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

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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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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.

Fig. 1. Map of locations from which cyanobacterial extracts were collected for the study; 1—Zarzęcin site; 2—Tresta site; 3—Smardzewice site.

2.2.2. Purified cyanobacterial extracts

For further purification, solid-phase extraction (SPE) on octadecyl columns (JT Baker) was performed on evaporated crude extracts. They were dissolved in 70% methanol and applied to methanol-preconditioned C18 columns. Elution was performed with 90% methanol with 0.1% trifluoroacetic acid. The obtained sample was evaporated, dissolved again in 75% methanol and filtered using Whatman solid-state PTFE filters. Prepared purified extracts (PE) were finally dissolved in DMSO with the concentration standardized to initial dry weight of cyanobacterial material. The SPE method was done according to Jurczaka et al. (2005).

2.3. Analysis of microcystins

Microcystins in crude and purified cyanobacterial extracts were determined as described by Mankiewicz-Boczek et al. (2011). Briefly, analysis was performed by HPLC with a diode array detection (DAD) detector (model 1100, Hewlett Packard) according to the method principle of Meriluoto and Codd (2005). The microcystins were analyzed in 75% methanol on chromatographic column LiChroCART^{TM} (55 mm × 4 mm) with Purospher^{TM} STAR RP-18e (3 μm), run at 40 °C. The mobile phase consisted of 0.05% trifluoracetic acid (A) and acetonitrile with 0.05% trifluoracetic acid (B) in the time gradient: 0 min 25% B, 5 min 70% B, 6 min 70% B, 6.10 min 25% B. Identification of microcystin peaks was based on the comparison of retention times and characteristic UV spectra with a maximum at 238 nm. Mass with standard microcystins (Enzo Life Sciences). The microcystin concentrations were calculated automatically by calibration curves prepared for standards of MC-RR and MC-LR (Calbiochem). The limit of detection (LOD) was 4 ng of microcystin per injection (20 μl). The limit of quantification (LOQ) was 10 ng of microcystin per injection (20 μl). The HPLC-DAD analysis of microcystins was done according to Jurczaka et al. (2005).

2.4. Protein phosphatase inhibition assay

The inhibition of protein phosphatase Type 1 (PP1) was assayed according to An and Carmichael (1994) with minor modifications (Meriluoto and Codd, 2005). Extract samples were incubated at 37 °C with 0.00167 U μl^{-1} of PP1 from rabbit skeleton muscle (New England BioLabs) and a 15 mM solution of the substrate p-nitrophenyl phosphate (pNPP) (Fluka) was used. The quantity of *p*-nitrophenyl (*p*NP) product after reaction of PP1 enzyme with pNPP substrate was measured after 2 h at 405 nm in a microplate reader (Labsystems). The product quantity decreased with increasing inhibition of PP1 activity.

2.5. Cell viability assay

Cell viability was assayed in all biosensor cell lines described below according to the resazurin method of O'Brian et al. (2000). Briefly, cells were seeded in 96-well plates at 10^4 per well and exposed to cyanobacterial extracts at varying concentrations for 24 h. Subsequently, cells were washed and resazurin was added to a concentration of 5 μM in culture medium with a further 1-h incubation to allow for resazurin reduction by viable cells. The fluorescence of reduced resorufin in the medium was determined using a microplate reader (PerkinElmer) at 530 nm excitation and 590 nm emission wavelengths. Fluorescence intensity was directly proportional to the percentage of remaining viable cells.

2.6. NHRTOX cellular biosensor assay

Generation of the biosensor cell lines for the NHRTOX assay is described in detail by Ratajewski et al. (2011). Briefly, hepatocellular carcinoma-derived cells (HepG2 cell line) were used for stable transfection with a reporter vector containing a minimal viral promoter and nine copies of a consensus PXR binding site (5′-GATCAGACAGTTCATGAAGTTCATCTAGATC-3′). A clonal cell line (NHRTOX-HepG2) was selected for the highest and most consistent rifampicin-induced (PXR-mediated) luciferase expression.

The PXR-responsive reporter cell line was used as a cellular biosensor of toxicity of cyanobacterial extracts. For a biosensor experiment, cells were seeded in 96-well plates at 1.5×10^4 cells per well and were incubated with various concentrations of cyanobacterial extracts or environmental toxins in cell culture

medium for 24 h. Subsequently, firefly luciferase reporter gene expression was determined by luminometric assay of luciferase activity in cell lysate on a microplate reader (PerkinElmer) as described in Ratajewski et al. (2006). The results were recalculated as percentage of control (cells exposed only to cell culture medium). As a control of biosensor functionality, each experiment also included cells exposed to 20 μM rifampicin (Sigma) and the induction ratio was verified to be stable. The environmental (fungal) toxin controls (aflatoxin B1, fumonisin and 9-cis retinoic acid) were all from Sigma.

