Aquatic Toxicology 168 (2015) 1–10

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Application of cellular biosensors for detection of atypical toxic bioactivity in microcystin-containing cyanobacterial extracts

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ARTICLE INFO

Article history:
Received 4 May 2015
Received in revised form 4 September 2015
Accepted 8 September 2015
Available online 10 September 2015

Keywords:
Cyanobacterial extract
Microcystins
Biological activity
Cellular biosensors
Reporter genes
Immunotoxicity

ABSTRACT

Despite the focus of most ecotoxicological studies on cyanobacteria on a select group of cyanotoxins, especially microcystins, a growing body of evidence points to the involvement of other cyanobacterial metabolites in deleterious health effects. In the present study, original, self-developed reporter gene-based cellular biosensors, detecting activation of the main human xenobiotic stress response pathways, PXR and NFkappaB, were applied to detect novel potentially toxic bioactivities in extracts from freshwater microcystin-producing cyanobacterial blooms. Crude and purified extracts from cyanobacteria containing varying levels of microcystins, and standard microcystin-LR were tested. Two cellular biosensor types applied in this study, called NHRTOX (detecting PXR activation) and OXIBIOS (detecting NFkappaB activation), successfully detected potentially toxic or immunomodulating bioactivities in cyanobacterial extracts. The level of biosensor activation was comparable to control cognate environmental toxins. Despite the fact that extracts were derived from microcystin-producing cyanobacterial blooms and contained active microcystins, biosensor-detected bioactivities were shown to be unrelated to microcystin levels. Experimental results suggest the involvement of environmental toxins (causing a response in NHRTOX) and lipopolysaccharides (LPS) or other cell wall components (causing a response in OXIBIOS) in the potentially harmful bioactivity of investigated extracts. These results demonstrate the need for further identification of cyanobacterial metabolites other than commonly studied cyanotoxins as sources of health risk, show the usefulness of cellular biosensors for this purpose and suggest a novel, more holistic approach to environmental monitoring.

1. Introduction

Microcystin-producing cyanobacterial blooms in freshwater constitute a significant public health threat to drinking water supplies as well as to bathers who use the afflicted water bodies for recreational purposes (WHO, 2003, 2011; Chorus, 2012). This phenomenon has been recognized by environmental quality regulatory authorities in many countries around the world (Chorus, 2012), by European Committee (Directive 2006/7/EC) and WHO (2003, 2011). Legislations and proposed limits relate primarily to the number of cells and biomass of cyanobacteria, and to the concentration of best known cyanotoxins, mainly microcystins and sometimes anatoxin or cylindrospermopsin. Microcystins directly from water samples are determined using several analytical and biochemical screening methods. The most widely used analytical methods include gas chromatography (GC/FID or GC/MS), thin layer chromatography (HPTLC), matrix-assisted laser desorption/ionization time of flight (MALDI-TOF), and different kinds of liquid chromatography (e.g. HPLC UV, PDA, FLD, LC/MS) (Lawton et al., 1994; Meriluoto et al., 1998; Sano et al., 2001; Welker et al., 2002; Jurczak et al., 2004; Zhang et al., 2004; Ortelli et al., 2008; Al-Sammak et al., 2013, 2014; Moreira et al., 2014). Additionally, immunobiotest ELIS, can be used as a screening method for estimation of microcystins concentrations (Metcalf and Codd, 2004; Lawton et al., 2010). Moreover, for

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http://dx.doi.org/10.1016/j.aquatox.2015.09.004
0166-445X/

the determination of biological activity (toxicity) of microcystins, the screening Protein Phosphatase Inhibition Assay was proposed (An and Carmichael, 1994; Sevilla et al., 2009; Garibo et al., 2014).

The Sulejów Reservoir, a major reservoir on the Pilica River in Central Poland, has been often used as a model in studies on cyanobacteria and their impact on water quality (Tarczyńska et al., 2001; Jurczak et al., 2004; Mankiewicz-Boczek et al., 2006; Izydorczyk et al., 2008; Gągała et al., 2014). Previous research in this waterbody focused on identification of cyanotoxins and toxin-producing cyanobacteria. Since *Microcystis* was identified as the predominant genus, and microcystins (a group of hepatotoxins) as toxins produced in the largest quantities, further work entailed development of methods for direct assay of microcystins and other main groups of cyanotoxins. Studies have also been performed on the biological effects at the cellular and biochemical level, devoted to cytotoxicity and genotoxicity of cyanobacterial extracts containing microcystins as well as inhibition of protein phosphatase (Mankiewicz et al., 2002, 2003; Palus et al., 2007). However, the cited studies have focused only on the role of major groups of cyanobacterial toxins, never on the possibility of interactions of other compounds included in the extracts, such as e.g., cell wall components: lipopolysaccharide (LPS), lipopeptides etc., with their respective molecular targets.

