxic effect of the mixture than concentrations of individual compounds. The total presence of compounds able to interfere with retinoid receptor in cyanobacteria has been assessed in our lab by means of a very sensitive *in vitro* reporter gene assay. The bioassay is based on the pluripotent embryonic carcinoma cell line P19 stably transfected with the firefly luciferase gene under the control of a retinoic acid–responsive element (clone P19/A15) (Novák et al., 2007).

Most existing papers present retinoid-like activity from a few species separately either from extracts (Jonas et al., 2015; Kaya et al., 2011) or from exudates (Jonas et al., 2014). Moreover, they report data from only one sample per species with no information regarding optimization of extract sample preparation (Jonas et al., 2015, 2014; Kaya et al., 2011). Only Javůrek et al. (Javůrek et al., 2015) optimized extraction procedures for field samples but this study was focused specifically on *Microcystis aeruginosa* dominated water blooms. Detectable retinoid-like activity was found in extracts and exudates (extracellular products) from several laboratory cultured species and field samples. Since different compounds can contribute to the activity and we are dealing with mixture of unknown composition it is necessary to test the efficiency of extraction procedures for the total retinoid-like activity to maximize the recovery of the bioactive compounds.

The major goal of this study was to simultaneously assess the intracellular and extracellular retinoid-like activity, *i.e.* in both extracts and exudates from several independent cultivations of selected widespread cyanobacterial species known to form dense water blooms in the environment. Specifically, the study aimed to characterize the variability in its production and distribution between biomass and exudates. An important goal was also to investigate the occurrence of nine retinoids in both extracts and exudate samples by LC-MS/MS analysis and assess their contribution to total retinoid-like activity detected in *in vitro* bioassays. The important necessary step was the optimization of the extraction process to maximize recovery of compounds with retinoid-like activity. The production of the compounds with retinoid-like activity in the independent cultivations of the same species was recalculated to biomass dry weight as well as to cell density to provide relevant data for risk assessment in relation to occurrence of massive cyanobacterial blooms in surface water bodies.

2. Materials and methods

2.1. Preparation of cyanobacterial samples

Five widespread cyanobacterial species representing several cyanobacterial orders were cultured and analysed: coccal *Microcystis aeruginosa* and filamentous *Aphanizomenon gracile, Cylindrospermopsis raciborskii, Limnothrix redekei,* and *Planktothrix agardhii*. All tested species were long-term cultivated in RECETOX labs in 50% (v/v) mixture of Zehnder and Bristol Bold medium. To characterize the variability in production and distribution of compounds with retinoid-like activity between biomass and exudates, three or four independent cultivations of each species were done during one year period. The total number of independent cultivations was 18. For each species, growth curve was characterized in preliminary experiments and starting inoculum densities were selected to ensure having cells in linear growing phase for the 21 days cultivations (Table S1). Organisms were grown in 1 L glass flasks 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 cells (biomass) by centrifugation (2350 g, 3 min, 23 °C) after 21 days of culture and vacuum filtered through 0.6 μm paper filter (Fisher Scientific, Czech Republic). Extract and exudate samples were prepared from all independent cultivations according to the same optimized procedures. Organic compounds present in the media (exudates) were concentrated by solid phase extraction (SPE). The SPE procedure was performed according to manufacturer's instructions for used columns HLB Oasis (Waters, Milford, USA). The samples were dosed to the cartridges through PTFE tubes with a flow rate of approximately 4–6 mL/min. After loading the samples, the cartridges were dried under nitrogen gas, and then eluted with 15 mL of methanol. Final concentration of exudate samples that corresponded to 4000-fold concentrated original media was prepared using evaporation of solvents by stream of inert gas (nitrogen) at room temperature.

2.2. Optimization of extraction process

The cyanobacterial cells (biomass) separated from growth medium by centrifugation were frozen and lyophilized prior to extraction.

In order to select the most efficient approach for the extraction of retinoid-like substances, extraction method was optimized in two steps. Firstly, based on the information from previous studies, selection of the most suitable solvent was conducted by the comparison of the extraction efficiency of 75% (v/v) methanol, 100% (v/v) methanol, ethyl acetate, hexane: chloroform (1:1 and 3:1, v/v) and acetone across the biomasses of the listed species. Approximately 200 mg of freeze-dried biomass for each species were extracted in glass test tube with 5 mL of each solvent by sonication for 2 × 2 min with addition of 1 g of washed glass beads to support complete cell disruption (Bandelin Sonopuls HD 2070, 100% power, cycle 0.9) in cooling bath. Cell disruption was checked by microscope during all experiments. Then, test tubes were centrifuged (3052 g for 3 min) and extracts were collected.

Further, extraction recovery was maximized by repeated re-extraction. After the first extraction and collection of the primary extract we added another 5 mL of 100% (v/v) MeOH into the glass vial with extracted biomass, sonicated for 2 min and left on orbital shaker protected from light for 2 h and continued by previously optimized extraction steps. The same procedure was repeated after 5, 24, and 48 h.