2.7. OXIBIOS cellular biosensor assay

Generation of the biosensor cell lines for the OXIBIOS assay is described in detail by Wagner et al. (2011). Briefly, an NFkappaB-responsive reporter plasmid was prepared containing an artificial promoter sequence with six copies of a consensus NFkappaB binding site (5′-GGGAATTTCC-3′) and a minimal core promoter in a firefly luciferase-encoding vector. Subsequently, this vector was used for stable transfection into two cancer cell lines of different tissues of origin (ovarian epithelial—Sk-Ov-3 and hepatocellular—Hep3B), with stable clonal cell lines derived by antibiotic selection and selected to yield the highest luciferase expression enhancement ratio upon treatment with NFkappaB-activating stimuli.

The NFkappaB-responsive reporter cell lines were used as cellular biosensors of bioactivity of cyanobacterial extracts. Results were obtained using the Hep3B-originated OXIBIOS-Hep3B reporter cell line, which allows for specific detection of NFkappaB ac tivation in a cellular background typical for liver cells, and also in an ovarian cell background (OXIBIOS-Sk-Ov-3) which shows greater sensitivity and signal enhancement due to higher endogenous expression of NFkappaB pathway components. For a biosensor experiment, cells were seeded in 96-well plates at 10^4 cells per well and were incubated with various concentrations of cyanobacterial extracts or bacterial cell wall-derived compounds in cell culture medium for 24 h. Subsequently, firefly luciferase reporter gene expression was determined by luminometric assay of luciferase activity in cell lysate on a microplate reader (PerkinElmer) as described in Ratajewski et al. (2006). As a control of biosensor functionality, each experiment also included cells exposed to 1 ng ml^{-1} human recombinant TNFalpha (Sigma) and the induction ratio was verified to be stable. The bacterial cell wall component controls (*Escherichia coli* LPS, Pam3CSK4 and FSL-1) were all from Invivogen.

2.8. Data analysis

Biosensor experiments were performed in sextuplicate ($n = 6$), with three independent experiments (with separate biosensor cell seeding, on different days) each with duplicate parallels in microtitre plate wells. Luminescence values were recalculated as percentage of basal (control, non-exposed) biosensor activity (relative luciferase activity). Statistical significance of results of biosensor exposure were verified by analysis of variance, with subsequent identification of significant differences by Tukey's post-hoc test.

3. Results

In order to verify whether the procedure of cyanobacterial extract preparation allows their analysis by cellular biosensors, the toxicity of extracts to biosensor cells was determined using the resazurin viability test. All studied extracts (1-3CE, 1-3PE) were found to exert no effect on cellular viability at concentrations up to 2000 mg l^{-1} (all concentrations are standardized to the initial amount of dry cyanobacterial material) for all three biosensor cell lines: NHRTOX-HepG2, OXIBIOS-Hep3B and OXIBIOS-Sk-Ov-3 (data not shown). Thus, it was decided to use this concentration range (the highest obtainable technically) in further experiments in the present study.

The NHRTOX biosensor cell line, which detects activation of the PXR transcription factor, was found to respond in a concentration-dependent manner to exposure to cyanobacterial extracts (Fig. 2). The biosensor was activated by all studied extracts (CE and PE) within the tested concentration range, with the strongest response at the concentration of 2000 mg l^{-1} being ca. 1.5-fold for crude extracts and ca. 1.8-fold for purified extracts. The highest extent of activation was weaker than biosensor response to the cognate control activator (20 μM rifampicin—relative luciferase activity 244 ± 4%). In comparison, some non-cyanobacterial (fungal) environmental toxins tested in parallel for NHRTOX biosensor activation exhibited a stronger effect (10 μM aflatoxin B1—282 ± 6%, 1 μm 9-cis retinoic acid—438 ± 2%), while others had no effect at all (10 μM fumonisin B1—99 ± 3%).

A different pattern of response was seen in OXIBIOS biosensor cell lines, which detect activation of the NFkappaB transcription factor. The OXIBIOS-Hep3B cell line showed a clear difference in effect of crude and purified extracts, with negligible differences between extracts from different sampling sites (Fig. 3). Specifically, crude extracts showed a modest biosensor activation (in the range of 1.25-fold) at concentrations up to 500 mg l^{-1}, with inconsistent and probably non-specific effects at higher concentrations; while purified extracts showed a consistent inhibition of basal biosensor activity (up to ca. 0.75-fold). The OXIBIOS-Sk-Ov-3 cell line presented a similar picture, with some differences in details (Fig. 4). Crude extracts caused activation of the biosensor by up to 1.3-fold (at concentrations higher than for the OXIBIOS-Hep3B cell line, i.e. 1000 and 2000 mg l^{-1}), two of the purified extracts (2PE and 3PE) caused very weak inhibition of basal activity, while the third (1PE) activated the biosensor to a similar extent as its crude counterpart. All of these effects were significantly weaker than the strong induction seen for both of these biosensor cell lines using the cognate ligand (20 ng ml^{-1} TNFalpha).

