Dissertation thesis

Biochemical mechanisms of cyanobacterial toxicity

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ABSTRACT

Massive development of cyanobacteria as result of water trophization by nutrients due to anthropogenic activity is a worldwide problem. These photoautotrophic organisms are able to produce wide spectrum of chemically and structurally different compounds with various biologically active properties such as cytotoxic, biocidous, genotoxic, tumor promoting or anticancer, where some of them are considered as human health hazardous. Most studies investigated identified cyanobacterial metabolites, especially cyanotoxins (microcystins and cylindrospermopsin). But there is a cumulating evidence of cyanobacterial toxicity not associated with known cyanotoxins, which indicates existence of novel metabolites with toxic properties similar to cyanotoxins.

This thesis is divided into two major thematic topics covering interactions between toxic cyanobacteria and other aquatic autotrophic organisms such as algae as well as carcinogenicity of both known cyanotoxins and unknown metabolites.

Presented results are based on four publications and two manuscripts, most of the studies deal with cyanobacterial toxicity as complex mixtures (intracellular and extracellular), which is more relevant scenario in contrary to traditional toxicological studies with isolated cyanobacterial metabolites.

In the first part of the thesis the role of microcystins as signaling molecule or allelochemical and their effects on growth and cell differentiation in phytoplanktonic species are discussed. Also comparison of modulations on biochemical markers caused by complex mixtures (intra- and extra-cellular) and pure microcystins in green alga were studied. The allelopathic property of microcystins was not confirmed by our results (no growth effects on green algae and cyanobacteria at environmentally relevant concentrations). Observed effects caused by extremely high concentrations (1000-25000 μg/l) were species-specific and demonstrated various responses of photoautotrophic organisms to two microcystin congeners (MC-LR and MC-RR). Interestingly, cyanobacterial extract with microcystins concentration at relevant levels (2-20 μg/l) influenced cell differentiation, thus partially play to microcystin signaling hypothesis. More natural situation, where is microcystin present in mixture of other metabolites, evinced experiments with complex cyanobacterial mixtures (intra- and extra-

cellular) and pure microcystins, where all samples altered measured biochemical parameters but with different effects.

Second part of the thesis is focused on evaluation of possible carcinogenic potential of complex mixtures of various cyanobacterial strains (intra- and extracellular) using *in vitro* bioassay with established tumor promotion marker - gap junctional intercellular communication (GJIC). Tumor promoting potentiation of co-exposure of cyanobacteria and anthropogenic pollutants as well as characterization and description of novel tumor promoting compound extracellularly produced by cyanobacterium *Cylindrospermopsis raciborskii* were also devoted in this work. Our results showed species–specific and strain-specific production of tumor promoting compounds different from known cyanotoxin (microcystins and cylindrospermopsin). The strongest effects were caused by extracellular mixture of *C. raciborskii* which was further fractionated and characterized causative agents. After the detailed analyses, a compound with m/z 321.2 was identified as potential agent causing inhibition of GJIC as it was present in active but not non-active fractions.

The present thesis contributes to our understanding of molecular, cellular and biochemical mechanisms behind the toxicity of various cyanobacterial metabolites including known toxins as well as newly identified metabolites. The importance of studying naturally relevant mixtures of toxic compounds is highlighted.

ABSTRAKT

Masivní rozvoj sinic je celosvětovým problémem zejména díky antropogenní trofizaci vod. Tyto photoautotrofní organismy jsou schopny produkovat široké spektrum rozdílných látek, jak chemickou strukturou, tak i biologicky aktivními vlastnostmi, např. antibakteriální, cytotoxické, biocidní, genotoxické, nádorově promoční nebo naopak protinádorové. Některé z těchto metabolitů jsou považovány za zdravotně nebezpečné, jedná se zejména o cyanotoxiny (microcystin a cylindrospermopsin). Avšak hromadící se studie predikují existenci nových metabolitů, které mají podobný toxický potenciál jako cyanotoxiny.

Tato dizertace je rozdělena do dvou tématických kapitol zaměřených na interakce mezi toxickými sinicemi s dalšími vodními autotrofními organismy (př. řasy) a na karcinogenitu způsobenou známými cyanotoxiny ale i neznámými metabolity.

Experimentální část práce je zpracována do čtyř vědeckých článků a dvou manuskriptů. Většina uvedených studií se přistupuje k toxicitě sinic jako ke komplexním směsím (intracelulární a extracelulární) ve srovnání s toxicitou jednoho cyanotoxinu (microcystinu nebo cylindrospermopsinu).

V první části dizertace je zkoumána role microcystinu jako signální molekuly nebo látky s alelopatickým účinkem. Také bylo sledováno ovlivnění biochemických parametrů u zelené řasy způsobené komplexními směsmi sinic (intra- a extracelulární směsi). Alelopatické vlastnosti microcystinů nebyly našimi výsledky potvrzeny (žádné ovlivnění růstu zelených řas a sinic na environmentálně relevatních koncentracích). Pozorované účinky byly způsobeny extrémně vysokými koncentracemi microcystinů (1000-25000 µg/l), odpovědi fotoautotrofních organismů se lišily mezi druhy a také dle typu microcystinu (MC-LR nebo MC-RR). Avšak sinicový extrakt s relevatními hladinami microcystinů (2-20 µg/l) vyvolal změny v buněčné diferenciaci jednoho druhu sinice, což částečně nahrává teorii o jeho funkci signální molekuly. Experiment reflektující reálnou situaci v přírodě, kde je microcystin přítomen ve směsi s dalšími metabolity, srovnává vliv komplexních směsí sinic (intra- a extracelulární) s účinkem čistého microcystinu na biochemické parametry u zelené řasy. Všechny

testované směsi ovlivnily sledované biomarkery, avšak byly pozorovány různé účinky.

Další experimentální část je zaměřena na zjištění možného potenciálu vyvolat rakovinu u komplexních směsí různých kmenů a druhů sinic pomocí biotestu založeného na ovlinění mezibuněčné komunikace štěrbinovými spoji (gap junctional intercellular communication-GJIC), jejíž inhibice je zavedený parametr vedoucí k promoci nádorů. Následuje studie sledující možnou potenciaci účinků směsi extraktů sinic a antropogenních polutantů pomocí GJIC testu. Další experiment detailně charakterizuje a popisuje neznámou látku/látky s nádorově promočními vlastnostmi, která/é jsou extracelulárně produkované sinicí *Cylidrospermopsis raciborskii*. Naše výsledky ukázaly, že produkce nádorově promočních látek (odlišných od známých metabolitů-microcystinu a cylindrospermopsinu) je druhově specifická a take se liší mezi různými kmeny sinic. Nejsilnější účinek na GJIC byl způsoben extracelulární směsí *C. raciborskii*, který byl dále frakcionován a charakterizován. Po podrobné chemické analýze byla objevena látka o molekulové hmotnosti 321,2, která je přítomna jen v aktivní frakci (inhibující GJIC) a také je pravděpodobně zodpovědna za utlumení GJIC.

Tato dizertace přispívá k pochopení molekulárních, buněčných a biochemických mechanismů spojených s toxicitou, která je způsobena různými metabolity sinic- nejen známými toxiny ale také nově identifikovanými metabolity. V této práci je také zmíněna důležitost studia zabývajícího se přírodně relevantními směsmi toxických látek produkovaných sinicemi.

LIST OF ABBREVIATIONS

ATP	adenosin triphosphate
BAC	biologically active compounds
CYN	cylindrospermopsin
CYP450	cytochrome 450
d.w.	dry weight
Da	Dalton
DNA	deoxyribonukleic acid
ERK	extracellular receptor kinase
GJIC	gap-junctional intercellular communication
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GST	glutathione-S-transferase
i.p.	intra peritoneal
LC_{50}	lethal concentration causing 50% of deaths
MAPK	mitogen-activated protein kinase
MCs	microcystins
MW	molecular weight
NRPS	non-ribosomal peptide synthetase
PAHs	polycyclic aromatic hydrocarbons
PAR	photosynthetically active radiation
PCBs	polychlorinated biphenyls
PKSs	polyketide synthases
PPs	protein phosphatases
ROS	reactive oxygen species
SOD	superoxide dismutase
UVR	ultra violet radiation

A LIST OF ORIGINAL ARTICLES AND THE AUTHOR´S CONTRIBUTIONS

This thesis is based on the publications listed below:

Paper I Babica, P., Hilscherova, K., Bartova, K., Blaha, L., and Marsalek, B. (2007). Effects of dissolved microcystins on growth of planktonic photoautotrophs. **Phycologia** 46, 137-142.

Kateřina Nováková participated in the experiment design and performation, and contributed to the manuscript revision and correction.

Paper II Bártová, K., Hilscherová, K., Babica, P., and Maršálek, B. (2011). Extract of Microcystis water bloom affects cellular differentiation in filamentous cyanobacterium Trichormus variabilis (Nostocales, Cyanobacteria). **Journal of Applied Phycology** 23, 967-973.

Kateřina Nováková performed the cultivations and cell differentiation experiments, evaluated and interpreted data, prepared and finalized the manuscript.

Paper III Bártová, K., Hilscherová, K., Babica P., Maršálek B., Bláha, L. (2011). Effects of microcystin and complex cyanobacterial samples on the growth and oxidative stress parameters in green alga Pseudokirchneriella subcapitata and comparison with the model oxidative stressor - herbicide paraquat. **Environmental Toxicology** 26, 6, 641-648.

Kateřina Nováková performed samples cultivation and preparation, provided the experiments, analysed biochemical parameters linked with oxidative stress, evaluated and interpreted data, prepared and finalized the manuscript.

Paper IV Nováková, K., Babica, P., Adamovský, O., and Bláha, L. (2011). Modulation of gap-junctional intercellular communication by a series of cyanobacterial samples from nature and laboratory cultures. **Toxicon** 58,1,76-84.

Kateřina Nováková performed samples cultivation and preparation of biomasses and exudates, provided the experiments, evaluated and interpreted data, prepared and finalized the manuscript.

Paper V Nováková, K., Kohoutek, J., Adamovský, O., Babica, P., Jálová, V., Hilscherová, K., Bláha, L. Towards identification and characterization of novel tumor promoting metabolites produced in *Cylindrospermopsis raciborskii*. **Manuscript in preparation for Toxicological Sciences**

Kateřina Nováková participated in experimental design and performation of *in vitro* experiments with inhibition of gap-junctional communication, carried out cyanobacterial cultivations, preparations of exudates and its analytical fractions, and prepared manuscript.

Paper VI Nováková, K., Babica, P., Blaha, L. (2011). Potentiation effects in mixtures of toxic cyanobacteria and anthropogenic contaminants fluorathene and PCB 153 on an *in vitro* marker of tumor promotion - gap junctional intercellular communication. **Manuscript submitted to T**.

Kateřina Nováková participated in experimental design, performed samples cultivation and preparation, provided the experiments, evaluated and interpreted data and prepared the manuscript.

TABLE OF CONTENT

OBJECTIVES AND AIMS

Cyanobacteria are well known due their world wide occurrence in eutrophicated water bodies. Why do cyanobacteria dominate in majority of water bodies world wide? This is the question which is often asked by public as well as scientists. In spite of various hypotheses, the exact reason of cyanobacterial ecological success is not fully elucidated. Their adaptive strategies are evolutionary uncontested.

Besides various factors underlying cyanobacterial ecological prosperity (e.g. fixation of atmospheric oxygen, photosynthetic efficiency, adaptability and buoyancy in water column), scientists are deeply fascinated with diversity of cyanobacterial metabolite production.

Identified cyanotoxins (microcystins and cylindrospermopsin and some others) belong to the most intensively studied cyanobacterial metabolites because of their toxicity and human health risks but recent studies had shown similar or stronger toxic effects also for unknown metabolites.

The main goal of this study was the endeavour to bring new information about unknown effects of identified compounds (microcystin and cylindrospermopsin) as well as to study and characterize new potentionally toxic metabolites and describe their effects and mode of action. The research addressed following tasks:

- examination of the disputable allelopathic properties of microcystin on green algae at different levels – molecular, cellular and organismal level and comparison of effects caused by various cyanobacterial mixtures (intra/extracellular) with pure cyanotoxins

- evaluation of tumor promoting potential of complex cyanobacteria, extracellulary produced metabolites in comparison to pure compounds using established *in vitro* bioassay (modulation of gap-junctional intercellular communication - GJIC)

- fractionation of cyanobacterial mixture with the strongest *in vitro* tumor promoting potency using GJIC bioassay, effect directed analyses and detailed characterization of causative agent/s

- assessment of possible interactions of cyanobacteria with other anthropogenic pollutants commonly presented in water bodies using *in vitro* assay (modulation of GJIC)

Chapter 1

BIOLOGICALLY ACTIVE COMPOUNDS – INTERACTION BETWEEN ALGAE AND CYANOBACTERIA

This chapter contains an introduction and original results, which are based on following publications:

Paper I Babica, P., Hilscherova, K., Bartova, K., Blaha, L., and Marsalek, B. (2007). Effects of dissolved microcystins on growth of planktonic photoautotrophs. **Phycologia** 46, 137-142.

Paper II Bártová, K., Hilscherová, K., Babica, P., and Maršálek, B. (2011). Extract of Microcystis water bloom affects cellular differentiation in filamentous cyanobacterium Trichormus variabilis (Nostocales, Cyanobacteria). **Journal of Applied Phycology** 23**,** 967-973.

Paper III Bártová, K., Hilscherová, K., Babica P., Maršálek B., Bláha, L. (2011). Effects of microcystin and complex cyanobacterial samples on the growth and oxidative stress parameters in green alga *Pseudokirchneriella subcapitata* and comparison with the model oxidative stressor - herbicide paraquat. **Environmental Toxicology** 26, 6, 641-648.

1.1 Cyanobacteria and production of biologically active compounds (BAC)

Cyanobacteria as evolutionary ancient organisms with effective strategies can grow in various environments including the extreme biotopes (Adhikary 1996). This ability can probably partly explain the variety of biologically active compounds produced by these organisms, which can act on eukaryotic cells/organisms as well as on viruses and bacteria/other cyanobacteria. Number of studies reported cyanobacterial secondary products (not used in primary metabolism) with various properties such as cancer promoting, mutagenic, anticancer, antibiotical, cytotoxic, genotoxic, hepatotoxic, neurotoxic, herbicidous, algicidous, virocidous and etc. (Gademann and Portmann 2008; Pearson, et al. 2010). Cyanobacterial metabolites are partially identified and studied, that is the reason why are still under high pharmaceutical and scientific attention.

The BAC production can differ among strains or species. Also one metabolite can be produced by different species (Svrcek and Smith 2004). For example microcystins can be produced by 7 species (Tab. 1) or aeruginosins by three species (*Microcystis, Planktothrix, Nodularia*) (Welker and von Dohren 2006). Also one cyanobacterium can produce more than one bioactive compound, e.g. *Anabaena* sp. can produce anabaenopeptins, cyanopeptolins and microcystins (Jaiswal, et al. 2008). However, cyanobacterial species both strains producing cyanotoxins as well as cyanotoxin non producers are often found in same water body (Bernard, et al. 2003).

Table 1 Main classes of cyanobacterial peptides. Synonyms refer to names in original publications (Welker and von Dohren 2006).

Class	***Synonyms***	***Origin***	***Number of variants***
aeruginosins	microcin, spumigin	*Microcystis, Planktothrix, Nodularia*	27
microginins	cyanostatin, scillaginin, nostoginin	*Microcystis, Planktothrix, Nostoc*	38
anabaenopeptins	oscillamide, ferintoic acid, keramamide, konbamide, mozamide, nodulapeptin, plectamide, schizopeptin	*Anabaena, Aphanizomenon, Microcystis, Planktothrix, Plectonema, Nodularia, Schizothrix, Theonella*	32
cyanopeptolins	aeruginopeptin, anabaenopeptilide, dolostatin, hofmannolin, microcystilide, micropeptin, nostocyclin, nostopeptin, oscillapeptilide, oscillapeptin, planktopeptin, scyptolin, somamide, symplostatin, tasipeptin	*Anabaena, Dolabellaw, Lyngbya, Microcystis, Planktothrix, Scytonema, Symploca*	82
microcystins	motuporin, nodularin	*Anabaena, Hapalosiphon, Microcystis, Nodularia, Nostoc, Planktothrix, Theonella*	89
microviridins		*Microcystis, Planktothrix, Nostoc*	10
cyclamides	aanyascyclamide, bistratamide, dendroamide, microcyclamide, nostocyclamide, obyanamide, raocyclamide, tenuecyclamide, ulongamide, westiellamide	*Lyngbya, Microcystis, Nostoc, Oscillatoria, Stigonema, Westelliopsis*	21

1.1.1 Biosynthesis of cyanobacterial bioactive compounds

The cyanobacterial secondary metabolites are mostly oligopeptides. Multienzyme complex of non-ribosomal peptide synthetase (NRPSs) and polyketide synthases (PKSs) can be involved in their biosynthesis (Welker and von Dohren 2006). NRPS are responsible for the production of several biomedically important cyclic and linear peptides (von Dohren, et al. 1997) and PKSs play role in antibiotics synthesis and other bioactive metabolites (Hopwood 1997).

Microcystins, nodularins, anabenopeptolins, nostocyclopeptide, nostopeptolide are examples of NRPS-PKS synthesized cyanobacterial peptides. On the other hand patellamides belongs to ribosomal synthesized peptides (Sivonen and Börner 2008).

Interestingly, also compounds such as cylindrospermopsin are synthesized by similar non-ribosomal peptide synthetase PKS pathway (Mihali, et al. 2008; Schembri, et al. 2001).

1.1.2 Factors affecting production of bioactive metabolites of cyanobacteria

The production of BAC by cyanobacteria is generally influenced by status of cyanobacterial cell and number of environmental factors such as light, temperature, time period, nutrition composition, pH (Jaiswal, et al. 2008).

Growth phase

Amounts of metabolites varied during different growth stadia of cyanobacteria. Possible ratio between intra and extracellular concentration in culture can help determine how is the metabolite released into environment - by cell lyses or extracellulary transported. Examples of specific cases are listed below.

Algicidous metabolite produced by *Oscillatoria* sp. firstly appeared in medium at mid-exponential phase and according to intracellular and extracellular differences of algicide concentrations, which possibly indicates extracellular transport (Ray and Bagchi 2001).

On the other hand, increasing extracellular concentration of paralytic shellfish toxins produced by *Aphanizomenon* with culture time, indicated toxin release after cell lyses, which can be also expected in real situation after collapse of cyanobacterial bloom (Dias, et al. 2002). For microcystins and anatoxin-a produced by *Anabaena* sp. the production is the highest during logarithmic phase and maximum of its concentration in cells is in the late logarithmic phase, where only about 10% of toxins is detected extracellulary (Jaiswal, et al. 2008).

However, cylindrospermopsin is also highly produced in exponential phase, but its release outside is 50% of CYN in stationary phase (20 days old cultures) (Saker and Griffiths 2000). Similar trends were reported in real water bodies with CYN producing water blooms, where 20-80% of CYN were present extracellulary (Griffiths and Saker 2003).

Interestingly, spectrum of produced metabolites can differ during culture age. Non toxic metabolites were produced by Nostoc insulare into medium during linear growth, whereas antimicrobial and cytotoxic compounds were excreted during stationary phase (Volk 2007).

Nutrients

Toxin production in cyanobacteria is affected by various nutritional factors like nitrogen and phosphorus concentration. Direct correlation of microcystin-RR concentration and nitrogen amount was documented in Oscillatoria agardhii (Sivonen 1996). Controversial results about phosphorus limitation or redundancy in case of microcystin production by M. aeruginosa were reached (reviewed in Jaiswal, et al. 2008). Surprisingly, change in phosphate levels resulted in change in the type of paralytic shellfish toxin produced by Aphanizomenon sp. (Dias, et al. 2002). Evidently, phosphorus influences productions of various toxins in cyanobacteria, but their responses differ among strains and genera. Recent study

reported evidence of sulfur requirement of M. aeruginosa for microcystin production (Long 2010).

Other environmental factors

Enhancement of microcystin production by *M. aeruginosa* correlated with increasing light intensity up to 40 $\mu E.m^{-2}.s^{-1}$, but further increase in light intensity suppress toxin production (Jaiswal, et al. 2008). Similarly, cylindrospermopsin production by two *Aphanizomenon flos-aquae* strains was stimulated by higher light (to 60 $\mu E.m^{-2}.s^{-1}$) (Preussel, et al. 2009). Changes in nodularin production were also reported after exposure to various irradiances of both photosynthetic active radiation (PAR, 400-700 nm) and ultraviolet radiation (UVR, 280-400 nm) (Pattanaik, et al. 2010).