Presently, following some new reports on the activity of unknown endogenous toxic substances contained in cyanobacterial blooms (Sedmak and Šuput, 2002; Bláha et al., 2010; Štěpánková et al., 2011; Nováková et al., 2011, 2012; Sychrová et al., 2012), a holistic approach to water quality monitoring based on physiological effect of combined toxins and xenobiotics present in the cyanobacterial bloom seems to be a necessary complement for regulatory and monitoring purposes. Such an approach must involve the assessment of toxicity mechanisms on the organism, cellular and molecular level which are physiologically relevant to living components of the environment, especially to human health. This requires the development of new tools for composite bioactivity and environmental threat detection.

The use of reporter gene assays in environmental toxicology is more than two decades old, but only recently has their potential been appreciated for versatile and economical assays based on stable analyte-reponsive cell lines. Such tools, called "cellular biosensors", combine three major advantages when applied for environmental risk analysis: integration of the cellular response from many possible physiological targets of a complicated toxicant mixture, ability to pinpoint at the molecular level the specific signaling pathway/transcription factor affected by the toxicant, and the possibility of direct testing of environmental samples without a complicated chemical processing in miniaturized sample sizes (Michelini et al., 2013). Recently, at the Institute of Medical Biology PAS, cellular biosensors for assaying hormone disrupting and immunotoxic properties of xenobiotics have been developed and applied successfully for the ecotoxicological analysis of natural (fungal) toxins and actual complex pollutant mixtures (airborne particulate matter) (Wagner et al., 2011; Ratajewski et al., 2011, 2015).

Some research results are available in the literature concerning the potential use of biosensors as a tool for monitoring of the possible threat of cyanotoxins emerging during the bloom occurrence. Unfortunately, most were directed at specific types of main groups of toxins, such as microcystins or cylindrospermopsin (see reviews by Singh et al., 2012 and Weller, 2013). Pioneering studies from the laboratories of Ludek Bláha and Klára Hilscherová applied reporter gene-based biosensors to evaluation of endocrine disruption potential of cyanobacterial cell components, with estrogen-like and retinoid-like activity detected (Štěpánková et al., 2011; Sychrová et al., 2012; Jonas et al., 2014, 2015).

The aim of the present study was to elaborate a proof of concept for the application of cellular biosensors to analysis of toxicity of microcystin-containing cyanobacterial extracts. The specific goal was to test the relevance of microcystin-LR as the most commonly assayed cyanobacterial toxin to actual physiological responses of cells of human origin at the molecular signalling level, within the context of two main xenobiotic stress detection pathways: PXR and NFkappaB. PXR is a nuclear receptor functioning as a direct multispecific sensor of potentially toxic organic compounds, and its activation results in the upregulation of numerous detoxification and xenobiotic metabolism enzymes (Koutsounas et al., 2013). The NFkappaB pathway, a complex set of intracellular interactions resulting in stabilisation and nuclear translocation of transcription factors from the NFkappaB family, plays a key role in detection and response to several types of environmental stress (including oxidative stress) and can be activated both by specific receptor ligands and by disruption of intracellular homeostasis, leading e.g. to proinflammatory response (Tergaonkar, 2006). These pathways were chosen for the present study as hallmarks of cellular signalling response to dangerous xenobiotics (especially bacterial-derived ones). This approach together with a selection of well-characterized cyanobacterial sample types (extracts) allows potentially also for the identification of non-microcystin-mediated effects of cyanobacterial cell components with a direct relevance to human toxicology.

2. Material and methods

2.1. Collection of cyanobacterial samples

The study material was collected from cyanobacterial blooms in Sulejów Reservoir (a dam reservoir on the river Pilica), Central Poland. Currently, this waterbody serves as a storage reservoir with recreational function and is an alternative source of drinking water for the Łódź agglomeration. The samples used in the present study were collected in the summer of 2002 from surface water levels of three recreational bathing sites on the Sulejów Reservoir including: Zarzęcin (1), Tresta (2) and Smardzewice (3) (Fig. 1). Cyanobacterial blooms were collected using a 65 μm plankton net and concentrated in a laboratory using separators. All three samples collected from different sites contained *Microcystis aeruginosa* as the dominant species (>95% of the sample). The concentrated samples were frozen, lyophilized, homogenized by grinding and stored at -20 °C until analysis.

2.2. Preparation of cyanobacterial extracts

2.2.1. Crude cyanobacterial extracts

Cyanobacterial extracts were prepared as in our previous studies (Mankiewicz-Boczek et al., 2011); specifically, 1000 mg of freeze-dried cyanobacterial material for each sample were dissolved in 60 ml of 75% methanol and sonicated on ice for 10 min to release toxic compounds from cyanobacterial cells. Afterwards, samples were centrifuged for 10 min at 10,000× *g* at 4 °C. At the end, the supernatants were filtered using Whatman solid-state PTFE filters. The samples were subsequently divided into several portions and evaporated to dryness using vacuum centrifuge SC 110A SpeedVac Plus (Thermo-Savant). Evaporated extracts were dissolved again in 75% methanol with a subsample being taken for chromatographic microcystins analysis, combined together and another round of PTFE filter filtration and evaporation was applied. Prepared crude extracts (CE) were finally dissolved in DMSO with the concentration standardized to initial dry weight of cyanobacterial material.