In order to provide suggestions about the molecular mechanism of observed OXIBIOS activation, cyanobacterial extracts (CE and PE) were applied to OXIBIOS-Sk-Ov-3 biosensor cells together with a low concentration of the cognate ligand acting by a well-defined pathway (1 ng ml^{-1} TNFalpha, activating the NFkappaB pathway via the TNFR receptor). The activating effect of crude extracts was found to be independent and additive to TNFalpha action, with enhancement of activation by a similar ratio (ca. 1.2-fold) as for non-TNFalpha-activated cells (Table 1). The purified extracts showed no activating potency in this test. Several standard bacterial cell wall components, known to activate innate immune signaling, also activated the OXIBIOS-Sk-Ov-3 biosensor cells, with *E. coli* LPS showing no statistically significant effect and synthetic lipopeptides having a much stronger effect (Table 2).

Since all extracts were obtained from cyanobacteria known to produce microcystins, in order to test the possibility that these cyanotoxins might be responsible for some or all of the observed biosensor effects, microcystin levels in extracts were assayed. All extracts contained measurable amount of various microcystin variants, however there were large differences (>10 fold) between samples (Table 3). As expected, the purification procedure led to a consistently lower content of microcystins in purified extracts compared to crude extracts. HPLC-DAD chromatograms of the specific crude and purified samples used in the present study are presented in Fig. 5. Microcystins in all extracts were found to be active in a phosphatase inhibition bioassay, with concentration-dependent increase in PP1 inhibition (Table 3).

Finally, standard microcystin-LR was applied to all tested biosensor cell lines to directly assay its potency in these tests. Pre-

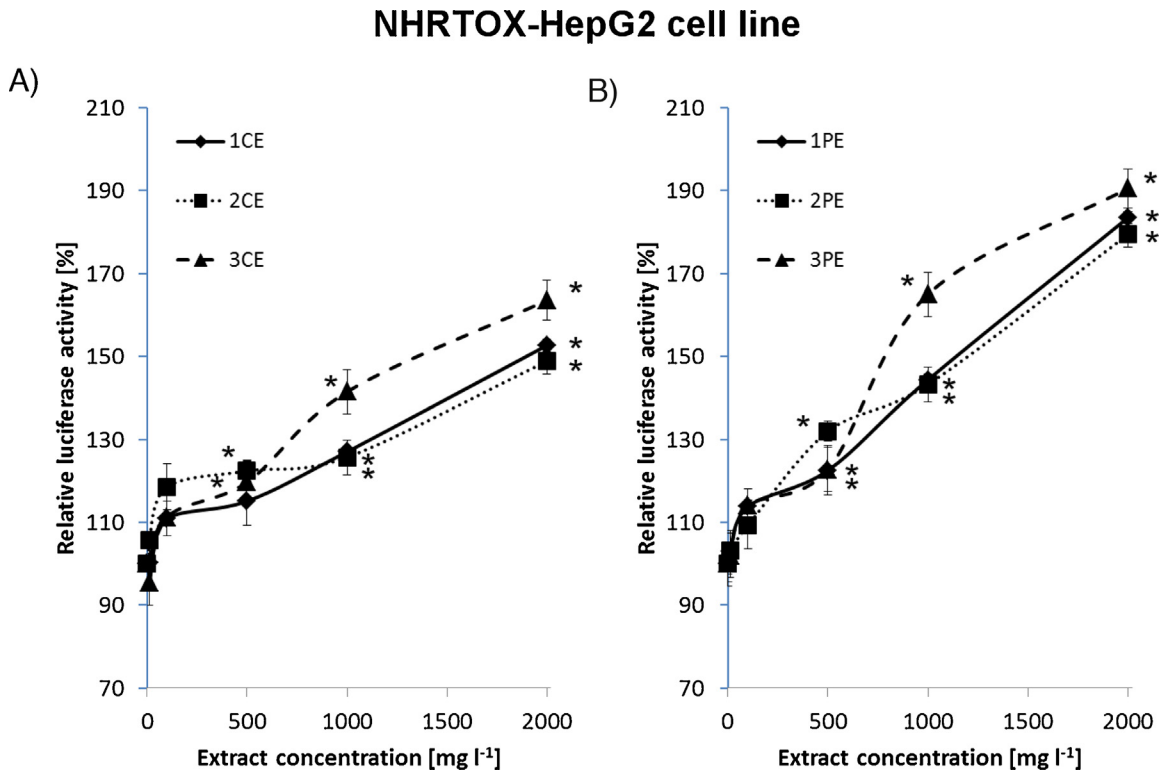

Fig. 2. Effect of exposure to cyanobacterial extracts on the activity of the NHRTOX biosensor (NHRTOX-HepG2 cell line), expressed as relative activity of the luciferase reporter gene in cellular lysates after 24 h of treatment. Panel A—crude cyanobacterial extracts (CE); panel B—purified cyanobacterial extracts (PE). Data presented as mean ± S.E.M., $n = 6$. *Significantly different from control, $p < 0.05$.