Other important factor influencing metabolite production in cyanobacteria is temperature. Cylindrospermopsin production by two *Aphanizomenon flos-aquae* strains was stimulated up to 20°C (Preussel, et al. 2009). In similar range (20-25°C) optimum formation of microcystin, saxitoxin and anatoxin was produced by *Oscillatoria* sp. (Sivonen 1990). This temperature seems to be best suited for toxin production in most cyanobacterial strains (Jaiswal, et al. 2008).

Also pH of growth medium/water play role in toxin production. Generally, higher toxin/metabolite concentrations are reported at lower and higher pH, where cells had grown slower in contrary to more optimal pH (pH 9) and maximum growth rate (Jaiswal, et al. 2008).

Another factor is also presence of other organisms, and fluctuations in metabolites and toxin production were reported as results of co-occurrence of cyanobacteria with grazers as well as bacteria (Shen, et al. 2010), green algae or other cyanobacteria (Jang, et al. 2008; Kearns and Hunter 2000; Pattanaik, et al. 2010) or stress situation (Kurmayer 2011).

1.1.3 Toxicity and mode of action of BAC

The production of bioactive metabolites needs large input of energy to initiate NRPS and PKS, thus their production should be advantageous to the cell. Although number of papers documented their structures, mechanisms of biological actions and occurrence in cyanobacteria, their function is not elucidated (see Tab. 2). The cyanobacterial toxins (cyanotoxins) thus seem to represent important group of chemical compounds which are scientifically interesting because of their possible participation in the interspecies competitions or communications (Pflugmacher 2002).

Table 2 Examples of less studied bioactive cyanobacterial metabolites and their properties (modified from Jaiswal, et al. 2008)

Toxin produced	**Type/Nature**	**Mode and/or site of action**	**Cyanobacterial genera**	**Reference**
Acutiphycin	Phospholipid compound	Antitumor properties, affects tissues	*Oscillatoria acutissima*	(Barchi, et al. 1984)
Cryptophycins A–F	Lipophilic peptides	Selectively inhibits tumors	*Nostoc spp*	(Trimurtulu, et al. 1994)
Fischerellin	Aminoacylpolyketide	Inhibits photosystem II activity	*Fischerella ambigua, Fischerella muscicola*	(Gross, et al. 1991; Papke, et al. 1997)
Hapalindole	Substituted indole alkaloid	Inhibits tumors	*Hapalosiphon fontinalis, Fischerella sp.*	(Etchegaray, et al. 2004; Moore, et al. 1984)
Laxaphycin A and B	Cyclic peptide	Inhibits tumor cell proliferation	*Anabaena laxa*	(Frankmolle, et al. 1992)
Linolenic acid	Polyunsaturated fatty acid	Decreases blood cholesterol levels, reduces the growth of cancers of the breast and colon	*Oscillatoria redekei, Fischerella sp.*	(Mundt, et al. 2001; Ashtana et al. 2006)
Norharmane	Indole alkaloid	Inhibits indoleamine 2,3- dioxygenase and nitric oxide synthase	*Nodularia harveyana*	(Volk 2005)
Nostocyclamide	Cyclic heptapeptide	Inhibits photosynthesis	*Nostoc sp. strain 31*	(Juttner, et al. 2001)
Spiroidesin	Lipopeptide	Inhibits cell growth	*Anabaena spiroides*	(Kaya, et al. 2002)

This dissertation thesis investigated toxic effects of two well studied toxins - microcystin and CYN, and following paragraphs briefly describe some major characteristics of these metabolites, which are often found also in the Czech Republic (Blahova, et al. 2008; Blahova, et al. 2009).

1.1.4 Microcystin (MC)

One of the most studied and worldwide detected cyanobacterial metabolites is a family of heptacyclic cyanotoxins - microcystins (MCs) (more than 90 structural variants) (Codd, et al. 2005; Sivonen and Jones 1999) which are produced by several strains of cyanobacteria (*Microcystis, Anabaena, Anabaenopsis, Aphanizomenon, Nostoc* etc.) (Carmichael 1997; Zurawell, et al. 2005). The most frequent structural congeners in the environment are microcystin-LR and microcystin-RR, and their desmethylated derivates (Gkelis, et al. 2005; Sivonen and Jones 1999). These intracellular cyanotoxins are released to the aquatic environment by senescence, cell death or application of algicide chemicals (Jones, et al. 1994).

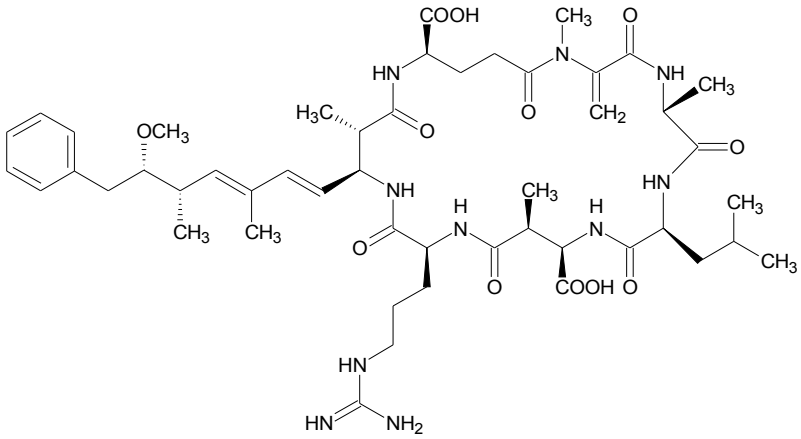

Figure 1 Structure of microcystin-LR, MW 995.2 (Sivonen and Jones 1999)

Mode of action

MC-LR and most of its congeners are hydrophilic and not able to penetrate vertebrate cell membranes (Svrcek and Smith 2004), therefore uptake via an adenosine triphosphate (ATP)-dependent transporter is required (van Apeldoorn, et al. 2007). The main known mode of action of microcystins is inhibition of protein phosphatases (PPs) 1 and 2A leading to higher phosphorylation of target proteins, which can result in cell proliferation, cell transformation and tumor promotion (Carmichael 1997; MacKintosh, et al. 1990) or growth inhibition in seedlings and blocking of root hair growth in plants (Kurki-Helasmo and Meriluoto 1998; Smith, et al. 1994).

Oxidative stress seems to be another important biochemical mechanism of microcystin toxicity. Microcystins have been shown to induce formation of reactive oxygen species (ROS) that might cause serious cellular damage such as peroxidation of lipid membranes, genotoxicity or modulation of apoptosis (Ding and Ong 2003). However, it has been proved that MCs could be detoxified via conjugation to glutathione (Kondo, et al. 1996) by glutathione-S-transferase (GST) (Pflugmacher, et al. 1998) in invertebrates (mollusks, crustacean), vertebrates (birds, fish, mammals) as well as in aquatic or terrestrial plants and microalgae (Paskova, et al. 2008; Pflugmacher, et al. 1998; Pietsch, et al. 2001).

Toxicity of microcystin

Microcystins are toxic to broad group of organisms, especially to mammals (Dawson 1998, Murphy *et al.* 2000). Toxic effects of microcystins have been observed with birds, fish and mollusks as well (Fischer & Dietrich 2000, Oberemm 2001, Malbrouck and Kestemont 2006, Zurawell *et al.* 2006). The acute toxicity of microcystins is in range LD_{50} 50 - 300 μg/kg of mouse body weight (Sivonen and Jones 1999). Even though the effects and concentrations of microcystins have been intensively studied, their natural role is not clear (Kaebernick & Neilan 2001). One of disputable hypothesis is allelopathic activity of microcystins to surrounding competing organism (Gross 2003; Legrand, et al. 2003; Pflugmacher 2002).

Microcystins interference with photoautotrophic organisms including both terrestrial and aquatic plants was observed. There were reported various changes in photosynthetic activity, germination of seedlings, growth, chlorophyll a/b ratios, root and leaves length, cell structures as well as necrosis, chlorosis and decrease in cell viability were documented (reviewed in Babica *et al.* 2006a). Also modulations of biochemical parameters such as superoxide dismutase (SOD), glutathione reductase, glutathione peroxidase (GPx), glutathione-S-transfrease (GST), glutathione, formation of reactive oxygen species (ROS) in plants and algae were documented after exposure to cyanobacteria and their metabolites (Hu, et al. 2005; Pflugmacher, et al. 2007; Saqrane, et al. 2007). Interestingly, less information is known about effects of MCs on algae and cyanobacteria themselves.

One of our experiments was focused on growth effects of several algal and cyanobacterial strains caused by two MC structural congeners in concentration range including environmentally relevant as well as extremely high levels of MCs (**Paper I,** paragraph 1.2). But no effects at environmental relevant concentration of both congeners (MC-LR and MC-RR) were observed. The species-specific growth inhibition was reported only after exposure to extremely high MCs concentration, thus did not imply allelopathic properties of microcystins.

Cyanobacterial differentiation and the role of microcystin

Filamentous cyanobacteria, especially orders *Nostocales* and *Stigonematales,* are capable to form specialized cells (heterocysts necessary for nitrogen fixation and akinetes – endospores used for survival and overwintering) in response to environmental conditions or stressors (see Fig. 2) (reviewed in Adams and Duggan 1999). Cell differentiation in cyanobacteria is complicated process controlled by huge amount of genes and enzymatic complexes which are partially elucidated, but exact mechanisms and signaling pathways are still not known (Flores and Herrero 2010).

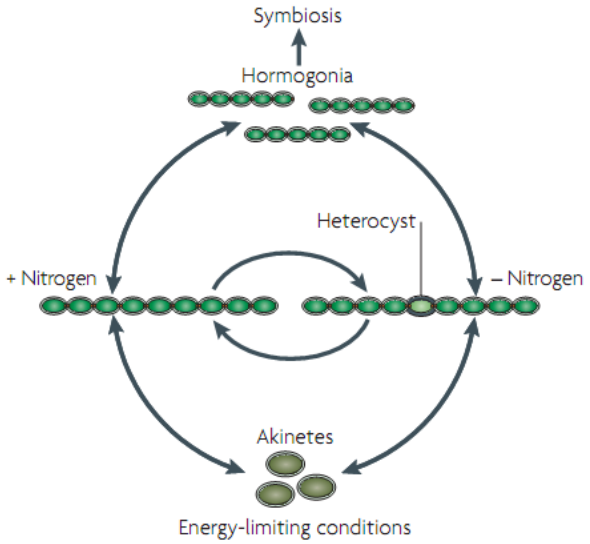

Figure 2 The complex life cycles of some heterocyst- forming cyanobacteria. Some filamentous cyanobacteria, such as Anabaena sp. PCC 7120 (also known as Nostoc sp. PCC 7120), form heterocysts in response to deprivation of combined nitrogen; when filaments are re-supplied with combined nitrogen, heterocysts become spaced apart as intervening vegetative cells grow and divide (Flores and Herrero 2010).

Several studies documented changes in cell differentiation of filamentous cyanobacteria after exposure to metabolites produced by cyanobacteria or algae (Hirosawa and Wolk 1979; Kearns and Hunter 2002). Thus experiments evidenced that cell differentiation is sensitive and can be regulated by extracellulary produced molecules of the same strain or also by other species.

Our next experiment investigated hypothesis of microcystin role as a signaling compound and its possible impact on cellular differentiation (**paper II,** paragraph 1.3). Observed concentration-dependent inhibition of cell differentiation was caused by environmental relevant concentrations (0.2-20 µg/l) of microcystin-LR in cyanobacterial biomass extract, which enlarge evidence of possible signaling function of microcystin.

1.1.5 Cylindrospermopsin (CYN)

Cylindrospermopsin is a cyclic guanidine alkaloid (Ohtani, et al. 1992) produced by tropically and subtropically occurring cyanobacterial species such as *Cylindrospermopsis raciborskii*, *Umezakia natans*, *Aphanizomenon ovalisporum, Aphanizomenon flos-aquae, Raphidiopsis curvata, Anabaena lapponica, Anabaena bergii, Lyngbya wollei* and *Aphanizomenon issatschenkoi* (Babica, et al. 2006; Davis, et al. 2009).

While cylindrospermopsin was originally found in tropical Australian waters (Hawkins, et al. 1997) it has since shown up in Hungary, Japan, and Israel, and the cylindrospermopsin producing genera has been found in other parts of Europe and the USA (Davis, et al. 2009). Nowadays CYN has been detected also in Czech Republic, and its production was associated with various *Aphanizomenon* strains (Blahova, et al. 2008).

CYN consists of a tricyclic guanidine moiety combined with hydroxymethyluracil (see Fig. 3) (Ohtani, et al. 1992) and is relatively stable in sunlight, and decomposes slowly in temperatures ranging from 4 to 50°C at a pH of 7 (Chiswell, et al. 1999). It is also very soluble, and boiling does not cause significant degradation. At the present time, three structural congeners of CYN are known: demethoxycylindrospermopsin and deoxycylindrospermopsin isolated from Australian strain of *C. raciborskii* (Norris, et al. 1999), 7-epi-cylindospermopsin is produced by *Aph. ovalisporium* (Banker, et al. 2001). Compared with other cyanotoxins releasing mostly after cell lyses, cylindrospermopsin is extracellular transported during the specific growth phase and can be found free in water in large concentrations than in cell biomass (Chiswell, et al. 1999).

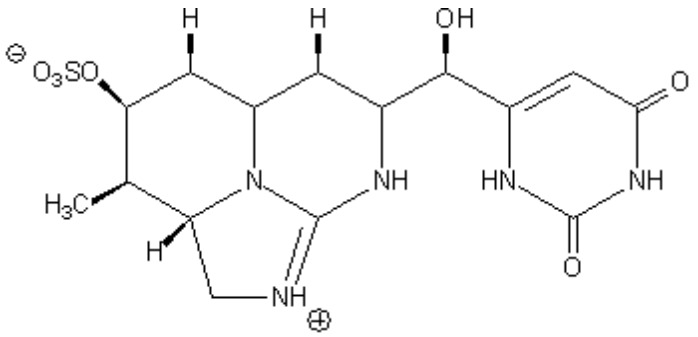

Figure 3 Structure of cylindrospermopsin; MW 415 (Sivonen and Jones 1999)

Mode of action

Unlike microcystins, cylindrospermopsins do not inhibit PP1 or PP2A. Their toxicity appears to result at least in part from the inhibition of protein synthesis. The translation step of protein synthesis is inhibited by the cylindrospermopsins, but the mechanism of this inhibition is not known yet (Runnegar and Lu 1994; Runnegar, et al. 2002). Probably the effect of CYN on protein synthesis is not caused only by its binding on ribosome, but rather on soluble proteins of translation (Froscio, et al. 2008).

Various *in vitro* models documented decrease of GSH levels after CYN exposition, which reduces detoxification in organisms/cells and can possible increase damages caused by reactive oxygen species (ROS) (Humpage, et al. 2000; Runnegar, et al. 1994), probably leading to genotoxicity. Also CYN biotransformation by cytochrome p-450 (CYP450) takes a part in acute toxicity (Humpage, et al. 2005).

The mechanism for cellular uptake is unknown, but it does require the bile acid transport system. Also diffusion was suggested as a possible uptake mechanism without help of a pore or transporter molecule (Chong, et al. 2002).

Toxicity of cylindrospermopsin

Cylindrospermopsins are classified as hepatotoxins and also cytotoxins, moreover there is also mounting evidence about genotoxic and neurotoxic properties of CYN (Humpage, et al. 2000; Kiss, et al. 2002), but definite proof does not exist now. While the toxicity for CYN has been thoroughly investigated in mammals, aquatic vertebrates and invertebrates (Platt, et al. 2008; Metcalf, et al. 2002; Saker and Eaglesham 1999), more research is needed for describing CYN toxicity for other organisms such as phototrophic macrophytes and algae.

The acute (24h) LD_{50} of CYN (via i.p. injection) was found to be 2.1 mg/kg in comparison to chronic lethal dose which LD_{50} (i.p.) is ten-fold lower 0.2 mg/kg after 5-6 day exposure for rats (Chong, et al. 2002; Ohtani, et al. 1992).

1.1.6 Toxicity of complex cyanobacterial samples

Cyanotoxins are not the only one responsible for cyanobacterial toxicity. The biomass extracts (intracellular content) or exudates (extracellular mixture) of various cyanobacteria may also cause toxic effects on organisms, observed effects are often stronger than after exposure to pure cyanotoxins (Pietsch, et al. 2001; Valdor and Aboal 2007). However, studies comparing effects of complex intra- and extra-cellular cyanobacterial mixtures are rather rare.

In previous studies, both biomass extracts as well as exudates from several cyanobacterial strains (*Microcystis aeruginosa* and *Plantkothrix planctonica*) were tested on growth of green alga *Ankistrodesmus falcatus* using co-occurrence type of experiments with various cell densities and growth inhibition was observed at very low densities of cyanobacterial strains (Vasconcelos and Almeida 2008). Similarly, growth inhibition was observed on green alga *Chlamydomonas* sp. after co-occurrence with cyanobacterium *Fischerella* sp. isolated from Florida water bodies, also photosynthetic inhibition and loss of ultrastructural cell organization were provided (Gantar, et al. 2008).

It was reported that no all of cyanobacterial strains can produce allelochemicals as was proved for cyanotoxin production. Exudates (extracellular mixture) from twenty two cyanobacterial strains were investigated on growth affection of two green algae and interestingly, only two cyanobacterial strains (*Cylindrospermopsis raciborskii* and *Oscillatoria* sp.) showed production of allelochemical with species-specific effect on growth in dose-dependent manner (Leao, et al. 2009). In general, it is assumed that cyanobacteria contain not only numerous metabolites whose activity is poorly understood, but also further currently unknown toxins.

Comparison of effects of pure microcystin-LR, intra- and extra-cellular cyanobacterial mixture from *Microcystis aeruginosa* on biochemical parameters linked to oxidative stress in green alga was evaluated in our next work (**Paper III,** paragraph 1.4). Oxidative stress in green alga was induced after exposure to each tested samples, but the effects of pure microcystin-LR differ from cyanobacterial mixtures, which are more complex and relevant in natural situation.

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1.2. Effects of dissolved microcystins on growth of planktonic photoautotrophs

Paper I

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Effects of dissolved microcystins on growth of planktonic photoautotrophs

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Effects of cyanobacterial toxins microcystin-LR and -RR on growth of five representatives of Chlorophyta (*Chlamydomonas reinhardtii, Chlorella kesslerii, Pediastrum duplex, Pseudokirchneriella subcapitata, Scenedesmus quadricauda*) and cyanobacterium *Microcystis aeruginosa* (Cyanophyta) were investigated in the concentration range 1–25,000 μg l^{-1} using microplate assays (evaluated after 4, 7 and 11 days). Our results demostrate different susceptibility of several planktonic organisms to microcystins. In some species (*C. reinhardtii, C. kesslerii, P. duplex, M. aeruginosa*), microcystin-RR induced more pronounced effects on growth than structural variant microcystin-LR. However, environmentally relevant concentrations of microcystins (1–10 μg l^{-1}) did not cause significant growth alterations. Also, concentrations 100–5000 μg l^{-1} were ineffective in most tested species with the exception of *P. subcapitata.* Growth of *P. subcapitata* was strongly inhibited at concentrations of microcystin-LR or -RR ≥ 1000 μg l^{-1} after 4 days of exposure, whereas *S. quadricauda* was affected only at concentration 25,000 μg l^{-1}. *C. reinhardtii* and *C. kesslerii* responded to both microcystins at the highest experimental concentration, but effects of microcystin-LR were weak and apparent only after 11 days of exposure, while microcystin-RR inhibited alga growth from day 4. Growth of *P. duplex* was also reduced at 25,000 μg l^{-1} of microcystin-RR from day 4, but only slightly inhibited by microcystin-LR (after 4 and 7 days of exposure). Growth of *M. aeruginosa* was only slightly affected by microcystin-LR (inhibition at 25,000 μg l^{-1} on day 7) and inhibited by microcystin-RR especially at the highest experimental concentration. Our results suggest that microcystin effects on phytoplankton are species specific and congener specific. However, microcystins are not likely to affect proliferation of planktonic photoautotrophs at environmentally occurring conditions. These findings do not support a hypothesis of possible direct allelopathic natural function of microcystins, at least as far as growth inhibition effects are concerned.