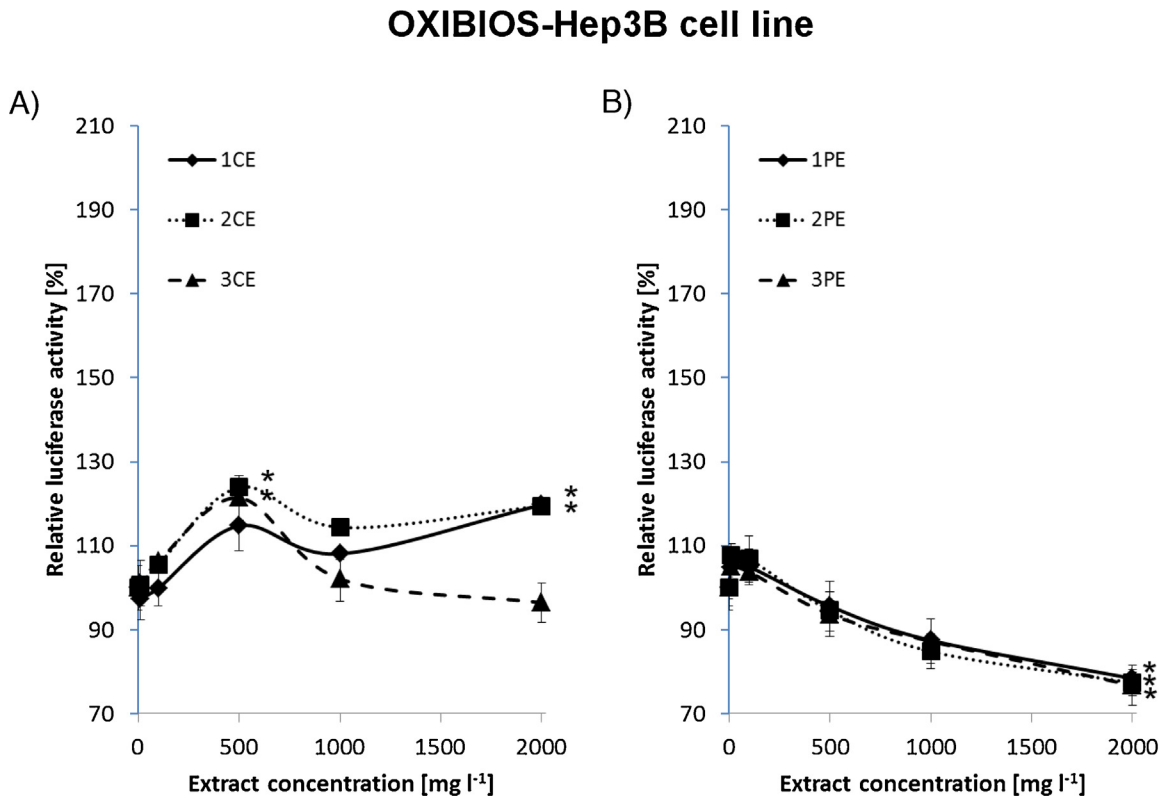

Fig. 3. Effect of exposure to cyanobacterial extracts on the activity of the OXIBIOS biosensor (OXIBIOS-Hep3B cell line), expressed as relative activity of the luciferase reporter gene in cellular lysates after 24 h of treatment. Panel A—crude cyanobacterial extracts (CE); panel B—purified cyanobacterial extracts (PE). Data presented as mean ± S.E.M., $n = 6$. *Significantly different from control, $p < 0.05$.

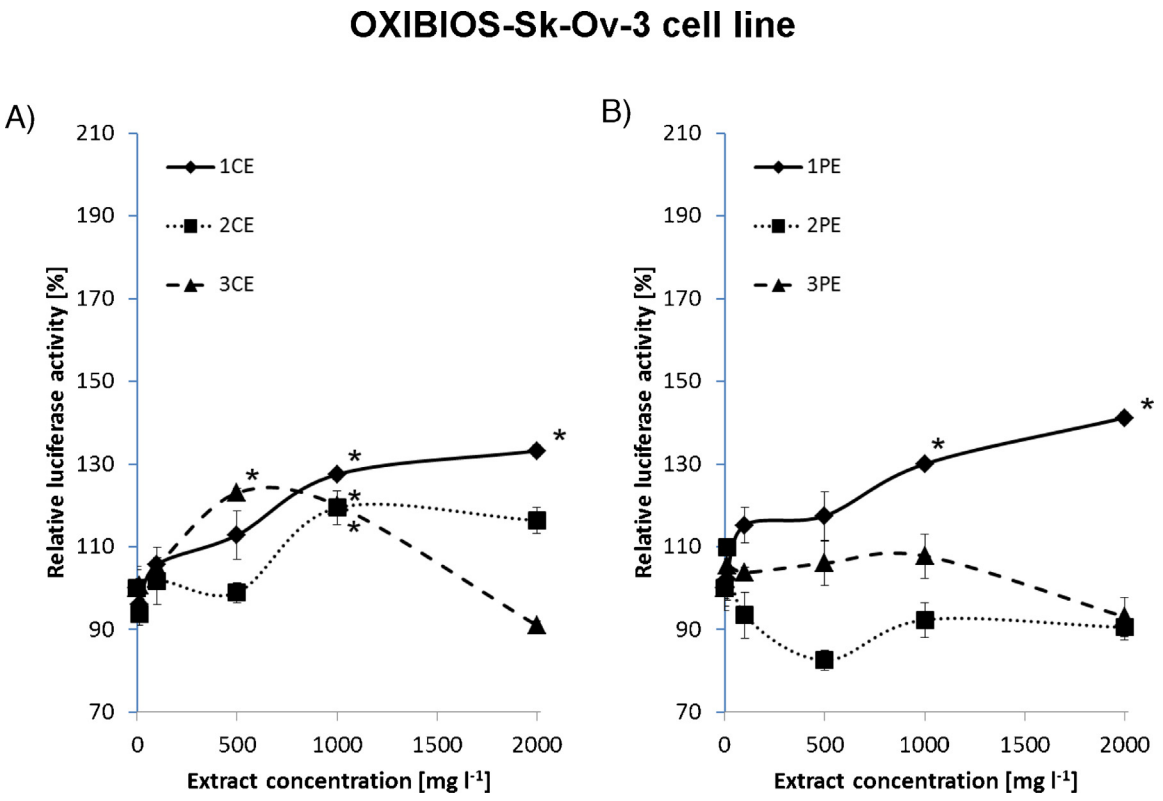

Fig. 4. Effect of exposure to cyanobacterial extracts on the activity of the OXIBIOS biosensor (OXIBIOS-Sk-Ov-3 cell line), expressed as relative activity of the luciferase reporter gene in cellular lysates after 24 h of treatment. Panel A—crude cyanobacterial extracts (CE); panel B—purified cyanobacterial extracts (PE). Data presented as mean ± S.E.M., $n = 6$. * Significantly different from control, $p < 0.05$.

Table 1
Activity of the OXIBIOS biosensor (OXIBIOS-Sk-Ov-3 cell line) upon exposure to TNFalpha alone or together with cyanobacterial extracts, expressed as relative activity of the luciferase reporter gene in cellular lysates after 24 h of treatment.

TNFalpha [1 ng ml^{-1}]	+	+	+	+	+	+	+
Cyanobacterial extract [1 g l^{-1}]	–	+(1CE)	+(2CE)	+(3CE)	+(1PE)	+(2PE)	+(3PE)
Relative luciferase activity [%]	188 ± 3	221 ± 7(*)	213 ± 8(*)	219 ± 5(*)	201 ± 6	191 ± 5	195 ± 7

CE—crude extract, PE—purified extract, "+"—present in treatment, "–"—absent from treatment. Data presented as mean ± S.E.M., $n = 4$.
* Significantly different from control (no extract), $p < 0.05$.

Table 2
Activity of the OXIBIOS biosensor (OXIBIOS-Sk-Ov-3 cell line) upon exposure to control bacterial cell wall components, expressed as relative activity of the luciferase reporter gene in cellular lysates after 24 h of treatment.

Treatment	Control	LPS1 μg ml^{-1}	Pam3CSK4 500 ng ml^{-1}	Pam3CSK4 5 μg ml^{-1}	FSL-1100 ng ml^{-1}	FSL-11 μg ml^{-1}
Relative luciferase activity [%]	100 ± 11	121 ± 8	171 ± 17(*)	278 ± 14(*)	397 ± 27(*)	4280 ± 128(*)

Data presented as mean ± S.E.M., $n = 6$.
* Significantly different from control, $p < 0.05$.

Table 3
Microcystin content in cyanobacterial extracts and PP1 inhibition. Concentration of various microcystin variants is presented in μg g^{-1} (dry weight); the result of phosphatase inhibition assay is presented as the percentage of inhibited activity of PP1 after treatment.

Sampling site	Cyanobacterial extract	Microcystins [μg g^{-1} dry weight]					PPIA*[%]
		MC-RR	MC-YR	MC-LR	Other	Total	
1	1CE	277.80	1080.02	572.12	405.30	2335.24	84
	1PE	163.88	612.73	330.16	232.12	1338.89	84
2	2CE	292.34	353.95	251.38	58.69	956.36	82
	2PE	144.59	175.21	130.23	20.21	470.24	72
3	3CE	23.90	40.85	41.37	0.00	106.12	66
	3PE	24.63	33.98	32.31	0.00	90.92	67

1—Zarzęcin site; 2—Tresta site; 3—Smardzewice site; CE—crude extract containing microcystins; PE—purified extract containing microcystins; MC-RR, MC-YR, MC-LR—microcystins variants; PPIA*—protein phosphatase inhibition assay in the extract at the total concentration of 2000 mg/L.