KEY WORDS: Algae, Allelopathy, Cyanotoxin, Cyanobacteria, Microcystin, Phytoplankton

INTRODUCTION

Microcystins form a family of cyclic heptapeptides (Codd *et al.* 2005) frequently produced by some strains of planktonic bloom-forming cyanobacteria (*Anabaena, Microcystis, Planktothrix*) or terrestric/benthic cyanobacterial genera (*Nostoc, Hapalosiphon*). Microcystins are extremely toxic for mammals (Dawson 1998; Dietrich & Hoeger 2005) as well as for other organisms including fish, amphibians, arthropods or molluscs (Wiegand & Pflugmacher 2005; Zurawell *et al.* 2005). Moreover, microcystins also have potential to affect various photoautotrophic organisms including terrestrial and aquatic plants or algae (Babica *et al.* 2006).

Microcystins are biosynthesized inside cyanobacterial cells and only about 10% of total microcystin content is usually found in extracellular space, i.e. dissolved microcystins (Pietsch *et al.* 2002; Zheng *et al.* 2004). Whereas the majority of microcystins is released after cell lysis and decay, active transport from growing cyanobacterial cells was also recently suggested (Pearson *et al.* 2004). Although concentrations of dissolved microcystins might reach up to several hundreds of micrograms per liter during the collapse of cyanobacterial bloom, they commonly do not exceed 10 μg l^{-1} and concentrations 0.05–5 μg l^{-1} are considered to be typical for aquatic ecosystems with cyanobacterial massive development (Sivonen & Jones 1999; Chorus 2001). Therefore, particular attention should be given to environmentally relevant concentrations of dissolved microcystins when discussing potential harmful effects or allelopathy of microcystins to aquatic photoautotrophs. In spite of several studies with higher aquatic plants, where negative effects of environmentally relevant concentrations of dissolved microcystins were described (Pflugmacher *et al.* 1999, 2001; Pflugmacher 2002; Romanowska-Duda & Tarczynska 2002; Pflugmacher 2004; Yin *et al.* 2005c), only few papers investigated toxicity of environmentally relevant concentrations of microcystins in planktonic photoautotrophs (Kearns & Hunter, 2000, 2001; Pietsch *et al.* 2001; Hu *et al.* 2004). The growth alterations of phytoplanktonic species were mostly observed only at high concentrations of microcystins (Christoffersen 1996; Sedmak & Kosi 1998; Singh *et al.* 2001; Hu *et al.* 2004, 2005; Ou *et al.* 2005).

More than 70 structural variants of microcystins have been described (Codd *et al.* 2005), but some congeners such as microcystin-RR and microcystin-LR seem to be more prevalent in the environment than the others (Gkelis *et al.* 2005). Consequently most studies with photoautotrophic organisms have dealt with either microcystin-RR or microcystin-LR (Babica *et al.* 2006). However, toxicity and toxicokinetics of different structural variants could differ substantially not only in animals (Blom *et al.* 2001; Gupta *et al.* 2003), but also in plants (McElhiney *et al.* 2001). Although the animal toxicity of microcystins has been widely investigated, the question of their natural function remains to be answered. One of the hypotheses suggests a role of microcystins in interspecific phy-

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toplankton interactions with possible allelopathic function (Kaebernick & Neilan 2001), but the data on microcystin toxicity to planktonic algae are controversial (Babica *et al.* 2006). Therefore, the aim of this study was to investigate inhibition of algal growth in the presence of a wide concentration range of dissolved microcystins including low, environmentally relevant concentrations, and to compare the effects of two common microcystins, -LR and -RR. The following species of green algae were selected for growth inhibition experiments: common planktonic alga *Chlamydomonas reinhardtii* Dangeard, which has been described as an organism sensitive to low concentration of microcystins (Kearns & Hunter 2000, 2001), *Pediastrum duplex* Meyen and *Scenedesmus quadricauda* (Turpin) Brébisson, two species that are frequently found in phytoplankton communities together with microcystin producers, and further *Pseudokirchneriella subcapitata* (Koršikov) Hindák and *Chlorella kesslerii* Fott & Nováková, the organisms commonly used in algal growth inhibition tests (Rojickova-Padrtova & Marsalek 1999); they also co-occur with planktonic cyanobacteria. The last investigated species was cyanobacterium *Microcystis aeruginosa* Kützing, because stimulation of its growth by microcystin-RR has been previously reported (Sedmak & Kosi 1998) and a possible role of microcystins in intraspecific signaling has been suggested (Dittmann *et al.* 2001; Kehr *et al.* 2006). The *M. aeruginosa* strain PCC 7806 used in the present study is a microcystin producer (Cremer *et al.* 1991).

MATERIAL AND METHODS

Test organisms and cultivation conditions

All studied species were obtained from Culture Collection of Algal Laboratory, Institute of Botany, Czech Academy of Sciences, Třeboň, Czech Republic: *Chlamydomonas reinhardtii* (UTEX 2246), *Pediastrum duplex* (CCALA 403), *Chlorella kesslerii* (CCALA 253), *Pseudokirchneriella subcapitata* (CCALA 433), *Scenedesmus quadricauda* (CCALA 463) and *Microcystis aeruginosa* (PCC 7806). Cultures of all organisms were grown at 22°C under continuous light (cool white fluorescent tubes, 3000 lux) in cultivation medium with the following composition: mixture of Zehnder medium (Schlosser 1994), Bristol (modified Bold) medium (Stein 1973) and distilled water (1 : 1 : 2). Cultures were aerated with ambient air sterilized by passing through 0.22 μm filter (Labicom, Olomouc, Czech Republic).

Algal growth inhibition assay

Algal growth inhibition assays were performed in 96-well polystyrene microplates sterilized by ultraviolet (UV) light (Gamedium, České Budějovice, Czech Republic). Microcystin solution of desired concentration (prepared in cultivation medium) was mixed with an aliquot of the stock culture and cultivation medium to achieve optical density approximately 0.04–0.05 (at 680 nm). Final concentrations of microcystin were 0 (negative control), 1, 10, 100, 1000, 5000 and 25,000 $\mu g\ l^{-1}$. Validity of the assays was controlled using positive controls (three concentrations of potassium dichromate). Each experimental concentration was tested in five replicates with the exception of the negative control (15 replicates). Test cultures (250 μl) were added into each microplate well. Microplates were incubated for up to 11 days at 22°C under continuous light (cool white fluorescent tubes, 1500 lux) without shaking. Algal growth was measured indirectly as optical density using microplate (spectrofluorophotometer) reader GENios Spectra Fluor Plus (Tecan Group, Männedorf, Switzerland). Before the measurement of optical density (680 nm on days 0, 4, 7 and 11), the well content was resuspended with a pipette. More detailed study was performed with *P. subcapitata,* where optical density was evaluated at more time points during the whole exposure period. Each experiment was repeated independently at least twice.

Microcystin extraction and purification

Microcystin-LR and -RR were extracted and purified from natural cyanobacterial bloom sample (reservoir Nove Mlýny, Czech Republic, summer 2003, dominated by *Microcystis aeruginosa*). Collected bloom biomass was repeatedly frozen and thawed and subsequently extracted three times with 10% (v/v) methanol under sonication (ultrasonic probe Bandelin Sonopuls HD2070, Bandelin Electronics, Berlin, Germany). Cell debris was separated by centrifugation after each extraction step. Combined supernatants were filtered (Whatman GF/C glass microfiber filter, Maidstone, UK) and passed through preconditioned SepPak 35cc 10 g C-18 cartridge (Waters, Millford, MA, USA). Step gradient of 9 × 100 ml of methanol : water (from 10% of methanol to 100% with 10% increment) was used for microcystin elution. Microcystin content in each fraction was analysed by high-performance liquid chromatography (HPLC) Agilent 1100 Series coupled with PDA detector (Agilent Technologies, Waldbronn, Germany) on Supelcosil ABZ+ Plus column, 150 × 4.6 mm, 5 μm (Supelco, Bellefonte, PA, USA) at 30°C. Binary gradient of mobile phase consisted of (A) H_2O + 0.1% trifluoroacetic acid (TFA) and (B) acetonitrile + 0.1% TFA (linear increase from 20% B at 0 min to 59% B at 30 min); flow rate was 1 ml min^{-1}. UV spectra were recorded from 200 to 300 nm and chromatograms were evaluated at 238 nm. Microcystins were identified by comparison of UV spectra and retention time with standards of microcystin-LR, -LF, -LW, -RR, -YR (Alexis Biochemicals, Läufelfingen, Switzerland). The fractions containing microcystins were combined, evaporated to dryness by rotary vacuum evaporator, redissolved in a small volume of 50% (v/v) methanol, filtered (0.45 μm nylon syringe filter, Labicom, Olomouc, Czech Republic) and used for final purification in the same chromatographic system with isocratic elution (27% of organic mobile phase). Fractions with microcystins were manually collected and analysed by HPLC as described above. Purity of both microcystin-RR and -LR exceeded 98% according to HPLC chromatograms at 238 nm.

Data analysis

Optical density at 680 nm reflecting algal growth was expressed as percentage (%) of mean optical density of negative control at each time point. To assess differences among the treatment groups, combined weighed data from independent experiments were evaluated by nonparametric Kruskal–Wallis test using Statistica 7.1 for Windows. Results were considered significant at $P < 0.05$ level.

trastructure and inductions of oxidative stress were detected in exposed alga (Ou *et al.* 2005). Our results showed only minor and mostly nonsignificant effects of microcystins at concentrations 100–5000 μg l^{-1} (with the exception of *P. subcapitata*) and mostly the growth inhibition was observed only at the highest tested concentration 25,000 μg l^{-1}.

The differences in results of various studies might be explained by different experimental conditions. For example, Sedmak & Kosi (1998) observed pronounced effects under low light conditions (4 μE m^{-2} s^{-1}) and the effects usually became apparent after prolonged (8–12 days) exposures. On the other hand, in our study we have used higher intensity of illumination and shorter exposure times. Three- to four-day exposures are recommended for algal growth inhibition tests according to several guidelines (OECD 1984; ISO 1989; US EPA 2002), and they were successfully used also for alternative microplate assays (Rojickova *et al.* 1998; Rojickova-Padrtova & Marsalek 1999). However, it is apparent that microcystin-related growth inhibition often occurs only after prolonged 11-day exposures (for example, effects of microcystin-LR on *C. reinhardtii* or *C. kesslerii* in the present study).

Further explanation of the differences in the results of various studies might also be different sensitivity of phytoplankton species. For example, in our study *C. reinhardtii, C. kesslerii,* and *P. duplex* as well as *M. aeruginosa* were affected only weakly by microcystin-LR at the highest tested concentration (25,000 μg l^{-1}), whereas growth of *S. quadricauda* was reduced more dramatically at the same concentration. *Pseudokirchneriella subcapitata* was inhibited in a concentration-dependent manner even at concentrations ≥1000 μg l^{-1} of microcystin-LR.

Although we do not have a clear explanation for the unusual variability observed in experiments with *M. aeruginosa* (Fig. 2), one of possible reasons could be an interference in effects of externally added microcystin-RR with endogenous production of microcystins, because the studied strain (PCC 7806) is a microcystin producer (Cremer *et al.* 1991). However, this observation will need more thorough investigation in the future.

Another important observation was significantly higher toxicity of microcystin-RR to *C. reinhardtii, C. kesslerii, P. duplex* and *M. aeruginosa* compared to microcystin-LR. Interestingly, no such difference was observed in our experiments with *S. quadricauda* and *P. subcapitata.* There was also no significant difference in some studies, such as growth experiments with *Poterioochromonas* sp. (Ou *et al.* 2005) or assessment of enzyme activities in *S. armatus* (Pietsch *et al.* 2001). Similarly, Sedmak & Elersek (2005) observed only minor differences among the effects of microcystin-LR, -RR and -YR on cell volumes of *S. quadricauda* and *M. aeruginosa.* On the other hand, microcystin-RR was found to be a slightly more potent inhibitor of growth of mustard *Sinapis alba* than microcystin-LR and it was much more potent than microcystin-LF (McElhiney *et al.* 2001). In general, microcystin-RR seems to be more toxic to photoautotrophs that might be correlated with its lower hydrophobicity in comparison with other microcystin variants (McElhiney *et al.* 2001). The more pronounced effects of microcystin-RR found in our study could be the result of prolonged exposure, differences in endpoints and species sensitivity. The interactions between photoautotrophs and microcystins seem to be highly species specific, and the detailed explanation will require further research.

In summary, our results demonstrate various effects of different microcystin variants on the growth of phytoplanktonic species. The effects were highly species specific and they occurred only at high concentrations. Hypothesis on allelopathic function or effects of microcystins has been discussed (Pflugmacher 2002; Hu *et al.* 2004, 2005; Sedmak & Elersek, 2005; Yin *et al.* 2005c) but our observations of generally minor microcystin effects do not support it. However, more complicated mechanisms of action could be involved in interactions between microcystin-producing cyanobacteria and other photoautotrophs, particularly when a wide range of physiological and biochemical modulations induced by microcystins in photoautotrophs as recently summarized Babica *et al.* (2006) or reported Sedmak & Elersek (2005), Yin *et. al* (2005a, b) is taken into consideration.

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Table 1. The effects of microcystins (at concentrations of 1000–25,000 μg l^{-1}) on growth of green algae *Chlamydomonas reinhardtii, Chlorella kesslerii, Pediastrum duplex, Pseudokirchneriella subcapitata* and *Scenedesmus quadricauda*. Data (expressed as percentage of control) are means ± standard deviations. Significant differences ($P < 0.05$) compared to control are indicated by asterisks.

Organism	Microcystin	Exposure time (days)	Concentration (μg l^{-1}) 1000	5000	25,000
Chlamydomonas reinhardtii	LR	4	101 ± 5	104 ± 5	97 ± 3
		7	97 ± 6	102 ± 4	96 ± 10
		11	94 ± 5*	95 ± 9	94 ± 7*
	RR	4	101 ± 8	101 ± 10	57 ± 18*
		7	93 ± 10	96 ± 12	46 ± 19*
		11	96 ± 8	98 ± 6	42 ± 13*
Chlorella kesslerii	LR	4	104 ± 6	100 ± 11	103 ± 13
		7	110 ± 16	97 ± 12	92 ± 14
		11	98 ± 9	96 ± 9	86 ± 8*
	RR	4	93 ± 7	97 ± 9	88 ± 12
		7	100 ± 12	99 ± 14	66 ± 9*
		11	99 ± 9	98 ± 13	50 ± 15*
Pediastrum duplex	LR	4	96 ± 6	90 ± 5*	91 ± 10*
		7	99 ± 6	96 ± 5	88 ± 11*
		11	101 ± 7	96 ± 6	100 ± 9
	RR	4	103 ± 13	101 ± 15	60 ± 14*
		7	98 ± 11	99 ± 14	28 ± 7*
		11	102 ± 16	102 ± 18	31 ± 5*
Pseudokirchneriella subcapitata	LR	4	76 ± 22*	67 ± 17*	68 ± 29*
		7	51 ± 12*	40 ± 8*	35 ± 12*
		11	65 ± 18*	43 ± 10*	26 ± 9*
	RR	4	89 ± 11	77* ± 11	54 ± 9*
		7	58* ± 5	42* ± 3	21 ± 4*
		11	67* ± 4	37* ± 4	12 ± 3*
Scenedesmus quadricauda	LR	4	107 ± 7	116 ± 8*	80 ± 8*
		7	102 ± 18	106 ± 25	23 ± 3*
		11	94 ± 10	92 ± 12	12 ± 3*
	RR	4	103 ± 7	106 ± 8	91 ± 8*
		7	95 ± 22	99 ± 23	39 ± 4*
		11	91 ± 11	89 ± 13	17 ± 4*

RESULTS

Our experiments showed different inhibitions of algal growth by the two microcystin variants and the effects became more pronounced within prolonged exposure time (Table 1). Remarkable differences were observed in response of several organisms. Some species also responded to microcystin-LR and -RR with different susceptibility.

Lower concentrations (1–100 μg l^{-1}) of microcystins caused only weak and usually statistically nonsignificant alterations of growth without any apparent concentration-dependent trend (data not shown). *Pseudokirchneriella subcapitata* was the most sensitive species and its growth was significantly inhibited from 1000 μg l^{-1} of both investigated microcystins. The effects were significant already after 4 days of exposure (Fig. 1). The growth of other green algae was usually affected only at the highest tested concentration (25,000 μg l^{-1}). At the highest concentration, growth of *S. quadricauda* was affected by both microcystins in a similar manner. Although slightly significant growth inhibition was observed after 4 days, the effect was more apparent on days 7 and 11 (Table 1).

We have observed different responses of *C. reinhardtii, C. kesslerii* and *P. duplex* to both investigated microcystin variants. Whereas microcystin-LR caused only minor growth inhibitions of *C. reinhardtii* and *C. kesslerii* at 25,000 μg l^{-1} after 11 days, much more pronounced effects were observed for microcystin-RR already after 4 days. The effects of microcystin-RR on *P. duplex* were very similar to those observed at *C. reinhardtii*. However, microcystin-LR reduced growth of *P. duplex* slightly on days 4 and 7, but there was no significant difference between control and 25,000 μg l^{-1} after 11 days of exposure in contrast to *C. reinhardtii* (Table 1), where there were no effects at shorter exposure times but very slight decrease at 11 days.

Also cyanobacterium *M. aeruginosa* displayed variable responses to different structural variants of microcystin. Micro-

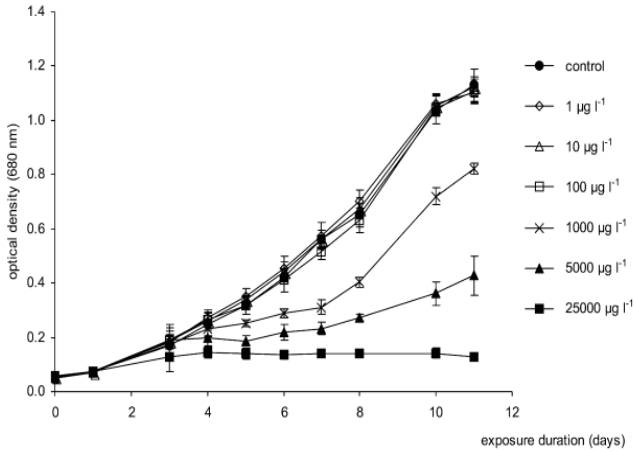

Fig. 1. Concentration-dependent growth inhibition of *Pseudokirchneriella subcapitata* by microcystin-RR.

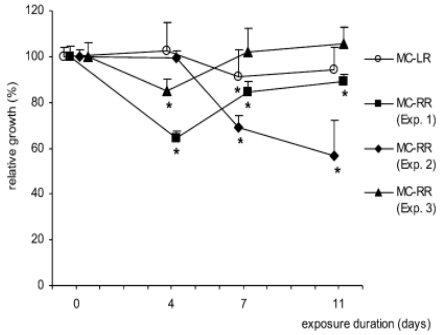

Fig. 2. The effects of microcystin-LR (mean data from independent experiments) and microcystin-RR (data from three independent experiments) at 25,000 μg l^{-1} on growth of *Microcystis aeruginosa.* Data (expressed as percentage of control) are means + standard deviations. Significant differences ($P < 0.05$) compared to control are indicated by asterisks.

cystin-LR caused either no significant effects (at concentrations 1–5000 μg l^{-1}, data not shown) or the growth inhibition was only weak (25,000 μg l^{-1}, 7 days, Fig. 2). More significant effects were observed for microcystin-RR, especially at the highest experimental concentration. Interestingly, the time-course effects of microcystin-RR differed considerably among independent experiments. Whereas initial growth reduction of *M. aeruginosa* (observed on day 4) was less pronounced at longer exposures during experiment 1 and experiment 3, there was significant growth inhibition after 7 days that further progressed during experiment 2 (Fig. 2). Moreover, stimulation of growth was observed after 11 days at a concentration of 1 μg l^{-1} of microcystin-RR in experiments 2 and 3 (120% or 107% of control, respectively). Concentrations 10–5000 μg l^{-1} of microcystin-RR caused only minor alterations of growth in all three experiments (data not shown).