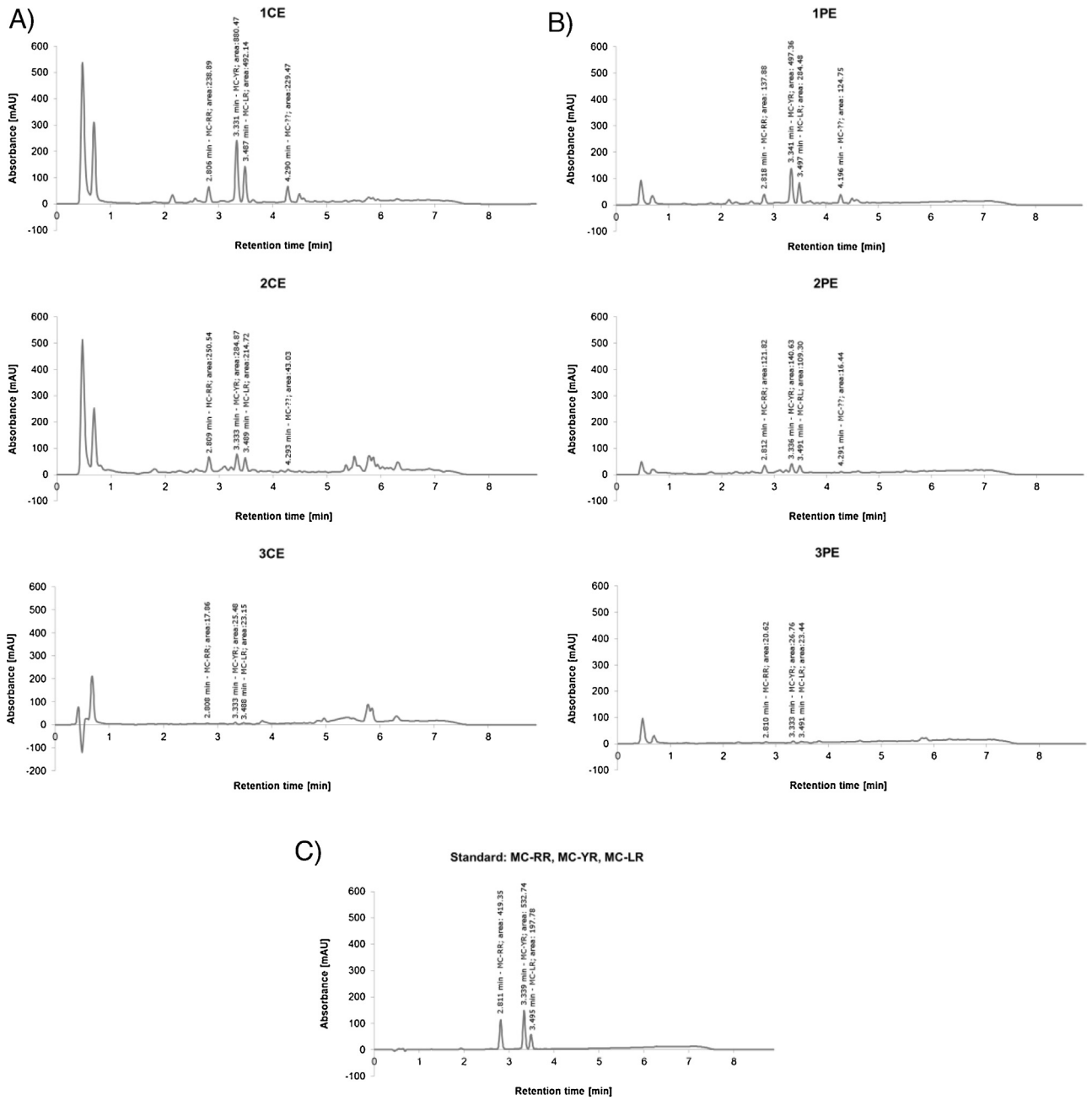

Fig. 5. Chromatograms of microcystins separated by HPLC-DAD for (A) crude extracts: 1C, 2CE, 3CE, and (B) purified extracts: 1PE, 2PE, 3PE, analysed in the present study. Additionally in (C) the separation of microcystin standards for MC-RR, MC-YR and MC-LR are provided.

liminary viability tests confirmed no toxicity of microcystin-LR for any of the three cell lines at concentrations up to 500 ng ml^{-1} (data not shown). At these concentrations, microcystin-LR exposure did not activate any of the biosensor cell lines: NHRTOX-HepG2, OXIBIOS-Hep3B and OXIBIOS-Sk-Ov-3 (Fig. 6).

4. Discussion

We have successfully applied reporter gene-based cellular biosensors for analysis of cyanobacterial extracts from environmental samples. This analytical approach is a cutting edge tool in aquatic toxicology and arouses increasing interest in ecotoxicological circles, as several recent publications have shown (Singh et al., 2012; Weller, 2013). Within the last two years, the cellular biosensor method has helped to identify potential developmental toxicity effects of cyanobacterial extracts due to their endocrine disruption potency (Jonas et al., 2014, 2015). Endocrine disruption has been exhaustively studied as molecular mechanism of environmental toxicity for several decades and reporter gene assays are an obvious tool for its evaluation (Baker, 2001). On the other hand, it is becoming increasingly clear that activation of cellular signaling pathways unrelated to classical hormones, especially stress response pathways and immune/xenobiotic response mechanisms, is another major area of environmental toxicity which needs to be studied (Gennari et al., 2005; Hartung and Corsini, 2013), and cellular biosensor tools may be developed for use in this topic as well. We have developed original biosensors for two major stress response pathways (NFkappaB and PXR) and proved their applicability for ecotoxicological studies (Wagner et al., 2011; Ratajewski et al., 2011). In this study, the approach to toxic cyanobacterial blooms,

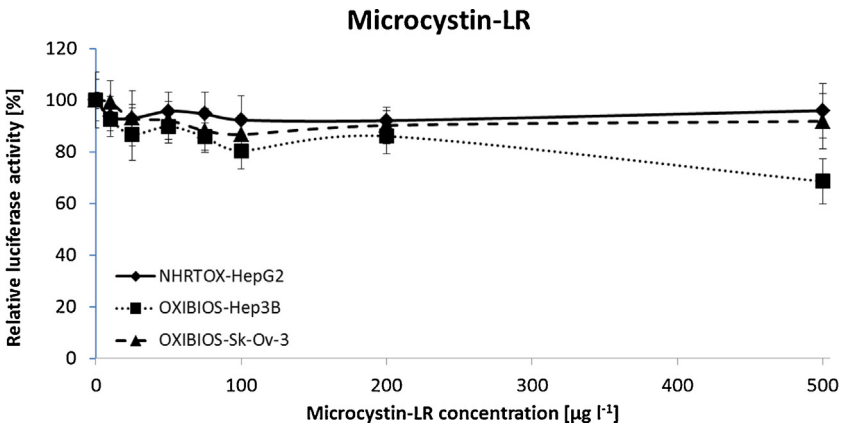

Fig. 6. Effect of exposure to microcystin-LR on the activity of NHRTOX biosensor (NHRTOX-HepG2 cell line) and OXIBIOS biosensor (OXIBIOS-Hep3B and OXIBIOS-Sk-Ov-3 cell lines), expressed as relative activity of the luciferase reporter gene in cellular lysates after 24 h of treatment. Data presented as mean ± S.E.M., $n = 6$.

where the paradigm of toxicity due to classical, well-studied toxin groups (especially hepatotoxins represented by microcystins) is recently giving way to a more physiologically oriented, complex bioactivity-based toxicity concept, has been extended.

Since cellular biosensors respond to actual biological stimuli rather than a chemical composition of the extract, they allow to identify unexpected activities even in the presence of compounds with another mode of action. This is especially important for cyanobacterial extract studies, where the majority of studies have dealt with chemical assays of microcystins, determining the relative abundance and bioactivity of their chemical variants (with microcystin-LR often cited as the strongest toxicity factor). The results presented in this study show unequivocally that it was possible to identify bioactivities with potentially toxic effects which are not related to microcystins or microcystin-like compounds. This strong conclusion could be drawn due to selection of the tested samples: microcystin-containing blooms with demonstrably different microcystin levels were assayed, and for each of them the bioactivity of purified and crude extracts enriched in microcystin-like compounds was compared. Thus, toxicity hallmarks due to bacterial cell components other than microcystins are evident in our results.

The molecular setup of the two biosensors used in this study, responding to different types of xenobiotic bioactivity, allowed for a potentially simultaneous identification of different bioactive molecular species among cyanobacterial metabolites. Moreover, the comparison of the results before and after the application of a strictly defined extraction procedure provides a ground for speculation on the biochemical composition of such metabolites. Specifically, it was possible to demonstrate the activation of NHRTOX by a compound which was present in similar amounts in all the tested extracts, regardless of a sampling site or a purification method (Fig. 2). Actually, the extracts with lowest microcystins content (3CE, 3PE) showed an even slightly stronger activatory potency that the remaining ones, again ruling out the identity of the bioactive compound as microcystin. Since all the samples derive from the same waterbody, the most probable explanation for the molecular identity of PXR ligand in this case is an environmental toxin which co-purifies with the cyanobacterial extract during its preparation (including the SPE phase). What strengthens the argument for the derivation of the bioactive compound from a source external to cyanobacteria themselves, is the fact that some fungal toxins have similar effects to cyanobacterial extracts at high concentrations. However, it is also possible that the observed effect is due to a hitherto unidentified toxin-like xenobiotic present in all *Microcystis* blooms at a stable level.