DISCUSSION

The results of our experiments demonstrate that microcystins at higher concentrations could inhibit growth of planktonic photoautotrophs. However, concentrations corresponding to environmental conditions (1–10 μg l^{-1}) were found to be either completely ineffective or causing only weak growth alterations in all studied species. There have been several studies investigating the effects of microcystins on eukaryotic microalgae or cyanobacteria within this environmentally relevant concentration range. Similarly to our results, only a few of them have reported significant effects on growth. Kearns & Hunter (2000) observed growth inhibition of *C. reinhardtii* by semipurified cyanobacterial extract from *Anabaena flos-aquae* (Lyngbye) Brébisson containing microcystins (10 μg l^{-1}). The same concentration of pure microcystin-LR caused settling and motility inhibition of *C. reinhardtii* (Kearns & Hunter 2001). However, in our experiments we did not observe any growth inhibition at *C. reinhardtii* exposed to pure microcystin-LR or -RR at concentrations lower than 25,000 μg l^{-1}. It is possible that growth inhibition effects observed by Kearns & Hunter (2000) could be caused by other bioactive compounds potentially present in the semipurified *Anabaena* extract (Codd *et al.* 2005; Welker & von Dohren 2006). Also the enhanced settling rate (after 1 h of exposure) or inhibition of motility (12-day experiment) observed after exposures to pure microcystin-LR (10 μg l^{-1}) by Kearns & Hunter (2001) does not necessarily have to correspond to growth reduction, as the authors have not reported any increased algal mortality. Escoubas *et al.* (1995) observed inhibition of chlorophyll *a* accumulation but no growth inhibition in green alga *Dunaliella tertiolecta* Butcher exposed to microcystin-LR (1–100 μg l^{-1}, 24 h) under low light conditions. In other short-term experiments, exposure to microcystin-LR or -RR (0.25 μg l^{-1}) for 1 h affected activities of peroxidase and glutathione *S*-transferase in green alga *Scenedesmus armatus* (Chodat) G. M. Smith (Pietsch *et al.* 2001). In an 8-day study with cyanobacterium *Synechococcus elongatus* (Nägeli) Nägeli (Hu *et al.* 2004), microcystin-RR (10 μg l^{-1}) caused only weak, non-significant stimulation of growth along with some other physiological effects (changes in the content of pigments, carbohydrates and proteins, inhibition of photosynthesis and nitrate reductase activity). Higher concentrations (100–1000 μg l^{-1}) had more pronounced and significant effects in their study (Hu *et al.* 2004). Taken together, there is evidence that microcystins are able to affect various physiological and biochemical parameters in planktonic photoautotrophs at low concentrations relevant to the environmental situation. However, alteration of growth was observed only rarely, which fully corresponds with our observations.

Higher concentrations of microcystins (>10 μg l^{-1}) were reported to affect a range of morphological, physiological and biochemical changes in planktonic photoautotrophs such as cell aggregation, changes in pigment contents, increase of cell volume and induction of oxidative stress (Vardi *et al.* 2002; Sedmak & Elersek 2005). There are also studies that reported growth inhibition (or stimulation) of various planktonic organisms exposed to elevated microcystin concentrations. In laboratory experiments with microcystin-RR at concentrations 104 and 519 μg l^{-1} (12–20 days of exposure), early growth stimulations followed by subsequent growth inhibition were observed in cryptophyte *Cryptomonas erosa* Ehrenberg, green alga *Coelastrum microporum* Nägeli and cyanobacterium *Chroococcus minutus* (Kützing) Nägeli. On the other hand, authors observed growth stimulation of green algae *Monoraphidium contortum* (Thuret) Komárková-Legnerová, *S. quadricauda* and cyanobacterium *M. aeruginosa* using the same experimental design (Sedmak & Kosi 1998). Growth inhibition and changes in biochemical parameters related to antioxidative system were recently described in *S. elongatus* exposed to 100 μg l^{-1} of microcystin-RR for 6 days (Hu *et al.* 2005). Nevertheless, only weak effect on growth was reported in *Nephroselmis olivacea* Stein exposed to 157 μg l^{-1} of microcystin-LR for 10 days (Christoffersen 1996). In another study with cyanobacteria *Nostoc muscorum* Agardh and *Anabaena* BT-1 (Singh *et al.* 2001), semipurified microcystin-LR inhibited growth (15-day experiment) as well as photosynthesis, nitrogenase activity and CO_2 uptake at very high concentrations ≥ 25,000 μg l^{-1}. On the other hand, growth of grazing chrysophyte *Poterioochromonas* sp. was stimulated by microcystin-LR or -RR at concentrations 100–4000 μg l^{-1} within 17 days of exposure, but negative changes in cell ul-

trastructure and inductions of oxidative stress were detected in exposed alga (Ou *et al.* 2005). Our results showed only minor and mostly nonsignificant effects of microcystins at concentrations 100–5000 μg l^{-1} (with the exception of *P. subcapitata*) and mostly the growth inhibition was observed only at the highest tested concentration 25,000 μg l^{-1}.

The differences in results of various studies might be explained by different experimental conditions. For example, Sedmak & Kosi (1998) observed pronounced effects under low light conditions (4 μE m^{-2} s^{-1}) and the effects usually became apparent after prolonged (8–12 days) exposures. On the other hand, in our study we have used higher intensity of illumination and shorter exposure times. Three- to four-day exposures are recommended for algal growth inhibition tests according to several guidelines (OECD 1984; ISO 1989; US EPA 2002), and they were successfully used also for alternative microplate assays (Rojickova *et al.* 1998; Rojickova-Padrtova & Marsalek 1999). However, it is apparent that microcystin-related growth inhibition often occurs only after prolonged 11-day exposures (for example, effects of microcystin-LR on *C. reinhardtii* or *C. kesslerii* in the present study).

Further explanation of the differences in the results of various studies might also be different sensitivity of phytoplankton species. For example, in our study *C. reinhardtii, C. kesslerii,* and *P. duplex* as well as *M. aeruginosa* were affected only weakly by microcystin-LR at the highest tested concentration (25,000 μg l^{-1}), whereas growth of *S. quadricauda* was reduced more dramatically at the same concentration. *Pseudokirchneriella subcapitata* was inhibited in a concentration-dependent manner even at concentrations ≥1000 μg l^{-1} of microcystin-LR.

Although we do not have a clear explanation for the unusual variability observed in experiments with *M. aeruginosa* (Fig. 2), one of possible reasons could be an interference in effects of externally added microcystin-RR with endogenous production of microcystins, because the studied strain (PCC 7806) is a microcystin producer (Cremer *et al.* 1991). However, this observation will need more thorough investigation in the future.

Another important observation was significantly higher toxicity of microcystin-RR to *C. reinhardtii, C. kesslerii, P. duplex* and *M. aeruginosa* compared to microcystin-LR. Interestingly, no such difference was observed in our experiments with *S. quadricauda* and *P. subcapitata.* There was also no significant difference in some studies, such as growth experiments with *Poterioochromonas* sp. (Ou *et al.* 2005) or assessment of enzyme activities in *S. armatus* (Pietsch *et al.* 2001). Similarly, Sedmak & Elersek (2005) observed only minor differences among the effects of microcystin-LR, -RR and -YR on cell volumes of *S. quadricauda* and *M. aeruginosa.* On the other hand, microcystin-RR was found to be a slightly more potent inhibitor of growth of mustard *Sinapis alba* than microcystin-LR and it was much more potent than microcystin-LF (McElhiney *et al.* 2001). In general, microcystin-RR seems to be more toxic to photoautotrophs that might be correlated with its lower hydrophobicity in comparison with other microcystin variants (McElhiney *et al.* 2001). The more pronounced effects of microcystin-RR found in our study could be the result of prolonged exposure, differences in endpoints and species sensitivity. The interactions between photoautotrophs and microcystins seem to be highly species specific, and the detailed explanation will require further research.

In summary, our results demonstrate various effects of different microcystin variants on the growth of phytoplanktonic species. The effects were highly species specific and they occurred only at high concentrations. Hypothesis on allelopathic function or effects of microcystins has been discussed (Pflugmacher 2002; Hu *et al.* 2004, 2005; Sedmak & Elersek, 2005; Yin *et al.* 2005c) but our observations of generally minor microcystin effects do not support it. However, more complicated mechanisms of action could be involved in interactions between microcystin-producing cyanobacteria and other photoautotrophs, particularly when a wide range of physiological and biochemical modulations induced by microcystins in photoautotrophs as recently summarized Babica *et al.* (2006) or reported Sedmak & Elersek (2005), Yin *et. al* (2005a, b) is taken into consideration.

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1.3 Extract of Microcystis water bloom affects cellular differentiation in filamentous cyanobacterium *Trichormus variabilis* (*Nostocales, Cyanobacteria*)

Paper II

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Extract of *Microcystis* water bloom affects cellular differentiation in filamentous cyanobacterium *Trichormus variabilis* (Nostocales, Cyanobacteria)

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Abstract Cyanobacteria produce a wide spectrum of biologically active compounds such as microcystins, whose effects on photoautotrophic organisms and role in the aquatic ecosystems have been most intensively discussed and studied. In our study, we examined effects of semipurified *Microcystis* extract containing microcystins (0.2–20 nM corresponding to 0.2–20 μg L^{-1}) on age-induced cell differentiation of filamentous cyanobacterium *Trichormus variabilis*. The heterocyst and akinete formation was significantly decreased after exposure to extract containing 2 or 20 nM of microcystins within 10 days of exposure. To our knowledge, this is the first information about effects of metabolites produced by planktonic cyanobacteria on cell differentiation in filamentous cyanobacteria. Since the effects were induced by environmentally relevant concentrations of microcystins, our observations may indicate not only that microcystins or other bioactive peptides could affect conservation, overwintering, and nitrogen-fixation in filamentous cyanobacteria under environmental conditions but also contribute to the understanding of possible signaling function of cyanobacterial metabolites.

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Keywords Akinete frequency · Cell differentiation · Heterocyst frequency · *Microcystis* extract · Microcystin · *Trichormus variabilis*

Introduction

Differentiation into specialized cell types, akinetes or heterocysts, is a characteristic feature of many filamentous cyanobacteria from the orders *Nostocales* and *Stigonematales*. Akinete is a specialized spore-like dormant cell, resistant to cold and desiccation, able to germinate to new filament under suitable conditions, whereas heterocysts, which may be evolutionarily derived from akinetes, provide a spatially separated environment suitable for nitrogen fixation (Adams and Duggan 1999; Herdman 1988). Differentiation into these specialized cell types is a process controlled by a particular group of genes (reviewed in Golden and Yoon 1998; Wolk 1996) and potentially affected by both abiotic and biotic factors including nutrients, light intensity, salinity, stress, toxicants, or aging (Dubey and Rai 1987).

Interestingly, it was reported that cyanobacterial differentiation can be modulated also by bioactive chemicals produced either autocrinely or by competing photoautotrophic organisms. Cyanobacterium *Cylindrospermum licheniforme* excretes a compound stimulating akinete formation in young cultures of the same species (Fisher and Wolk 1976; Hirosawa and Wolk 1979), whereas allelochemical produced by green alga *Chlamydomonas reinhardtii* suppresses heterocyst differentiation in *Anabaena flos-aquae* (Kearns and Hunter 2002). These studies indicate that the process of cellular differentiation in cyanobacteria may be regulated by production and release of chemical signals (quorum sensing) or be a subject of allelochemical actions of competitors.

Indeed, cyanobacteria synthesize a wide range of biologically active compounds, whose natural ecological or physiological role is unclear, although many of these chemicals have been implicated in intra- or interspecific chemical interactions and signaling (Leao et al. 2009). Many bioactive compounds from cyanobacteria are non-ribosomally synthesized linear or cyclic oligopeptides, with microcystins being one of the most prominent groups. Cyclic heptapeptides microcystins are produced by many planktonic bloom-forming cyanobacterial genera like *Anabaena*, *Microcystis*, or *Planktothrix*, and they were identified also in several cyanobacterial species isolated from phytobenthos, soil, or lichens (Heath et al. 2010; Sivonen and Jones 1999). Microcystins are, therefore, commonly found not only dissolved in water column but also have been detected also in cyanobacterial mats (Heath et al. 2010) or freshwater sediments (Babica et al. 2006b; Ihle et al. 2005; Schmidtkunz et al. 2009). Thus, both planktonic and benthic cyanobacterial species can be easily exposed under environmental conditions to microcystins released either by themselves or by co-occurring cyanobacteria. Laboratory experiments with different cyanobacterial species demonstrated that exposure to microcystins or to other cyanobacterial oligopeptides might affect various physiological and biochemical responses, and allelochemical, signaling, or regulatory function of these compounds is often discussed (Babica et al. 2006a; Sedmak et al. 2008; Schatz et al. 2007; Vassilakaki and Pflugmacher 2008). However, possible effects of microcystins on cellular differentiation of cyanobacteria have not been previously studied.

Therefore, in our study, we investigated effects of cyanobacterial extract containing microcystins on filamentous cyanobacterium *Trichormus variabilis* occurring in soil and phytobenthic littoral communities (Komárek 1992). The major aim of this study was to examine if cyanobacterial extract with environmentally relevant concentrations of microcystins can affect age-induced cyanobacterial differentiation to akinetes and heterocysts and thus contribute to understanding of ecological impact of cyanobacterial oligopeptides and their natural function. We observed inhibition of cellular differentiation into heterocysts and akinetes in cyanobacterium *T. variabilis* caused by cyanobacterial extract with microcystins.

Materials and methods

Microcystis biomass extraction

Cyanobacterial water bloom dominated by *Microcystis aeruginosa* was extracted with aqueous methanol and semipurified with solid-phase extraction (SepPak 35 cc 10 g C-18 cartridge, Waters, Millford, USA). The solid phase extraction cartridge was washed and eluted using step-gradient of aqueous methanol. The content of microcystins in the eluted fractions was determined by HPLC Agilent 1,100 Series coupled with PDA detector (Agilent Technologies, Waldbronn, Germany) using Supelcosil ABZ+ Plus column, 150×4.6 mm, 5 μm (Supelco, Bellefonte, USA) at 30°C. Binary gradient of mobile phase consisted of (A) H_2O+0.1%TFA and (B) acetonitrile+0.1%TFA (linear increase from 20% B at 0 min to 59% B at 30 min), flow rate was 1 $mL\,min^{-1}$. UV spectra were recorded from 200 to 350 nm and chromatograms were evaluated at 238 nm. Microcystins were identified by comparison of UV spectra and retention time with standards of microcystin-LR, microcystin-LF, microcystin-LW, microcystin-RR, microcystin-YR (Alexis Biochemicals, Läufelfingen, Switzerland). The eluted fractions containing microcystins were combined, evaporated to dryness using rotary vacuum evaporation, and the solid phase extraction and evaporation was repeated two more times. The final microcystin fraction used in the experiments was dissolved in distilled water.

Cultivation of *T. variabilis*

The cyanobacterium *T. variabilis* (Kützing ex Bornet & Flahault) Komarek & Anagnostidis, strain Greifswald/92 (syn. *Anabaena variabilis* Kütz. ex Born. et Flah;.CCALA 204) obtained from Culture Collection of Algal Laboratory (Institute of Botany, Czech Republic) was cultivated on 100 mm glass Petri dishes on agar-solidified (1.5%, *w*/*w*) mixture of two cultivation media and water: Zehnder medium (Schlosser 1994), Bristol (modified Bold) medium (Stein 1973), and distilled water (1:1:2). Organism was grown at 22±2°C under continuous light (cool white fluorescent tubes 2,000 lx). Experiments with microcystins were carried out under the same conditions after 10 days of precultivation of *T. variabilis*.

Determination of microcystins in biomass of *T. variabilis*

Biomass of cyanobacterium *T. variabilis* was collected by centrifugation and then lyophilized. Freeze-dried biomass (200 mg) was extracted with 50% aqueous methanol (*v*/*v*) and the extract was concentrated using Supelclean LC-18 (Supelco) cartridges. Cartridges were eluted with 90% methanol+0.1%TFA and the content of microcystins was determined by HPLC-PDA as described above. Method limit of detection for each individual microcystin variant was 0.1 $\mu g\,g^{-1}$ dry weight.

Experimental design

Ten-day-old culture of *T. variabilis* on Petri dish was exposed to aqueous solution of semipurified extract of

Microcystis water bloom with the final experimental concentration of microcystins 0.2, 2 and 20 nM (control variant was treated with the equal volume of distilled water). Each experimental treatment was tested in five replicates. The organisms were exposed for 10 days. On the 3rd, 5th, 7th, and 10th day, a sample of cyanobacterium was taken from each dish and homogenized mechanically in a test tube with sterile cultivation medium for microscopic observation. Three independent repetitions of the experiment were performed.

Heterocyst and akinete frequency

The heterocyst and akinete frequency was analyzed by counting vegetative cells and akinetes in interheterocyst distances in randomly chosen filaments by microscopic observation (×400 magnification) according to Smith et al. (1987). Thousand cells were counted for every treatment and replicate.

Statistical analysis

The heterocyst and akinete frequency was recorded as the percentage of the total cell count (the sum of heterocysts, akinetes, and vegetative cells). The software Statistica 7.0 for Windows (StatSoft, Tulsa, OK) was used for statistical analyses. Homogeneity of variances was evaluated by Levene's test. Since some of the treatment groups have shown nonhomogeneity of variance, nonparametric Kruskal–Wallis test was used to assess the differences among the treatment groups. Values $p<0.05$ were considered statistically significant for all tests.

Results

In our study, *T. variabilis* was exposed to cyanobacterial extract containing microcystins. The final semipurified extract of *Microcystis* water bloom used in the experiment contained three prominent microcystin variants, microcystin-LR (22%), microcystin-RR (68%), and microcystin-YR (10%), whereas only a minor chromatographic peaks were observed for other compounds at wavelengths 220, 238, and 280 nm (Fig. 1).

Cyanobacterial extract was applied on cultures after 10 days of cultivation. This period represented the end of exponential growth and beginning of the stationary phase of growth characterized by elevated cell differentiation into specialized cells. The increase of cell differentiation in the control cultures during the 10-day experiment was documented by decline of relative number of vegetative cells (Fig. 2). The frequency of vegetative cells in the culture treated with extract containing 0.2 nM microcystins did not differ significantly from the control variant and decreased from 97% to 94% during the experiment. In the cultures exposed to 2 and 20 nM microcystins, the frequencies of vegetative cells remained relatively stable (96–97%) throughout the experiment and thus became significantly higher than in the control culture after 7 and 10 days of exposure (Fig. 2).

Heterocyst frequency showed only minor variations in both control and culture treated with extract containing 0.2 nM microcystin, but a significant increase in heterocyst frequency from days 7 to 10 was observed in these experimental variants (Mann–Whitney test, $p<0.05$). Relative numbers of heterocysts in the cultures treated with 2 and 20 nM microcystin remained unchanged during the experiment and the heterocyst frequency after 10 days of exposure to the extract with 20 nM microcystin was significantly lower than in the control culture (Fig. 3).

Akinete formation in the control increased during the experiment from 0.43% on day 3 to 2.8% on day 10 (Fig. 4). This age-induced formation of akinetes was

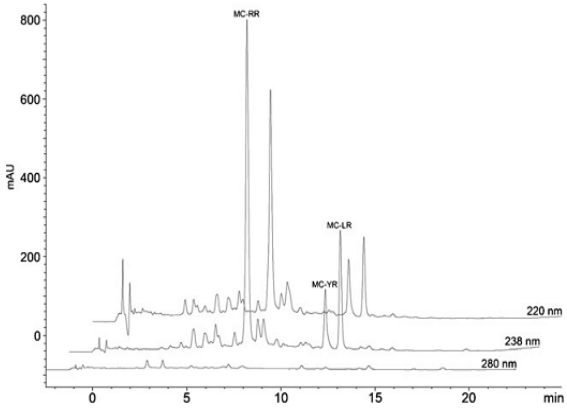

Fig. 1 HPLC chromatograms of semipurified extract of *Microcystis* bloom recorded at 238 nm (*solid line*), 220 nm (*dotted line*), and 280 nm (*dashed line*). The overlaid chromatograms are shifted by 5% on both *x*- and *y*-axis. *MC* microcystin

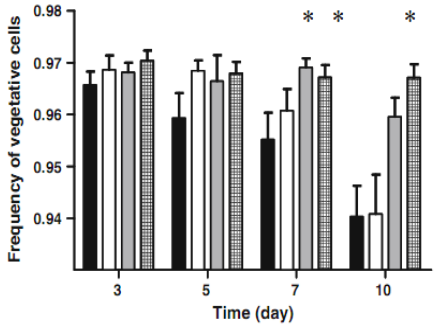

Fig. 2 Effect of extract of *Microcystis* bloom on frequency of vegetative cells (% of total cells counted) in *T. variabilis* over 10-day exposure. The columns represent: *black* control, *white* 0.2 nM MCs in extract, *gray* 2 nM MCs in extract, and *patterned* 20 nM MCs in extract. Values represent means±SEM (n=15). *Asterisks* indicate significant differences from control (Kruskal–Wallis test, p<0.05), *MCs* microcystins

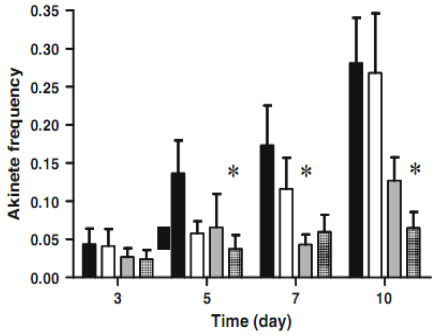

Fig. 4 Effect of extract of Microcystis bloom on akinete frequency (% of total cells counted) in *T. variabilis* over 10-day exposure. The columns represent: *black* control, *white* 0.2 nM MCs in extract, *gray* 2 nM MCs in extract, and *patterned* 20 nM MCs in extract. Values represent means±SEM (n=15). *Asterisks* indicate significant differences from control (Kruskal–Wallis test, p<0.05), *MCs* microcystins

inhibited by the exposure to *Microcystis* extract in concentration-dependent manner, which resulted in significantly lower akinete frequency in the cultures treated with the extract containing 20 nM microcystin, where only 0.24–0.65% akinetes was observed during the experiment.

We did not find any significant differences in total cell counts between the control and any treatment group (data not shown) and also no endogenous production of microcystins by the used strain of *T. variabilis* was detected by HPLC (data not shown).

In summary of our results, higher experimental concentrations of extract (containing 2 and 20 nM microcystin) suppressed cell differentiation consistently in all three independent repetitions of the experiment as indicated by all three measured parameters, i.e. higher relative number of vegetative cells, lower frequencies of heterocysts and akinetes.

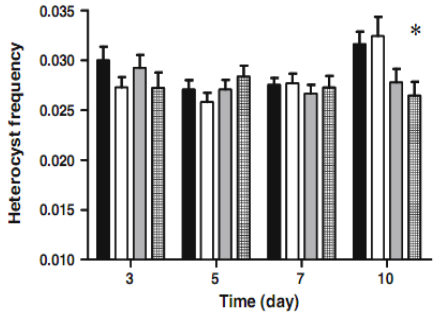

Fig. 3 Effect of extract of Microcystis bloom on heterocyst frequency (% of total cells counted) in *T. variabilis* over 10-day exposure. The columns represent: *black* control, *white* 0.2 nM MCs in extract, *gray* 2 nM MCs in extract, and *patterned* 20 nM MCs in extract. Values represent means±SEM (n=15). *Asterisks* indicate significant differences from control (Kruskal–Wallis test, p<0.05), *MCs* microcystins

Discussion

Under our experimental protocol, culture of filamentous cyanobacterium *T. variabilis* underwent cellular differentiation characterized by increase of akinete frequency and by complementary decrease of relative number of vegetative cells, the process induced by natural aging process of cyanobacterial culture (Fay and Van Baalen 1987). Furthermore, the relative number of heterocyst significantly increased in the control cultures from days 7 to 10 of experimental period. We may speculate that this occurred because of nitrogen starvation after total 10 days of precultivation and 10 days of the experiment.

Exposure of cyanobacteria to microcystins may result in various effects ranging from alteration of growth and cellular morphology, photosynthesis, changes in pigment, protein, and carbohydrate contents, modulation of enzyme activities, changes in glutathione pool, increased formation of reactive oxygen species, activation of gene expression, or cell lysis (Babica et al. 2007; Hu et al. 2004, 2005, 2008; Li et al. 2009; Sedmak and Elersek 2005; Sedmak and Kosi 1998; Singh et al. 2001; Valdor and Aboal 2007; Vassilakaki and Pflugmacher 2008). However, these effects are usually observed after exposure to microcystin at concentrations higher than 50 μg L^{-1}. Correspondingly, the treatment of *T. variabilis* with semipurified *Microcystis*

extract containing microcystins (0.2–20 nM corresponding to 0.2–20 μg L^{-1}) did not induce any differences in absolute cell numbers between the experimental variants and also no apparent changes of cell morphology or cell lysis were observed in our experiment. However, the treatment of *T. variabilis* with microcystins at concentrations 2 and 20 nM clearly inhibited the process of cellular differentiation observed in the control cultures and resulted in significantly higher frequency of vegetative cells and lower frequency of both heterocysts and akinetes in cultures treated with *Microcystis* extract.

To the best of our knowledge, this is the first report on effects of cyanobacterial extract containing microcystins on cyanobacterial cellular differentiation, but molecular mechanisms responsible for the observed effects are not known. Although possible indirect effects of microcystin on cultured organisms, e.g. formation of complexes with metals and alternation of nutrient bioavailability (Saito et al. 2008) or microcystin utilization as a source of nitrogen (Orr and Jones 1998) cannot be ruled out, other mechanisms of suppression of cell differentiation by microcystins may involve more specific interactions of microcystins with intracellular signaling pathways controlling formation of akinetes and/or heterocysts. Microcystins are known as potent inhibitors of eukaryotic serine/threonine protein phosphatases 1 and 2A. Genes encoding homologues of serine/threonine protein phosphatases were recognized in cyanobacteria and for instance, protein phosphatase PrpA characterized in *Anabaena* sp. is involved in regulation of nitrogen fixation and cellular differentiation. Inactivation of PrpA causes changes in structure of heterocysts resulting in non-functional nitrogen fixation (Zhang et al. 1998). Protein hetR, which is required for both heterocysts and akinete differentiation, is dephosphorylated by IphP phosphatase and represents another signaling pathway controlling cell differentiation via reversible protein phosphorylation (Potts et al. 1993). However, the information whether cyanobacterial phosphatases can be actually inhibited by microcystins is very limited. Shi et al. (1999) described that protein phosphatases isolated from microcystin-producing and non-producing strain of *M. aeruginosa* were not inhibited by known inhibitors of eukaryotic protein phosphatases, microcystin-LR and okadaic acid.

Besides inhibition of protein phosphatases, microcystins are known to induce an oxidative stress. Consequences of extensive formation of reactive oxygen species include not only oxidative damage of cellular components (DNA, proteins, membrane lipids) and growth inhibition but also alteration of intracellular redox-dependent signaling, which was shown to be involved in regulation of heterocyst differentiation (Jiang et al. 1997). Actually, formation of reactive oxygen species and altered expression of genes encoding antioxidant enzymes has been recently demonstrated in cyanobacteria exposed to microcystins (Hu et al. 2005; Li et al. 2009; Vassilakaki and Pflugmacher 2008). Importantly, the study of Vassilakaki and Pflugmacher (2008) reported formation of reactive oxygen species (hydrogen peroxide) and activation of different antioxidant enzymes in cyanobacterium *Synechocystis* after exposure to microcystins at environmentally feasible concentrations (0.05–1 μg L^{-1}), and the authors proposed that microcystin-induced oxidative stress may be a part of communication between cyanobacterial species (Vassilakaki and Pflugmacher 2008).

Although the observed effects on heterocyst and akinete formation have been so far discussed as a consequence of microcystin presence in the extract, we need to stress out that the extract of *Microcystis* water bloom was only semipurified using repeated solid-phase extraction and step-gradient elution. The extract contained three prominent microcystin variants, microcystin-RR, microcystin-YR, and microcystin-LR, and minor quantities of other unidentified microcystins. Since different microcystin variants can sometimes induce quantitatively different responses in phototrophic organisms (Babica et al. 2007), the use of such extract may better simulate the situation in the environment, where cyanobacteria are exposed to the mixture of different microcystin variants and also to other metabolites of cyanobacterial origin. Although the recorded HPLC chromatograms show prominent microcystin peaks and there is a lack of any other significant non-microcystin peaks at wavelengths 220 and 280 nm, which are the absorption maxima of some other cyanobacterial oligopeptides like anabaenopeptins or cyanopeptolines (Sedmak et al. 2009), we cannot rule out that such chemicals co-produced and co-eluted with microcystins might be also present in the extract and simply not being detected by our HPLC method. Therefore, these chemicals could have also contributed to or been responsible for modulation of cellular differentiation observed in our study. Since many cyanobacterial oligopeptides are known to inhibit eukaryotic serine-type proteases (Bubik et al. 2008), we can speculate that they might affect cell differentiation by inactivation of autoregulatory serine-type protease HetR, which is required for heterocyst differentiation (Dong et al. 2000). Although there is only limited information about effects of cyanobacterial oligopeptides on prokaryotic proteases (Ghosh et al. 2008), some cyanobacterial oligopeptides other than microcystins have been recently demonstrated to affect cyanobacteria themselves, although the underlying molecular mechanisms are unclear. Exposure of *Microcystis* sp. to microcystin, micropeptin, and microginin increased cellular content of McyB, a protein involved in microcystin biosynthesis, and the role of these compounds in intraspecies communication and infochemical function has been proposed (Schatz et al. 2007).

Planktopeptin BL1125, anabaenopeptin B, and anabaenopeptin F were shown to alter growth and activate lytic cycle in *M. aeruginosa* (Sedmak et al. 2008, 2009). It is evident from these studies that natural function and ecological role of microcystin should not be studied separately from the other non-ribosomally synthesized cyanobacterial oligopeptides, which are probably evolutionary and functionally closely related to microcystins (Rantala et al. 2004). It may also indicate that the effects of *Microcystis* on akinete and heterocyst formation might be still interpreted as a result of intraspecific signaling despite the absence of endogenous production of microcystins by *T. variabilis* in our study, since microcystins or other cyanobacterial oligopeptide from the *Microcystis* extract could just mimic an effect of some oligopeptide(s) naturally produced by *T. variabilis* and regulating cell differentiation.

Regardless, the effects observed in our study resulted from natural intra- or interspecies signaling function of cyanobacterial peptides, or they were just a side effect of oligopeptides production for other purposes, the inhibition of cellular differentiation in *T. variabilis* was induced by extract containing microcystins at concentrations which can be commonly found in the environment. Concentrations of extracellular microcystins, which are freely dissolved in water and thus represent a fraction bioavailable for potential uptake by cyanobacteria, range typically from 0.1 to 10 $\mu g\ L^{-1}$ (Sivonen and Jones 1999). A recent survey of dissolved microcystins in Czech water reservoirs reported a median concentration of dissolved microcystins 0.67 $\mu g\ L^{-1}$ and maximal concentrations about 9–37 $\mu g\ L^{-1}$, and these results are well comparable with data obtained from other countries worldwide (Blahova et al. 2007). About 2.5 $\mu g\ L^{-1}$ of microcystins was found in cyanobacterial benthic mats from Australian lake (Dasey et al. 2005) and microcystin concentrations in surface layer of sediments in eutrophic reservoirs could reach 100–300 $\mu g\ L^{-1}$ (Babica et al. 2006b; Ihle et al. 2005). However, detailed distribution and fate of microcystins in dynamic microenvironment of benthic communities or sediments is still under investigation; therefore, it remains unclear what portion of total microcystin content could be actually bioavailable for phytobenthic organisms.

Conclusions

Our study describes for the first time an effect of *Microcystis* extract on differentiation of a filamentous cyanobacterium (*T. variabilis*) into akinetes and heterocysts. The effect was achieved using the extract containing environmentally feasible concentrations of microcystins, indicating that our observations could have an ecological significance. The suppression of cell differentiation may contribute to structural and functional changes of phytoplankton or phytobenthic communities by affecting conservation and overwintering of filamentous cyanobacteria as well as processes of nitrogen-fixation, especially in eutrophicated water bodies. Whether the observed effects of cyanobacterial extract on the cell differentiation are a manifestation of an intraspecific signaling, competitive interspecific chemical interactions, or an evolutionary unintentional consequence of cyanobacterial oligopeptides production, needs to be addressed in the future studies, as well as the confirmation of the active compounds and responsible molecular mechanisms.

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1.4 Effects of microcystin and complex cyanobacterial samples on the growth and oxidative stress parameters in green alga *Pseudokirchneriella subcapitata* and comparison with the model oxidative stressor - herbicide paraquat

Paper III

Bártová, K., Hilscherová, K., Babica P., Maršálek B., Bláha, L. (2011). Effects of microcystin and complex cyanobacterial samples on the growth and oxidative stress parameters in green alga Pseudokirchneriella subcapitata and comparison with the model oxidative stressor - herbicide paraquat. **Environmental Toxicology** 26, 6, 641-648.

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Effects of Microcystin and Complex Cyanobacterial Samples on the Growth and Oxidative Stress Parameters in Green Alga *Pseudokirchneriella subcapitata* and Comparison with the Model Oxidative Stressor—Herbicide Paraquat

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ABSTRACT: Oxidative stress is one of the biochemical mechanisms involved in toxicity of cyanobacterial toxins microcystins (MC), but its role in the effects of complex water blooms is elusive. The aim of thisstudy was to investigate effects of pure MCs and different complex mixtures of cyanobacterial metabolites on the growth and biochemical markers of oxidative stress and detoxification in green alga *Pseudokirchneriella subcapitata*. Pure MCs at high concentrations (300 μg/L) had no effects on the growth of *P.subcapitata* (up to 10 day exposures) but stimulated activity of glutathione reductase (GR) after short 3 and 24 h exposures. Other biomarkers (levels of glutathione, GSH, and activities of glutathione-*S*-transferase, GST, and glutathione peroxidase, GPx) were not affected by pure MCs). Crude extract of the laboratory culture of cyanobacteria *Microcystis aeruginosa* (containing 300 μg/L of MCs) had no effects on algal growth or any of the biomarkers. Weak growth stimulations after 4–7 days were observed after exposures to the growth-spent medium of the *M. aeruginosa* culture, which also inhibited activities of GST after prolonged exposures. Other investigated parameters (reduced GSH and activity of GPx) were not affected by any of the cyanobacterial samples. The results were compared with effects of model oxidative stressor herbicide paraquat, which exhibited variable effects on both algal growth and biomarkers (decrease in reduced GSH, stimulations of GR). Taken together, although pure MCs induce oxidative stress in green alga, the effects of cyanobacterial mixtures, which are more relevant to the natural situation, are more complex and they differ from the pure toxin. High variability in the biochemical responses to the oxidative stress makes the interpretation of results complicated, which limits the use of these biomarkers as early warnings of toxicity under natural conditions. © 2010 Wiley Periodicals, Inc. Environ Toxicol 26: 641–648, 2011.

Keywords: microcystin; *Microcystis aeruginosa*; growth-spent medium; crude extract; laboratory cultures; reactive oxygen species; green alga; *Pseudokirchneriella subcapitata*

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INTRODUCTION

Cyanobacteria are photoautotrophic organisms capable to produce structurally variable range of secondary metabolites with different biological activities such as anticancer properties (cryptophycins, borophycin, calothrixin), biocide effects (fischerellins, nostocyclamide), protease inhibitors (cyanopeptolins, micropeptins, nostopeptin), iron chelators (schizokinen, synechobactins, anachelins), or number of cyanotoxins with adverse effects in organisms (microcystins (MCs), cylindrospermopsin, saxitoxins, anatoxins, etc.; reviewed for example in Gademann and Portmann, 2008).

Especially cyclic heptapeptides MCs are highly toxic for animals, and they were extensively studied. Nevertheless, natural role of MCs is not clear, and one of the disputable hypotheses of MCs functioning is allelopathy, i.e., modulations of other photoautotrophs (Pflugmacher, 2002). However, most of the studies that focused on MCs effects in aquatic phytoplankton, algae and plants, showed relatively high-effective concentrations, which are nonrelevant in the environment (Sivonen and Jones, 1999; Babica et al., 2006; Blahova et al., 2007).

One of important biochemical mechanisms by which MCs affect other organisms is an oxidative stress, which is often accompanied by modulations of subcellular parameters–biomarkers. Numbers of oxidative stress markers were modulated in different organisms by MCs (Ding and Ong, 2003), but the mechanism of reactive oxygen species (ROS) overproduction is still not known (Babica et al., 2006). It was also shown that MCs are detoxified via conjugation with glutathione (GSH) by glutathione-S-transferase (GST) (Pflugmacher et al., 1998), and stimulations of GST by MC-LR were also reported in microalgae (Pietsch et al., 2001).

Although effects of pure MCs were intensively studied, much less attention has been paid to more relevant environmental situations, i.e., mixture effects of MCs with other cyanobacterial metabolites, which can be released extracellularly during cyanobacterial growth or after the cell lyses (Welker and von Dohren, 2006). Some studies reported effects of cyanobacterial extracts or extracellular products (exudates) on other phytoplankton organisms (Pietsch et al., 2001; Valdor and Aboal, 2007) but role of MCs in these effects remain elusive.

The aim of this study was to investigate effects of different complex mixtures produced by cyanobacteria on growth and biochemical parameters in green alga *Pseudokirchneriella subcapitata*, and compare the results with effects of isolated MCs and model oxidative stress inducer, herbicide paraquat. We have studied extracellular mixtures (growth-spent media containing cyanobacterial exudates) and intracellular content (crude extract of cyanobacterial cells) of *Microcystis aeruginosa* laboratory culture. Besides growth, a set of biochemical markers related to oxidative stress and detoxification were monitored including concentrations of reduced GSH and activities of GST, glutathione peroxidase (GPx), and glutathione reductase (GR).

MATERIALS AND METHODS

MC Extraction and Characterization

MC-LR and MC-RR were isolated from the biomass of cyanobacterial water bloom (collected in the reservoir Nové Mlýny, Czech Republic, summer 2003, dominated by *Microcystis aeruginosa*) by solid phase extraction followed by HPLC purification and fractionation as described in Babica et al. (2007). MC content in the spent medium and crude extract of laboratory culture of *M. aeruginosa* was determined by HPLC coupled with UV/DAD (Babica et al., 2007).

Test Organisms and Culture Conditions

Green alga *Pseudokirchneriella subcapitata* (Syn.: *Selenastrum capricornutum* or *Raphidocelis subcapitata*) was obtained from the Culture Collection of Algal Laboratory, Institute of Botany, Academy of Sciences, Třeboîn, Czech Republic (strain CCALA 433). Cyanobacterium *Microcystis aeruginosa* producing MCs was obtained from the Pasteur Culture Collection of Cyanobacteria (Paris, France, strain PCC 7806). Both microorganisms were cultured in the mixture of Zehnder medium (Schlosser, 1994), Bristol (modified Bold) medium (Stein, 1973), and distilled water in the ratio 1:1:2 (v/v/v). Organisms were grown at 22°C ± 2°C under continuous light (cool white fluorescent tubes, 3000 lux) and aerated with sterilized air by passing through 0.22 μm filter (Labicom, Olomouc, Czech Republic). Exposures to studied samples were performed under same conditions as described for cultivations.

Preparation of Growth-Spent Media and Crude Extracts

Extracellular material produced by cyanobacterial culture (14-day-old growth-spent media of *M. aeruginosa*-producing MCs) was collected by the centrifugation (2000 × *g*, 5 min) and freeze-dried. The resulting pellets were reconstituted in 10% of its original volume (i.e., 10× concentrated) in the algal cultivation medium, sonicated and filtered through 0.22 μm filter (Labicom).

Biomass of laboratory culture of *M. aeruginosa* was collected by centrifugation and freeze-dried. Extracts in distilled water were prepared by sonication on ice (Bandelin Sonopuls HD2070, Berlin, Germany). Samples were diluted to reach 300 μg/L final concentrations of total MCs (MC-LR: 223 μg/L, MC-YR: 75 μg/L, and MC-RR: 2 μg/L) to obtain comparable design to the studies with purified MCs.

Algal Growth Inhibition Assay

Algal growth inhibition assays were performed in sterile 96-well microplates. Tested samples were mixed with an aliquot of the stock algal culture and cultivation medium to achieve optical density ~0.04–0.05 (at 680 nm). Final tested concentrations of extracts and spent medium of *M. aeruginosa* were 0 (negative control), 100, and 300 μg/L of MCs. Validity of the assays was controlled by a positive toxicant (three concentrations of the potassium dichromate). Each experimental concentration was tested in at least five replicates. Inhibition of algal growth was determined by microplate spectrophotometer (PowerWave, BioTek, Winoosa, USA) as changes of the optical density at 680 nm after 4, 7, and 10 days of exposure. Each experiment was repeated two times independently.

Determination of Biochemical Markers

To study modulations of oxidative stress biomarkers, green alga was exposed in the stationary phase for up to 168 h to 300 μg/L of purified MCs (concentration, which is high or extreme under natural conditions), spent medium of *M. aeruginosa*, crude extract of *M. aeruginosa* (both samples contained 300 μg/L of MCs), or herbicide paraquat as a positive control (5, 10, 20, and 200 mg/L). After the exposure, 50 mL of algal suspension was collected by centrifugation (10 min at 2000 × *g* at 4°C). Extracts were prepared by intensive mixing of algal material with glass beads for 5 min on ice in 500 μL phosphate-buffered saline (PBS; pH 7.2). Supernatant was collected after 15 min centrifugation (2000 × *g* at 4°C) and stored frozen at –80°C until biochemical analyses.