On the other hand, the OXIBIOS biosensor was consistently activated by a compound present in crude extracts and removed during the SPE procedure, except for a single SPE-purified extract (1PE) for the OXIBIOS-Sk-Ov-3 cell line (Fig. 3 and 4). The level of this bioactive compound varies slightly from sample to sample. The fact that it was able to activate the NFkappaB signaling pathway in cells from two different cellular backgrounds as well as the level of activation suggests NFkappaB activation being due to the engagement of pattern recognition receptors—specialized cell membrane proteins interacting with microbial metabolites, especially bacterial cell wall components (Johannessen et al., 2013). Since cyanobacterial cell wall is expected to make a large contribution to the biomass of a crude extract, it makes the hypothesis of its responsibility for the observed effect very plausible. It has to be taken into account that the NFkappaB signaling pathway is a general cellular stress response pathway, so the interpretation of results is complicated by non-specific effects at higher extract concentrations. Also, in one of the purified extracts (1PE) a compound was still present activating one of the biosensor cell lines (Fig. 4B). Since this extract was richest in various microcystin variants (Table 3), this result may be caused by microcystins other than microcystin-LR. Alternatively, another environmental toxin may be specifically present only in this extract or its collection site. It is worth noting that inhibition of the NFkappaB activity in the OXIBIOS-Hep3B cell line (Fig. 3B) was observed after removal of the potential activatory compound mentioned above (1PE-3PE). This might suggest the presence of another cell-type-specific bioactive compound and provide an explanation for differences in extent of activation of both OXIBIOS biosensor types by crude extracts. Since natural products, including microbial-derived compounds, often show an inhibitory effect towards the NFkappaB axis (Wang et al., 2015), this effect is not unexpected, and its apparent cell type specificity may be related to larger complexity of NFkappaB signaling in cells of hepatic origin (Ko et al., 2013).

Cyanobacteria are Gram-negative eubacteria, and the best characterized cell wall component of these microbes activating pattern recognition receptors is lipopolysaccharide (LPS). Thus, the present result may, with high probability, be interpreted as pointing to the identity of the NFkappaB-activating compound in crude cyanobacterial extracts as LPS, an LPS-like molecule or a different cell wall component, such as a lipopeptide. Of course, without a detailed chemical fractionation study, it is impossible to exclude any of these possibilities. Several lines of evidence, including further experiments in this study, support this conclusion. Both OXIBIOS cell lines applied here respond to authentic bacterial LPS and express the necessary pattern recognition receptor TLR4 (d'Adhemar et al., 2014; Wang et al., 2013). In our study, the scale of their response

to *E. coli* LPS was smaller than observed response to cyanobacterial extracts (Table 2), in contrast to other cell wall components which bind to other pattern recognition receptors and provoke a much stronger response. Another typical way of environmental toxins triggering NFkappaB activation would be to interfere with TNFalpha signaling (Johannessen et al., 2013)—however, it was shown here that cyanobacterial extracts activate the NFkappaB pathway in an additive manner to TNFalpha, demonstrating a mechanism distinct from TNFalpha receptor action (Table 1). Finally, the SPE procedure used to prepare the purified extract may most probably be expected to result in the removal of most or all of LPS-like substances, explaining the conspicuous difference in the bioactivity of crude and purified extracts.

Since late 2000s there has been a growing body of evidence that many toxic effects of cyanobacteria are independent on classical toxins. Numerous studies using bioassays (Bláha et al., 2010), as well as recent work with cellular biosensors of endocrine disruption (Jonas et al., 2014, 2015) have also concentrated on demonstrating that microcystins are not responsible for the biological effects observed in these experiments. The present results also showed that focusing on one toxic compound (in environmental monitoring practice this is most commonly microcystin-LR) is not a valid approach to the holistic evaluation of environmental risk for human health from cyanobacterial blooms. Even microcystins consist of more than 100 different variants which differ between each other in terms of their toxicity for organisms (Vesterkvist et al., 2012). There are other main groups of cyanotoxins apart from hepatotoxic microcystins (neurotoxins or cytotoxins), but they are irrelevant for experiments described here since they are never produced by *Microcystis* blooms in Sulejów Reservoir (Jurczak, 2006). Thus, the emerging paradigm shift in environmental toxicity monitoring is supported by conclusions of the present study, especially since data presented here point towards a major role of cell wall components—and specifically LPS—in potential health threats (such as immunotoxicity) from cyanobacteria. This result strengthens the idea that regulatory approaches are in strong need of revision, and cyanobacterial blooms classified for practical purposes as "microcystin-producing toxic blooms" may have severe health impacts that are not dependent on microcystins concentration (measured as recommended by regulatory agencies including WHO).

5. Conclusions

Two cellular biosensor types applied in this study (NHRTOX detecting PXR activation and OXIBIOS detecting NFkappaB activation) successfully detected potentially toxic or immunomodulating bioactivities in cyanobacterial extracts. 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 (NHRTOX) and LPS or other cell wall components (OXIBIOS) in the potentially harmful bioactivity of investigated extracts. These results demonstrate the need for further identification of cyanobacterial metabolites other than main groups of 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.

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