Biomarker methods for algal samples were modified according to our previous study with plant material (Paskova et al., 2006). Briefly, GST activity was measured spectrophotometrically at 340 nm determining GST-catalyzed formation of conjugate of 1-chloro-2,4-dinitrobenzene (2 mM) and reduced GSH (2 mM) (Habig et al., 1974). Activity was expressed as nanomoles of evolved product per minute per milligram of protein.

Assessment of GR activity was based on the oxidation of nicotinamide adenine dinucleotide phosphate, NADPH, recorded as a kinetic decline of absorbance at 340 nm (Carlberg and Mannervik, 1975). The reaction mixture contained sample, 50 mM potassium phosphate/1 mM EDTA buffer (pH 7.0), 1 mM glutathione oxidized disodium salt (GSSG), and 0.1 mM NADPH.

Activity of GPx was also determined from the rate of NADPH oxidation (Flohé and Gunzler, 1984). The initial reaction mixture (sample, 1.2 mM butylhydroperoxide, 3 mM GSH, and 1 U of GR, 1 U was defined as amount which reduces 1.0 μmole of oxidized glutathione per min at pH 7.6 at 25°C) was incubated for 5 min in the room temperature. Then NADPH was added (0.15 mM in 0.1 M potassium phosphate/1 mM EDTA buffer pH 7) and decline in absorbance at 340 nm was monitored. Activities of GR and GPx were expressed as nmoles of NADPH oxidized per minute per milligram protein.

The concentrations of reduced GSH were determined by spectrophotometric reaction using 5,5′-dithiobis-2-nitrobenzoic acid as a substrate (Ellman, 1959). Proteins from the algal extracts were removed with trichloracetic acid (25% w/v) followed by centrifugation (8000 rpm for 10 min at 4°C). Supernatant was mixed with 0.01 M 5,5′-dithiobis-2-nitrobenzoic acid in 0.8 M Tris (tris[hydroxymethyl]aminomethane)-HCl buffer (pH 8.9) and incubated for 5 min at room temperature. Absorbance was then measured at 420/680 nm, and the concentrations of GSH (nmol GSH/mg protein) were calculated according to the calibration of GSH.

The protein concentrations were measured according to Lowry et al. (1951) by spectrophotometric detection (680 nm) of colored complex formed by reaction with Folin-Ciocalteu phenol reagents and proteins. Bovine serum albumin was used as a standard for protein calibration.

Biochemicals and enzymes used in biomarker methods were purchased from Sigma-Aldrich (Prague, Czech Republic).

Statistical Evaluations

Normality and equal distribution of variances among variants were evaluated by the Kolmogorov–Smirnov test and Levene's tests, respectively. Differences from the negative control were compared by one-way analysis of variance (ANOVA) followed by the Dunnet's test. In all statistics, *P*-values less than 0.05 were considered significant.

RESULTS

Isolated MCs MC-RR and MC-LR at 300 μg/L had no effects on the growth of *P. subcapitata* during 4, 7, and 10 day exposures [Fig. 1(A)]. Similarly, crude extract of *M. aeruginosa* containing 300 μg/L MCs did not affect *P. subcapitata* growth [Fig. 1(B)]. The only effect (stimulations at 4 and 7 days) was observed at algae exposed to *M. aeruginosa* spent medium [Fig. 1(B)].

With the exception of paraquat, none of the other tested samples (i.e., pure MCs, extract of *M. aeruginosa* cells or growth-spent medium) affected numbers of *P. subcapitata* cells during the stationary phase (Fig. 2). Paraquat showed dose- and time-dependent effects. The highest concentration (200 mg/L) caused lyses of cells with rapid effects during 24 h, lower concentrations (5–20 mg/L) caused lyses after prolonged 96–168 h exposures [Fig. 2(B)].

Purified MC-LR stimulated activity of GR in *P. subcapitata* after 3 and 24 h [Fig. 3(A)], whereas it had no effects

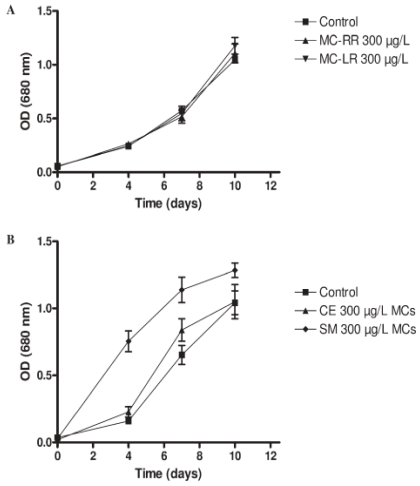

Fig. 1. Effects of MCs (A) or complex cyanobacterial samples (B) on the growth of *Pseudokirchneriella subcapitata*. Data represent means with standard errors (N = 3 replicates). CE, crude extract of cyanobacterial cells; SM, spent medium containing extracellular exudates.

on other parameters of oxidative stress and detoxification [Fig. 3(B–D)]. Crude cell extracts of *M. aeruginosa* did not significantly modulate any of the investigated biochemical parameters, and all treatments were comparable to the control during the entire experimental period. Growth-spent medium had the only effect on GST activity after 96 and 168 h [Fig. 3(C)], while other biochemical parameters were not affected.

Exposures to paraquat resulted in pronounced modulations of majority of studied biomarkers in *P. subcapitata* after 3 and 24 h exposures (Fig. 4). Prolonged 96–168 h exposures to all tested doses (5–200 mg/L) resulted in direct cytotoxicity [correlated with the effects on growth, Fig. 2(B)]. After short 3 h exposures, GSH concentration was statistically significantly increased at 5 and 10 mg/L [Fig. 4(B)], which also correlated with the stimulation of GR activity observed at the lower paraquat dose [Fig. 4(A)]. Downregulations of GST were detected but without clear dose-response relationship [Fig. 4(C)], and the activity of GPx was not affected [Fig. 4(D)].

DISCUSSION

MCs represent important group of waterborne toxins displaying various negative effects on aquatic organisms including photoautotrophs (Wiegand and Pflugmacher, 2005; Babica et al., 2006). Interestingly, the information of MCs effects in phytoplankton, especially eukaryotic algae, is still limited. MCs were shown to induce both growth inhibitions (Babica et al., 2007, Mohamed, 2008, Kearns and Hunter, 2000) and growth stimulations (Kearns and Hunter, 2000; Ou et al., 2005). Some studies reported modulations of photosynthetic pigments (Sedmak and Elersek, 2005; Mohamed, 2008), morphology (Sedmak and Elersek, 2005) or detoxification and antioxidant responses (Pietsch et al., 2001; Mohamed, 2008). Most of these effects might be attributed to two major toxicity mechanisms of MCs at molecular level, i.e., inhibitions of regulatory enzymes protein-phosphatases 1A and 2 (MacKintosh et al., 1990) or oxidative stress (Ding and Ong, 2003).

In this study, pure MCs had no significant effects on algal growth during 10 day exposures, although the toxin concentrations (300 μg/L) were relatively high compared to the concentrations commonly detected in the environment. Occasionally, several hundreds of μg/L can be detected during collapses of toxic cyanobacterial blooms or in the surface scum of water blooms (Sivonen and Jones 1999), but most often environmental concentrations of MCs are below 10 μg/L (Blahova et al., 2007).

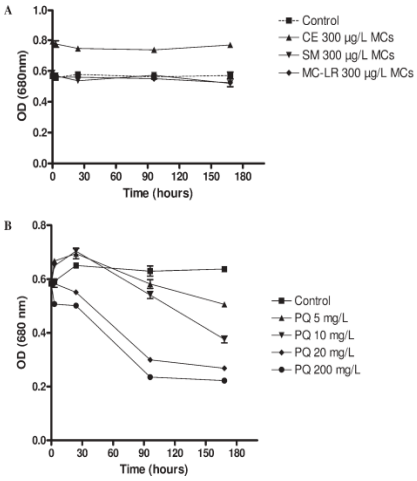

Fig. 2. Effects of cyanobacterial samples (A) and herbicide paraquat (B) on cell densities (determined at 680 nm) during the stationary phase of *Pseudokirchneriella subcapitata* growth. Data represent means with standard errors (N = 3 replicates). CE, crude extract of cyanobacterial cells; SM, spent medium containing extracellular exudates; MC-LR, microcystin-LR (all samples contained MCs at concentration 300 μg/L).

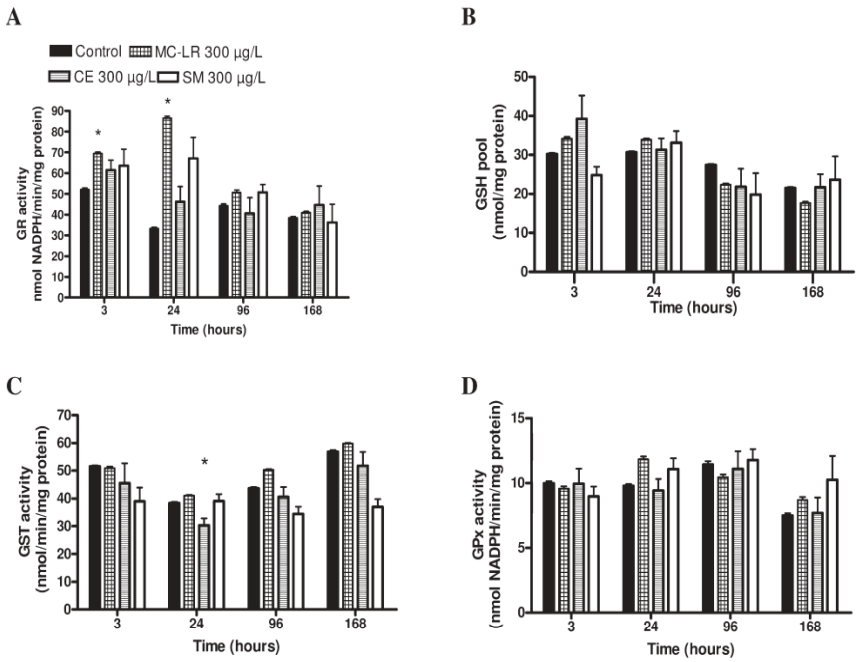

Fig. 3. Effects of pure MC-LR (300 μg/L), crude extract of *M. aeruginosa* cells (CE), and growth-spent medium of *M. aeruginosa* (SM) on oxidative stress biomarkers in *Pseudokirchneriella subcapitata* after 3–168 h exposures. (A) Activity of GR, (B) concentrations of GSH, (C) activity of GST, and (D) activity of GPx. Data represent means with standard errors (N = 3 replicates).

Weak effects of pure MCs on the growth and viability of green alga observed in this study correspond to our previous study with several algal strains exposed to wider concentration ranges of MC-LR and MC-RR (Babica et al., 2007). On the contrary, there are reports that showed effects of MCs (present in the form of crude cyanobacterial extract) on the growth of algae at concentrations as low as 10 μg/L (Kearns and Hunter, 2000; Mohamed, 2008).

It is apparent that effects of pure MCs may differ from the effects of cyanobacterial extracts or spent mediums with the same MC content (Pflugmacher et al., 2006; Valdor and Aboal, 2007) as also confirmed in our experiments. Although pure MC-LR (300 μg/L) had no effects on the cell growth, we have observed stimulations by the growth-spent medium (containing 300 μg/L MC-LR). This might be related to the presence of other organic products or residual nutrients in the complex spent medium mixture. Different results were reported from the studies with cyanobacteria *Cylindrospermopsis raciborskii*, i.e., inhibition of growth of various phytoplanktonic species after 24 h exposures (Figueredo et al., 2007). In another study, growth of green alga *Chlamydomonas reinhardtii* was inhibited by exudates from cyanobacterium *Anabaena flos-aquae*, and authors demonstrated reciprocal inhibitory effects of the green algae on the toxin production (MCs and anatoxins) in the cyanobacterium (Kearns and Hunter, 2000).

Besides the effects on growth, we have also studied modulations of oxidative stress biomarkers in green alga, which are considered early warnings of the chronic stress (Ferrat et al., 2003). Oxidative damage caused by cyanobacteria and MCs was previously reported from the studies with various phytoplankton species (Pietsch et al., 2001; Ou et al., 2005). Our experiments covered both short and prolonged exposures because biochemical changes are known to have variable temporal profiles (Ferrat et al., 2003). Oxidative stress was confirmed by observed transient stimulations of GR activities in the presence of pure MC-LR (3 and 24 h), while no

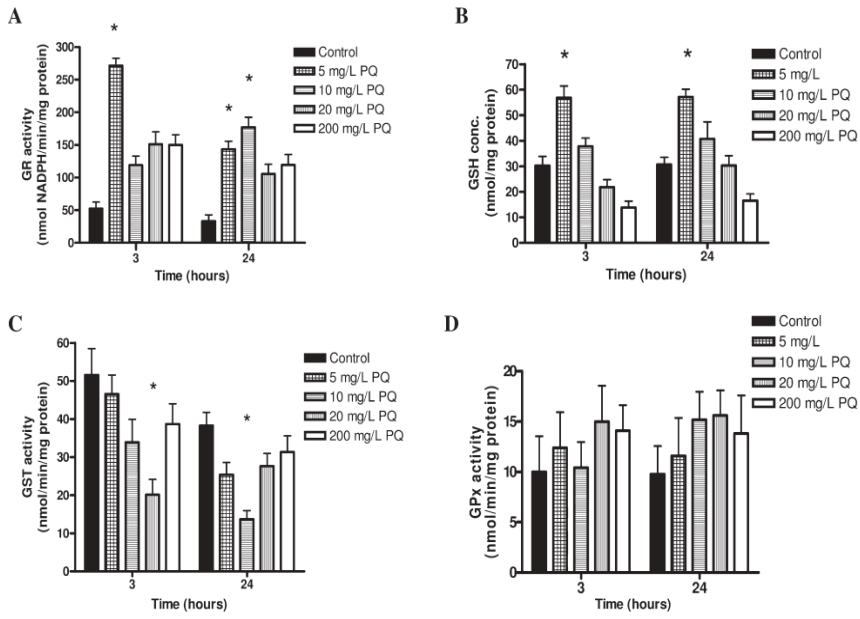

Fig. 4. Concentration-dependent effects of model oxidative stressor herbicide paraquat on biochemical parameters in *Pseudokirchneriella subcapitata* after 3 and 24 h exposures. (A) Concentrations of GSH, (B) activity of GR, (C) activity of GST, and (D) activity of GPx. Data represent means with standard errors ($N = 3$ replicates).

differences from controls were recorded after prolonged exposures. Similar profile was observed also for paraquat (elevations of GR activity at lower concentrations and short exposures) indicating that both paraquat and MC-LR induce similar biochemical responses related to the oxidative stress. Our findings spond to the studies with higher plants such as alfalfa *Medicago sativa* where MC-LR (5.0 μg/L MCs) as well as paraquat induced upregulated of GR activities (Pflugmacher et al., 2006). Inductions of GR (and also GST and GPx) were observed in green cress *Lepidium sativum* after 4 day exposures to pure MC-LR (Stuven and Pflugmacher, 2007), and comparable findings were reported in the study with common wheat *Triticum aestivum* (Pflugmacher et al., 2007).

While pure toxins induce relatively uniform biochemical responses, effects of complex cyanobacterial samples were variable. Thus, no modulations of GR were observed in algae exposed to cyanobacterial extract or spent-medium containing MCs in contrast to effects of pure MC-LR as discussed earlier. Similar, more potent effects of the pure toxin (in comparison with complex extract) were reported by Stuven and Pflugmacher (2007) but another study demonstrated more pronounced effects at complex extracts (Pflugmacher et al., 2006).

Such differences may be explained by possible interactions (antagonism or synergism) of MCs with other compounds present in the extract or exudate and also species-specific responses in different studies. For example, carotenoid pigments present in the complex mixture are known antioxidants (Mallick and Mohn, 2000; Ferrat et al., 2003), which may suppress the oxidative stress induced by MCs. Interactions of MCs with other compounds were addressed also in other studies with cyanobacterial lipopolysaccharides, LPS (Best et al., 2002) or anatoxin-a (Rogers et al., 2005).

In our study, we did not observe any effects on GPx but this enzyme appeared to be sensitive in other autotrophs including *Lepidium sativum*, where stimulations were observed after exposures to both MC-LR 10 μg/L and crude extract (Gehringer et al., 2003). Similar trend was observed in wheat *Triticum aestivum* after exposures to 0.5 μg/L MC-LR and MC-RR and crude cyanobacterial extract (Pflugmacher et al., 2007).

Also upregulations of GST, an important conjugation enzyme, by cyanobacterial samples were previously

reported (Pflugmacher et al., 2007). However, we observed GST inhibitions in algae exposed to the *M. aeruginosa* growth spent medium with MCs (no effects at pure MC-LR). While the stimulations represent specific biomarker response, inhibitions may reflect general toxicity (Halliwell and Gutteridge, 1999). Decrease in the GST activity was also documented in previous studies with green alga *Scenedesmus armatus* exposed for 1 h to cyanobacterial extract with 0.25 μg/L MCs, and these authors also reported stimulations of GST by pure MC-LR and MC-RR (Pietsch et al., 2001). Also paraquat temporarily inhibited GST in rat liver (Xi and Chen, 1997) or brown mussel *Perna perna* (Dafre et al., 2004).

It should also be noted that besides biomarkers investigated in this study, other authors also reported cyanobacteria-induced modulations of other parameters in photoautotrophs such as superoxidedismutase, catalase, peroxidase, or guajacol peroxidase (Mitrovic et al., 2005; Ding et al., 2009). MCs and cyanobacteria were also shown to cause oxidative membrane damage, i.e., lipid peroxidation, in several agricultural plants (Peuthert et al., 2007).

In summary, effects of complex cyanobacterial mixtures, which are relevant to natural situations, are complex and they apparently differ from the effects of pure MCs. Our experiments confirmed that pure MCs act via oxidative stress toxicity mechanisms in green alga *P. subcapitata*. On the other hand, complex crude aqueous extracts or spent media did not evoke modulations of studied oxidative stress parameters. GR was the most sensitive biochemical marker. It is an enzyme, which is induced by higher demands of reduced glutathione during oxidative stress situation. In complex mixtures (biomass or extracellular mixtures–growth-spent media), effects of pure MCs were modulated (or masked) by possible interactions with other nonidentified compounds. Our findings and literature demonstrate that studied biomarkers highly depend on the exposure regimes, concentrations, and they may be species-specific (Halliwell and Gutteridge, 1999). Biochemical responses to oxidative stress and detoxification are certainly useful in controlled laboratory studies investigating toxicity mechanisms. However, field measurements and straightforward interpretations of these biomarkers are complicated.

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Chapter 2

CARCINOGENIC POTENTIAL OF CYANOBACTERIAL METABOLITES

This chapter contains an introduction and original results, which are based on following publications:

Paper IV Nováková, K., Babica, P., Adamovský, O., and Bláha, L. (2011). Modulation of gap-junctional intercellular communication by a series of cyanobacterial samples from nature and laboratory cultures. **Toxicon** 58,1,76-84.

Paper V Nováková, K., Kohoutek, J., Adamovský, O., Babica, P., Jálová, V., Hilscherová, K., Bláha, L. (manuscript). Towards identification and characterization of novel tumor promoting metabolites produced in *Cylindrospermopsis raciborskii*. **In preparation for publication in Toxicological Sciences**

Paper VI Nováková, K., Babica, P., Blaha, L. (submitted). Potentiation effects in mixtures of toxic cyanobacteria and anthropogenic contaminants fluorathene and PCB 153 on an *in vitro* marker of tumor promotion - gap junctional intercellular communication. **Toxicology**

2.1. Carcinogenesis and cyanobacterial metabolites

Carcinogenesis can be initiated and promoted by many endogenous and external factors. The first group mentions immune system damage and inflammation caused by uncertain aetiology, genetic makeup, age, endocrine balance and physiological condition (reviewed in Oliveira, et al. 2007). The external factors includes nutritional habits (food preservation and preparation), socio-economic status, lifestyle (diet, cigarette smoking and betel chewing, alcohol drinking), physical agents (ionizing and non-ionizing radiation), biological infection (e.g. human papilloma virus and hepatitis B virus) and chemical compounds (natural and synthetic) (Wogan, et al. 2004). Contact with toxic compounds present in the air, water or food contamination may also enhance a risk of developing cancer (Oliveira, et al. 2007), and experimental and also epidemiological studies showed co-incidences of cancer after exposure to various anthropogenic contaminants such as aromatic hydrocarbons (PAHs), polychlorinated dibenzodioxins and polychlorinated dibenzofurans (PCDD/Fs) and polychlorinated biphenyls (PCBs) (Brody, et al. 2007).

Moreover, not only anthropogenic compounds can contribute to the development of cancer, higher prevalence of cancer were also linked with massive development of cyanobacteria in nutrient contaminated surface and coastal marine waters because of cyanobacterial production of bioactive and toxic metabolites (Pearson, et al. 2010; Svircev, et al. 2009). It is also necessary to consider possible interactions among cyanobacteria/metabolites and anthropogenic compounds co-occurred in water reservoir, but experimental studies are still insufficient and poor.

2.1.1 Cancer and carcinogens

Carcinogenesis is a multistage, multifactorial process consisting of three operational stages: initiation, promotion, and progression.

The one of the main characterization of cancer cells is incorrect control over cell proliferation caused by mutations. This situation can be caused by enhanced expression/hyperactivity of proteins initiated divisions (oncogens, e.g. Ras gene)

or blockage of antiproliferative mechanisms (tumor-suppressor genes/antioncogenes, e.g. p53) which stop proliferation, especially apoptosis.

According to mechanisms of action, carcinogens are commonly classified into two categories. Genotoxic carcinogens, for which mutations are supposed to be one of the first step in the development of cancer (DNA-reactive carcinogens, for example PAHs). Non-genotoxic or epigenetic carcinogens that do not bind covalently to DNA, do not directly cause DNA damage (e.g. phenoxy acid derivatives, alkylcarboxylic acids and phthalate esters) and are usually negative in the standard mutagenicity assays and act through a large variety of different specific mechanisms (Benigni and Bossa 2011).

Another class of nongenotoxic carcinogen acts through induction of hormonal imbalance and subsequent overproduction of hormones. For example, persistently increased hormonal levels stimulate cell proliferation and eventually tumors development (Benigni and Passerini 2002). Long time exposure to estrogenic hormones has been associated with increased cancer risk in hormone-sensitive tissues. The major mechanism of their carcinogenic effect is considered to be the stimulation of cellular proliferation through nuclear estrogen receptor-mediated signaling (Feigelson and Henderson 1996).

Oxidative stress is another possible mechanism by which non-genotoxic carcinogens may function. It derives from an imbalance between ROS production and the antioxidant capacity of the target cell. ROS may interact with critical cellular components such as protein, lipids, and DNA. Furthermore, it has been demonstrated that ROS interfere with signal transduction pathways and regulation of gene expression (Upham and Wagner 2001).

It has been also demonstrated that other mechanisms play role during abnormal cell growth and development including carcinogenicity such as down-regulation of gap-junctional intercellular communication (GJIC) in the tissues (Trosko and Goodman 1994).

Experiments in this part of the dissertation thesis were based mostly on *in vitro* bioassay testing GJIC, and this mechanism is explained in detail below.

2.1.2 Gap-junctional intercellular communication (GJIC)

Gap junctions are intercellular channels in the cytoplasmic membranes formed by proteins (connexins) that connect the cytoplasm of the neighboring cells. Connexins are four pass transmembrane proteins, six of which assemble to form continuous aqueous channel with 1.5 nm diameter, a connexon. Molecules with a molecular weight up to 1.2 kDa (e.g. cyclic AMP and Ca^{2+}) can pass through these channels and thus coordinate the functions of the cells. Various forms of connexins exist; their expression depends on the cell type. The ability to abolish and to restore functioning of GJIC represents one of the systems of intercellular regulation within tissues. Direct communication between cells through gap junctions constitutes a key regulatory system maintaining homeostatic balance of cell differentiation, proliferation, and death at the tissue level (Alberts, et al. 2002).

Functioning of GJIC is decreased in the majority of tumors in comparison to normal homologous tissues; in those tumors where the level of GJIC between tumor cells is high, GJIC is absent between tumor cells and the cells of adjacent normal tissue (reviewed in Krutovskikh and Yamasaki 1997). Thus, tumors with preserved functioning of GJIC between the cells inside the tumor remain insensitive to the regulatory influence of normal cells mediated by GJIC (Sharovskaya, et al. 2006).

Many nongenotoxic carcinogens exhibit an inhibition or reduction in GJICs, such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls or organochlorine pesticides as well as nickel and arsenic compounds (Blaha, et al. 2002; Gakhar, et al. 2009).

Inhibition of GJIC seems to be critical and central signaling events in the cell proliferative phases of human disease, such as cancer, also is considered as important *in vitro* biomarkers of tumor promoting potential (Naus and Laird 2010; Upham, et al. 2009).

2.1.3 Cyanobacteria and production of BAC with tumor promoting properties

Among various metabolites produced by cyanobacteria belong also compounds, which have been specifically associated with tumor promotion (reviewed in Sainis, et al. 2010; Zegura, et al. 2011). The most investigated and in legislation implemented is hepatotoxin microcystin, known a potent inhibitor of eukaryotic regulatory enzymes protein serine/threonine phosphatases PP1 and PP2A, and its tumor promoting activity has been demonstrated in both *in vitro* and *in vivo* experiments (Campos and Vasconcelos 2010; Humpage and Falconer 1999) More detailed information on microcystin toxicity and properties were discussed in the previous sections of this dissertation (see paragraph 1.1.4).

Another well studied hepatotoxin nodularin showed genotoxic potential and acts as tumor promoter due to inhibition of protein phosphatases 1 and 2A *in vitro* (Lankoff, et al. 2006) as well as *in vivo* (Ohta, et al. 1994), similarly as microcystins. On the other hand, no genotoxic or carcinogenic potential was documented about anatoxin-a, anatoxin-a(S) and no experimental data about mutagenicity, genotoxicity or carcinogenity are available for saxitoxins (reviewed in Zegura, et al. 2011).

Cylindrospermopsin is another cyanotoxin implicated in carcinogenesis, which irreversibly inhibits proteosynthesis and has been shown to induce genotoxic effects *in vitro* (Humpage, et al. 2000) as well as *in vivo* (Shen, et al. 2002) and initiate tumors in vivo (Falconer and Humpage 2001). Some of the experiments discussed in the following chapters were focused on cylindrospermopsin and its effect in comparison with complex cyanobacterial mixtures, and details about CYN properties and toxicity were discussed in the previous section of this dissertation (see paragraph 1.1.5)

2.1.4. Unknown cyanobacterial metabolites with tumor promoting potential

There is cumulative evidence that other cyanobacterial metabolites than well recognized cyanotoxins (microcystins or cylindrospermopsins) significantly contribute to cyanobacterial toxicity (Berry, et al. 2009; Vasconcelos and Almeida 2008). Recently novel tumor promoting metabolites different from known microcystins and cylindrospermopsin have been reported to inhibit gap junctional intercellular communication and activate mitogen-activated protein kinases bioassays (Blaha, et al. 2010). Inhibition of GJIC and MAPK activation effects were specifically induced by cyanobacterial but not bacterial or green algal extracts and interestingly no effects on both parameters were observed after pure microcystin and cylindrospermopsin.

However, not only the character of compound(s) responsible for the observed effects remains unknown, but so far there is limited knowledge whether GJIC-inhibitory effects are associated with particular cyanobacterial genera or strains and how often they can be detected in natural water blooms. Thus extracts and exudates from laboratory cultures with higher purity and lower complexity represent better material for investigation of tumor promotion **(paper IV)**. Inhibition of GJIC was caused by both intracellular (up 2 mg/ml d.w.) as well as extracellular mixtures (up 33times concentrated original extracellular mixture). Interestingly, no correlations of observed effects with toxin production (CYN and MCs) were found.

Also our further investigation was focused on characterization, isolation as well as screen on possible mechanisms involved in GJIC inhibition of causative agent/s extracellulary produced by *Cylindrospermopsis raciborskii* **(paper V)**. After detailed analyses compound (321.2 m/z) was found. It is supposed to be responsible for inhibition of GJIC and act with different mode of action from known tumor promoters such as PAHs and PCBs.

Several recent papers brought evidence about effect/toxicity enhancement due to interactions among cyanobacteria, other non-cyanobacterial contaminants and biological agents such as heavy metals or viral infection (Pikula, et al. 2010) or pesticides (Cerbin, et al. 2010).

Another study presented in this dissertation was focused on co-occurrence of cyanobacteria with other anthropogenic compounds such as polycyclic aromatic hydrocarbons-PAHs and polychlorinated biphenyls-PCBs **(paper VI)**. Binary mixture of anthropogenic pollutants fluoranthene and 2,2 ',4,4 ',5,5 '-hexachlorobiphenyl (PCB 153) showed additive effects as well as binary mixture of two cyanobacterial extracts. Surprisingly, synergistic interactions were reported only after exposure to binary mixtures of cyanobacterial extracts with anthropogenic pollutants (fluoranthene-PAHs or PCB 153).

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2.2. Modulation of gap-junctional intercellular communication by a series of cyanobacterial samples from nature and laboratory cultures.

Paper IV

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Modulation of gap-junctional intercellular communication by a series of cyanobacterial samples from nature and laboratory cultures

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ABSTRACT

Cyanobacterial extracts have been recently shown to alter two *in vitro* biomarkers of tumor promotion, namely to cause inhibition of gap-junctional intercellular communication (GJIC) and activation of mitogen-activated protein kinases (Blaha et al., 2010a). In the present study, we investigated GJIC-inhibitory potencies of 10 laboratory strains representing common water bloom-forming cyanobacteria (*Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis* and *Planktothrix*) and six natural water bloom samples (dominated by *Aphanizomenon* sp. or *Microcystis*). The most pronounced inhibitions of GJIC in a model rat liver epithelial cell line WB-F344 were caused by methanolic extracts of *Anabaena flos-aquae* UTEX 1444, *Aphanizomenon flos-aquae* SAG 31.87, *Aphanizomenon gracile* RCX 06, *Microcystis aeruginosa* PCC 7806, *Cylindrospermopsis raciborskii* SAG 1.97, *Planktothrix agardhii* CCALA 159 and SAG 32.79, whereas weaker effects were induced by *Aphanizomenon klebahnii* CCALA 009 and no inhibition was induced by extracts of *Aph. flos-aquae* PCC 7905 and *Aph. gracile* SAG 31.79. Exudates of the laboratory cultured strains concentrated by solid phase extraction also induced species-specific inhibitory effects, but they did not necessarily correlate with the inhibitory potencies of extracts from the corresponding species. Interestingly, the GJIC-inhibitory effects may not be restricted to cyanobacteria, since exudates of two green alga species also affected GJIC, although their extracts caused no effects. The extracts from different natural water blooms inhibited GJIC with different potencies without apparent relation to bloom-species composition. Since the observed effects on GJIC did not correlate with the content of cyanotoxins microcystins and cylindrospermopsin in the tested samples, they were most likely induced by unknown compound(s). Our results indicate that putative tumor promoting compound(s) could be associated with different species of bloom-forming cyanobacteria, but their production is probably species- and strain-specific.

1. Introduction

Cyanobacteria are known producers of toxic compounds, which have been linked to tumor promotion and chemical carcinogenesis (Blaha et al., 2010b). The most intensively studied cyanotoxin, microcystin-LR, is a potent inhibitor of eukaryotic regulatory enzymes protein serine/threonine phosphatases PP1 and PP2A, and its tumor promoting activity has been demonstrated in both *in vitro* and *in vivo* experiments (Humpage and Falconer, 1999; Ito et al., 1997; Nishiwaki-Matsushima et al., 1992). Moreover, a chronic exposure of human populations to drinking water contaminated with microcystins is being discussed as a factor increasing the prevalence of liver or colorectal cancer (Fleming et al., 2002; Svircev et al., 2009; Yu, 1995;

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Zhou et al., 2002). Another cyanotoxin implicated in carcinogenesis is cylindrospermopsin, which irreversibly inhibits proteosynthesis and has been shown to induce genotoxic effects *in vitro* (Humpage et al., 2000, 2005) and *in vivo* (Shen et al., 2002), increase morphological transformation *in vitro* (Maire et al., 2010), and initiate tumors *in vivo* (Falconer and Humpage, 2001).

Besides well-recognized cyanotoxins, cyanobacteria are capable of producing wide range of structurally diverse secondary metabolites (Welker and von Dohren, 2006), and there is cumulating evidence that cyanobacterial metabolites other than microcystins or cylindrospermopsin are significantly contributing to toxicity of cyanobacteria (Berry et al., 2009; Falconer, 2007). Interestingly, we have demonstrated recently that compounds present in cyanobacterial extracts but different from microcystins and cylindrospermopsin might elicit cellular responses known to be involved in the tumor promotion step of cancer (Blaha et al., 2010a). Specifically, biomass extracts of *Aphanizomenon flos-aquae* and *Microcystis aeruginosa* inhibited gap-junctional intercellular communication (GJIC) and activated mitogen-activated protein kinases (MAPKs) ERK1/2 in a pluripotent rat liver epithelial cell WB-F344. These effects were potently induced with cyanobacterial extracts but to much weaker extent with bacterial and green algal extracts (Blaha et al., 2010a).

Direct communication between cells through gap junctions constitutes a key regulatory system maintaining homeostatic balance of cell differentiation, proliferation, and death at the tissue level. Downregulation of GJIC is a common response of the cells to growth factors, oncogene activation or tumor promoters (Trosko, 2007; Trosko and Upham, 2005), including various environmental contaminants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls or organochlorine pesticides (Vinken et al., 2009). Although inhibition of GJIC may be a necessary step in the removal of a quiescent cell from the growth suppression, other epigenetic events such as the actual activation of a mitogenic-signaling pathway are also required (Trosko and Upham, 2005). Indeed, tumor promoters and chemicals inhibiting GJIC often activate MAPKs (Machala et al., 2003; Ruch et al., 2001; Rummel et al., 1999; Upham et al., 2009), another intracellular signaling mechanism regulating cell proliferation, differentiation, survival and adhesion (Alberts et al., 2002). Inhibition of GJIC and activation of MAPKs thus seem to be critical and central signaling events in the cell proliferative phases of human diseases, such as cancer, and they are considered as important *in vitro* biomarkers of tumor promoting potential (Upham et al., 2009).

Our recent study indicated that tumor promotion might be a process affected by cyanobacterial metabolites (Blaha et al., 2010a). However, not only the character of compound(s) responsible for the observed remains unknown, but so far there is limited knowledge whether GJIC-inhibitory effects are associated with particular cyanobacterial genera or strains and how often they can be detected in natural water blooms. It cannot also be ruled out that the observed effects on GJIC are result of general cell response to a mixture of chemicals present in complex cyanobacterial extracts (Palikova et al., 2007). Also it should be taken into account that natural cyanobacterial blooms may bioaccumulate other toxic compounds from water (Awasthi and Rai, 2004; Wang et al., 1998) or may be associated with bacteria contributing to their toxicity (Bernardova et al., 2008). Thus, samples with higher purity and lower complexity (such as fractionated extracts or exudates of laboratory cultures) could represent better material for more detailed investigation of the tumor promoting activity and characterization of the causative agents.

Therefore, the goal of the present study was to bring new information on the occurrence of GJIC-inhibitory effects among different cyanobacterial strains and natural water bloom samples. The focus of our study on laboratory cultured cyanobacteria aimed to eliminate eventual effects of non-cyanobacterial compounds potentially present in natural water bloom samples, and to investigate the specificity of the observed effects to cyanobacteria by comparing effects of cyanobacterial and green algal extracts. Our study also aimed to identify the strains inducing the strongest responses, which may become promising candidates for future isolation and chemical characterization of the putative GJIC-inhibiting compound(s). In order to obtain less complex material than biomass extracts, effects of partially purified and concentrated exudates (i.e. growth-spent media) of corresponding laboratory cultured strains were also investigated.

2. Materials and methods

2.1. Cyanobacterial strains and culture conditions

The list, identification and source of investigated cyanobacterial and algal strains as well as their production of cyanobacterial toxins microcystin and cylindrospermopsin are given in Table 1. All strains were cultivated in the mixture of Zehnder medium (Schlosser, 1994), Bristol (modified Bold) medium (Stein, 1973) and distilled water in the ratio 1:1:2 (v/v/v). The exception was *Aph. flos-aquae* PCC 7905 cultured in the nitrogen low BG11 medium (medium composition according to recommendation of Pasteur Institute). Organisms were grown for 14 days at 22 ± 2 °C under continuous light (cool white fluorescent tubes, 3000 lux) and aeration with air filtered through 0.22 μm filter (Labicom, Olomouc, Czech Republic). The cultivations were started with the 20% (v/v) of the inoculum with optical density 0.3 at 680 nm.

2.2. Biomass extract preparation

Biomasses of laboratory strains of the cyanobacteria and algae (Table 1) were collected by centrifugation (2880×g, 10 min, 25 °C) and freeze-dried. Supernatants were further processed to prepare exudate samples (see below). Biomasses of natural water blooms (Table 1) were collected and concentrated by plankton net (20 μm) and freeze-dried. Freeze-dried biomasses (100 mg) were twice reconstituted in 3 ml of 50% methanol and sonicated on ice (Bandelin Sonopuls HD2070, Berlin, Germany) for 5 minutes. After the sonication, samples were centrifuged

Table 1
List of strains and natural water blooms used in our study, their cyanotoxin content, and effects of extracts and exudates on GJIC. CCALA – Culture Collection of Autotrophic Organisms, Institute of Botany, Academy of Sciences of the Czech Republic; RCX – RECETOX Culture Collection of Cyanobacteria and Algae; SAG – Sammlung von Algenkulturen Goettingen; UTEX – The Culture Collection of Algae at the University of Texas at Austin; PCC – Pasteur Culture Collection of Cyanobacteria; MCs – microcystins; CYN – cylindrospermopsin; n.d. – not detected; n.a. – not analysed. Reported GJIC modulations were observed after exposure to 4 mg d.w./ml for extracts of laboratory strains and 10 mg d.w./ml for natural water bloom extracts.

Species	Source[a]	Cyanotoxin concentration (μg/g d.w.)[b]		GJIC after 30 min exposure (FOC)[c]		Total organic carbon (g/l)
		MCs	CYN	Extract (4 or 10 mg d.w./ml)	Exudate (50×)	Exudate (50×)
Non-treated control	–	–	–	1.00 ± 0.01	1.00 ± 0.10	
Cyanobacteria (Nostocales)						
Anabaena flos-aquae	UTEX 1444	n.d.	n.d.	0.02 ± 0.01 (0.84)	0.97 ± 0.11 (>100)	3.26
Aphanizomenon flos-aquae	PCC 7905	n.d.	3100 (29.8 μM)	0.93 ± 0.13	0.23 ± 0.09	1.79
Aph. flos-aquae	SAG 31.87	n.d.	n.d.	0.01 ± 0.00	0.28 ± 0.06	3.42
Aphanizomenon gracile	RCX 06[d]	n.d.	n.d.	0.02 ± 0.00 (1.05)	0.98 ± 0.19 (>100)	3.23
Aph. gracile	SAG 31.79	n.d.	n.d.	0.97 ± 0.18	0.45 ± 0.09	3.29
Aphanizomenon klebahnii	CCALA 009	n.d.	n.d.	0.54 ± 0.08	0.49 ± 0.07	3.14
Cylindrospermopsis raciborskii	SAG 1.97	n.d.	n.d.	0.00 ± 0.00 (1.55)	0.11 ± 0.01 (31.75)	3.22
Cyanobacteria (Chroococcales)						
Microcystis aeruginosa	PCC 7806	2500 (10 μM)	n.d.	0.20 ± 0.05 (1.38)	0.54 ± 0.09 (51.1)	3.52
Cyanobacteria (Oscillatoriales)						
Planktothrix agardhii	CCALA 159	170 (0.7 μM)	n.d.	0.02 ± 0.00 (1.70)	0.97 ± 0.19 (>100)	4.51
P. agardhii	SAG 32.79	200 (0.8 μM)	n.d.	0.01 ± 0.01	0.99 ± 0.16	3.24
Green algae (Sphaeropleales)						
Desmodesmus quadricauda	CCALA 463	n.d.	n.d.	0.98 ± 0.20	0.02 ± 0.00	2.13
Green algae (Chlorellales)						
Chlorella kessleri	CCALA 253	n.d.	n.d.	0.96 ± 0.15	0.43 ± 0.10	2.88
Natural water blooms[e]						
Aph. flos-aquae (80%)	Dehtář, 26.6.2007	8.5 (0.1 μM)	n.a.	0.43 ± 0.04	n.a.	n.a.
Aph. flos-aquae (80%)	Křižanovice, 3.9.2007	445 (4.5 μM)	n.a.	0.18 ± 0.02 (3.28)	n.a.	n.a.
Aph. gracile (80%)	Letovice–Křetínka, 18.9.2007	417 (4.2 μM)	n.a.	0.01 ± 0.03 (2.03)	n.a.	n.a.
Aph. klebahnii (80%)	Dehtář, 29.8.2007	n.d.	n.a.	0.65 ± 0.04	n.a.	n.a.
Aph. klebahnii (30%) and *Anabaena* sp. (50%)	Opatovický, 26.6.2007	n.d.	n.a.	0.30 ± 0.08	n.a.	n.a.
Microcystis sp. (70%)	Lednice – Zámecký rybník, 27.8.2007	1832 (18.4 μM)	n.a.	0.31 ± 0.05 (6.04)	n.a.	n.a.

[a] Culture collection ID for laboratory cultured strains or sampling site and date for natural water blooms.
[b] Concentration of cyanotoxins in biomass (method limit of detection was 5 μg/g d.w. for microcystins and 10 ng/g d.w. for cylindrospermopsin). Concentrations of cyanotoxins in the experiments with extracts are given in parentheses.
[c] Data are means of fraction of the solvent control (FOC) from three independent experiments ± standard deviations. Where available, IC50 values are given in parentheses.
[d] This species originates from CCALA (strain 008), but has been long-term cultivated at RECETOX.
[e] Percentage given in parentheses represents relative abundance of the species.

(2880×*g*, 10 min, 4 °C), collected supernatants were evaporated to dryness and redissolved in 50% methanol to reach the concentration equivalent to 400 mg d.w./ml for laboratory strains and 250 mg d.w./ml for natural water bloom samples.

2.3. Exudate preparation

Growth-spent media were separated from the cyanobacterial cells (biomass) by centrifugation (2880×*g*, 10 min, 25 °C) and filtered through 0.6 μm glass fiber filter (Fisher Scientific, Pardubice, Czech Republic). Organic compounds present in the media were concentrated by solid phase extraction (SPE) using two columns connected in the tandem: Oasis HLB column (Waters, Milford, MA, USA) and Carbograff column (Alltech, Deerfield, Illinois USA). SPE columns were eluted by 100% methanol and eluates evaporated to dryness. Residual material was redissolved in 100% methanol to reach the final volume that corresponded to 2000-times concentrated original media and sonicated for 5 min. Concentrations of total organic carbon (TOC) of exudates were measured by a LiquiTOC II analyzer (ELEMENTAR Analysensysteme, Hanau, Germany) using high temperature catalytic oxidation detection method.

2.4. Cyanotoxin analyses

Microcystins were analysed by HPLC Agilent 1100 Series coupled with PDA detector (Agilent Technologies, Waldbronn, Germany) using C18 Supelcosil ABZ+Plus column, 150 × 4.6 mm, 5 μm (Supelco, Bellefonte, PA, 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, -LW, -LF (Enzo Life Sciences, Lausen, Switzerland) and quantified using external calibrations (method limit of detection was 5 μg/g d.w. for microcystin). For cylindrospermopsin analyses, the extracts were reconstituted in Milli-Q water and analysed by the Agilent 1200 system coupled to 6410 Triple-Quad mass spectrometer with the electrospray interface. Separation was achieved on C18 Supelcosil ABZ+Plus column at 35 °C using a gradient elution with methanol acidified with 5 mM ammonium acetate. The mass spectrometer was operated in a multiple reaction monitoring mode (MRM) by monitoring the cylindrospermopsin transition ions m/z 416.2 ($M + H^+$) to 194.2 and m/z 416.2 to 176.1 for 25 ms dwell time. Identification and quantification was achieved using the product ions (194.2 and 176.1 transition) based on external calibration of cylindrospermopsin standard (Enzo Life Sciences, Lausen, Switzerland). Method limit of detection was 10 ng/g d.w. (Blahova et al., 2009).

2.5. Cell culture

The WB-F344 rat liver epithelial cell line was isolated by Drs. J.W. Grisham and M.S. Tsao of the University of North Carolina (Chapel Hill, NC) (Tsao et al., 1984). The cells (passage number between 6 and 26) were cultured in DMEM/Ham's F-12 supplemented with 5% fetal bovine serum (PAA, Pasching, Austria) at 37 °C in a humidified atmosphere containing 5% CO_2. For experiments, the cells were seeded at density 35×10^3 cell/cm^2 on 24-well tissue culture plates (TPP, Trasadingen, Switzerland). The bioassays for GJIC were conducted with confluent cell cultures obtained after two or three days of growth.

2.6. Gap-junctional intercellular communication (GJIC) assay

GJIC was assessed by scrape loading-dye transfer technique adapted from Elfouly et al. (1987). The samples were added directly to the cell culture medium from the concentrated stock solutions. Appropriate solvent controls (methanol) were tested in each experiment and did not induce any responses significantly different from the non-treated control (final methanol concentration in the well did not exceed 2.5%). After the exposure (5, 15 or 30 min), cells were washed with phosphate-buffered saline (PBS) and Lucifer Yellow dye (1 mg/ml of PBS; Sigma–Aldrich) was added. This membrane non-permeable dye was introduced into the cells with 10 scrapes through the monolayer of confluent cells using a surgical steel scalpel blade. The transfer of the dye through gap junction channels was allowed for 3 min, followed by a thorough rinse of the cells with PBS to remove extracellular dye. Cells were fixed using a 4% formaldehyde solution, and the dye transfer through the cell monolayer was observed at 100× magnification using a Zeiss OBSERVER.Z1 epifluorescence microscope equipped with a Zeiss AxioCam HRc camera. The image of the whole scrape was acquired by AxioVision software and quantified using the image analysis program created by Mr. Zdenek Kuna using MATLAB. Each experiment was performed three times independently in two replicates. Images were acquired from 6 scrapes for each replicate. The results were expressed as a fraction of the dye spread in the solvent control (fraction of the control, FOC).

2.7. Statistical evaluations

The mean values ± standard deviations from three independent experiments were evaluated by one-way ANOVA followed by Dunnett's post hoc test. P values less than 0.05 were considered statistically significant. The IC50 values and 95% confidence intervals were calculated using non-linear regression.

3. Results and discussion

In the first set of experiments, we investigated concentration-dependent effects of five cyanobacterial strains representing common bloom-forming species (*Anabaena flos-aquae* UTEX 1444, *Aphanizomenon gracile* RCX 06, *M. aeruginosa* PCC 7806, *Cylindrospermopsis raciborskii* SAG 1.97, *Planktothrix agardhii* CCALA 159) and also three natural water bloom samples dominated by *Aph. flos-aquae* (Křižanovice), *Aph. gracile* and *Microcystis* sp. The selected exposure period 30 min was previously demonstrated to be sufficient to achieve complete inhibition of GJIC by various chemicals as well as by cyanobacterial extracts in our cellular model (Blaha et al., 2010a; Machala et al., 2003).

After 30 min exposure, all studied extracts of laboratory strains significantly downregulated GJIC with the effective concentrations within the range of 1–4 mg d.w./ml (Fig. 1A). Although the extract of *A. flos-aquae* UTEX 1444 inhibited GJIC most potently, the observed differences in concentration-dependent GJIC inhibition between different strains were rather minor (IC50 values ranged between 0.84 and 1.70 mg d.w./ml) and extracts of all five strains inhibited GJIC at concentrations ≥4 mg d.w./ml almost completely (FOC < 0.20). These data are in a good agreement with the original results reporting similar effective concentrations for extracts of *Aph. flos-aquae* var. *gracile* CCALA 008 and *M. aeruginosa* PCC 7806 (Blaha et al., 2010a). The effects of exudates on GJIC were much less uniform than the effects of extracts prepared from corresponding cyanobacterial strains (Fig. 1B), despite the similar levels of TOC present in all five exudates (Table 1). The strongest inhibition of GJIC was induced by exudate of *C. raciborskii* SAG 1.97 with IC50 value corresponding to 31.75-times concentrated exudate, followed by *M. aeruginosa* PCC 7806 with IC50 value corresponding to 51.10-times concentrated exudate. The exudates of the other three strains did not affect GJIC at the experimental concentration range.

Extracts of natural water blooms differed in their GJIC-inhibitory potencies (Fig. 1C). The extract of *Aph. gracile* bloom was the most potent of the three tested samples and inhibited GJIC with efficiency similar to the extract of *A. flos-aquae* UTEX 1444 or *Aph. gracile* RCX 06 (IC50 2.03 mg d.w./ml). Inhibition of GJIC induced by the extract of *Aph. flos-aquae* bloom from water reservoir Křižanovice was less effective (IC50 3.28 mg d.w./ml) but still close to the responses elicited by the extracts of laboratory cultured strains, whereas inhibitory effects of *Microcystis* sp. bloom

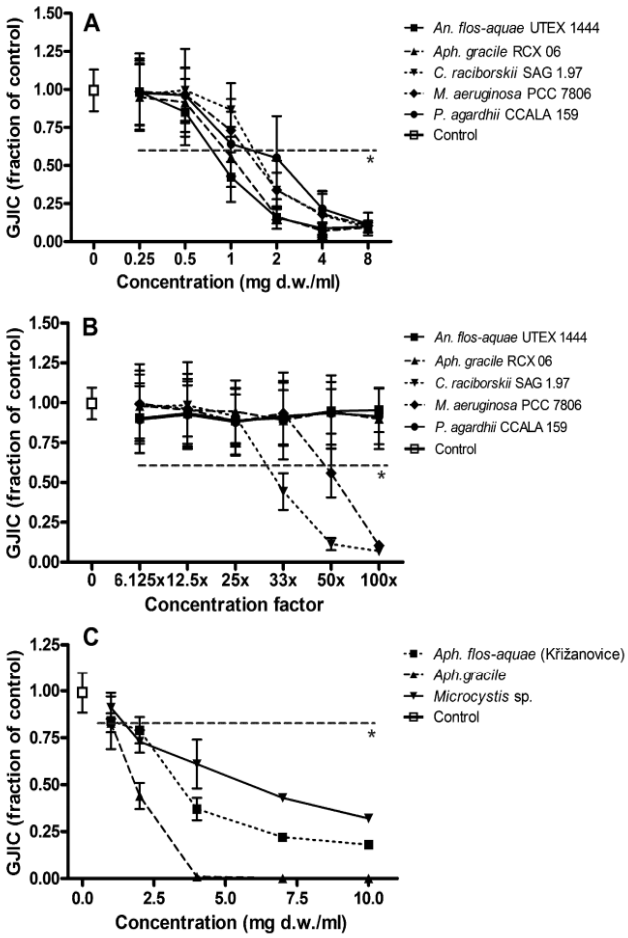

Fig. 1. Concentration-dependent inhibition of gap-junctional intercellular communication (GJIC) in WB-F344 cells caused by laboratory cultured strains or natural water blooms after 30 min exposure. (A) Effects of laboratory cultured strain extracts. (B) Effects of laboratory cultured strain exudates. (C) Effects of natural water bloom extracts. Data are means ± standard deviations of three independent experiments. *Mean values below the grey line were significantly different from control (ANOVA, $P < 0.050$, Dunnett's test).

extract were weaker (IC50 6.04 mg d.w./ml). These findings correspond to the results of Blaha et al. (2010a), who also reported greater differences between GJIC-inhibitory potencies of different water bloom extracts, with *Aphanizomenon* sp. bloom extract inducing stronger inhibition of GJIC and activation of MAPKs than *Microcystis* sp. bloom extract (Blaha et al., 2010a).

In the second set of experiments, time-response of GJIC inhibition induced by the same five laboratory cultured strains of cyanobacteria (*A. flos-aquae* UTEX 1444, *Aph. gracile* RCX 06, *M. aeruginosa* PCC 7806, *C. raciborskii* SAG 1.97, *P. agardhii* CCALA 159) was investigated. Since some chemicals (e.g. fluorinated fatty acids) were shown to completely inhibit GJIC even within 5 min of exposure (Upham et al., 1998), the cells were exposed to cyanobacterial samples for 5, 15 and 30 min. At the tested concentration (4 mg d.w./ml), the extracts rapidly inhibited GJIC and the observed effects became more pronounced with increasing exposure time (Fig. 2A). The time-course experiments revealed more remarkable differences between the studied extracts and clearly showed that *A. flos-aquae* UTEX 1444 extract induced the strongest effect when complete inhibition of GJIC occurred already after 5 min exposure (FOC 0.12), whereas 15–30 min exposure was required by the extracts of the other strains to close GJIC. The effects of exudates of laboratory strains were also

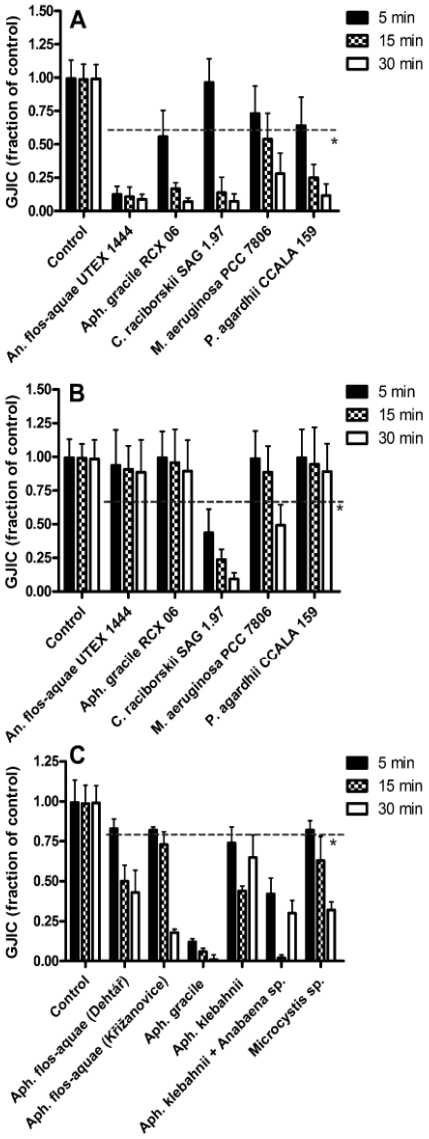

Fig. 2. Time-course inhibition of gap-junctional intercellular communication (GJIC) in WB-F344 cells caused by laboratory cultured strains and natural water blooms after different exposure times (5, 15 and 30 min). (A) Effects of laboratory strain extracts (4 mg d.w./ml). (B) Effects of laboratory strain exudates (50-times concentrated in comparison to the original growth-spent mediums). (C) Effects of natural water bloom extracts (10 mg d.w./ml). Data are means ± standard deviations of three independent experiments. *Mean values below the grey line were significantly different from control (ANOVA, $P < 0.050$, Dunnett's test).

time-progressive, but only *M. aeruginosa* PCC 7806 and *C. raciborskii* SAG 1.97 induced significant inhibition of GJIC within the experimental exposure regimes (Fig 2B), which corresponds well to the results of concentration–response experiments.

Since recent study of Blaha et al. (2010a) as well as our present results indicate that extracts of *Aphanizomenon* sp. and *Anabaena* sp. might be the most potent inhibitors of GJIC and therefore the samples of our particular interest, three additional extracts of complex water blooms dominated by *Aph. flos-aquae* (Dehtář), *Aph. klebahnii*, and *Aph. klebahnii* + *Anabaena* sp. were included into the time-response experiments. Higher experimental concentrations (10 mg d.w./ml) were used in these experiments with respect to weaker inhibitory potencies observed in some bloom samples. The experiments confirmed that extract of *Aph. gracile*, which inhibited GJIC almost completely after 5 min (FOC 0.12), was the most potent water bloom sample in our study (Fig. 2C). The effects of *Aph. flos-aquae* (Křižanovice and Dehtář) extracts and *Microcystis* sp. extract were less pronounced but progressing with exposure time, with maximum inhibition achieved after 30 min exposure. However, notably different time-course of inhibition was observed in the extracts of *Aph. klebahnii* bloom or *Aph. klebahnii* + *Anabaena* sp. bloom, which caused the maximum inhibition of GJIC after 15 min exposure (FOC 0.44 or 0.02) followed by restoration of communication after 30 min (FOC 0.65 or 0.30) (Fig. 2C). The observed differences in the time-responses may indicate that different extracts inhibited GJIC by different compound(s) with variable mode of action. For example, cells do not restore GJIC during exposure to polychlorinated biphenyl PCB 153, a chemical inhibiting communication by a different mechanism than tumor promoting phorbol ester or epidermal growth factor (Machala et al., 2003), which are known to downregulate GJIC only transiently (Rivedal and Opsahl, 2001).

The last part of our study focused on additional laboratory strains of cyanobacteria, namely *Aph. flos-aquae* PCC 7905, *Aph. flos-aquae* SAG 31.87, *Aph. gracile* SAG 31.79, *Aph. klebahnii* CCALA 009 and *P. agardhii* SAG 32.79. Based on the results from dose- and time-response experiments (Figs. 1 and 2), the effects of 30 min exposure to a single concentration of extracts (4 mg d.w./ml) and exudates (50-times concentrated) were studied. The results obtained from all 10 cyanobacterial strains under the study are compiled in the Table 1. Extracts of seven laboratory cultured strains inhibited GJIC completely, which suggests that the GJIC-inhibitory effects are in fact being induced by some compound(s) produced by cyanobacteria and not caused by other pollutants possibly bioaccumulated or biosorbed by cyanobacterial biomass, as it could be assumed in the case of natural water bloom samples. However, our experiments revealed apparent differences in GJIC-inhibitory potencies of various strains. The extract of *Aph. klebahnii* CCALA 009 induced only a partial inhibition at the tested concentration (FOC 0.54), and no effects on GJIC were elicited by extracts of *Aph. flos-aquae* PCC 7905 and *Aph. gracile* SAG 31.79. Also, the extracts of green algae *Desmodesmus quadricauda* CCALA 463 and *Chlorella kessleri* CCALA 253 did not affect GJIC. Considering the presence of such species-specific or strain-specific differences in GJIC-inhibitory potencies, it seems

that the effects were caused by unknown bioactive chemical(s) produced in particular strains rather than a result of general cell response to a complex mixture of cyanobacterial or algal primary and secondary metabolites. Moreover, GJIC was inhibited also by exudates concentrated and partially purified by SPE, which represent less complex mixture containing mostly organic compounds. No correlation between TOC concentrations in different exudates and their effects on GJIC was observed. Most exudates contained similar levels of TOC corresponding to 58–70 mg/L in non-concentrated samples, even though they inhibited GJIC to different extents (Table 1). The exudate of *P. agardhii* CCALA 159 with the highest concentration of TOC (90 mg/L in non-concentrated sample) did not cause inhibition of GJIC, whereas exudates of *Aph. flos-aquae* PCC7905 and *D. quadricauda* CCALA 463 containing the lowest TOC levels (36 and 43 mg/L in non-concentrated samples) were among the samples inducing the most profound inhibition of GJIC (FOC 0.23 and 0.02). These results suggest that metabolic activity and total amount of organic compounds extracellularly released during cyanobacteria or algae cultivation might have been similar, but production and release of causative agents of GJIC inhibition differed between species and strains.

Interestingly, the effects of exudates did neither correlate with tumor promoting activity of extracts of corresponding cyanobacterial strains (Table 1) and various scenarios were observed in our study: (A) GJIC was inhibited by both extract and exudate (*C. raciborskii* SAG 1.97, *Aph. flos-aquae* SAG 31.87, *Aph. klebahnii* CCALA 009, *M. aeruginosa* PCC 7806); (B) only the extracts but not the exudates downregulated GJIC (*A. flos-aquae* UTEX 1444, *Aph. gracile* RCX 06, *P. agardhii* CCALA 159, *P. agardhii* SAG 32.79); and (C) GJIC was inhibited only by the exudate and not by the extract (*Aph. flos-aquae* PCC 7905, *Aph. gracile* SAG 31.79). To our knowledge, there is only limited number of studies that investigated and compared effects of metabolites extracted from cyanobacterial cells and produced extracellularly. For example Bártová et al. (2010) reported that extract and exudate of *M. aeruginosa* PCC7806 induced quantitatively different oxidative stress-related responses in a green alga, even though the experimental concentrations of extract and exudate were adjusted to the same level of microcystin equivalents. The absence of such adjustment in the present study along with different growth characteristics of the studied strains could affect production and extracellular release of putative bioactive metabolites. This could then explain the lack of clear relationship between toxicities of extracts and exudates observed in our study. However, the possibility that different agents were responsible for the effects of extracts and exudates cannot be excluded. The importance of future toxicological and chemical characterization of exudates is stressed out by the finding that also exudates of the two green algae, *D. quadricauda* CCALA 463 and *C. kessleri* CCALA 253, downregulated GJIC, although their extracts had no effect (Table 1). These two green algal species represent environmentally common genera of plantkonic green algae (Reynolds, 2006) and therefore were chosen as relevant species for comparison with common species of planktonic cyanobacteria, however, more representative information on distribution of GJIC-inhibitory potencies and chemicals among green algae should be obtained by screening of more species in future studies.

Our study also demonstrated that the ability to inhibit GJIC can be found across different cyanobacterial orders and genera (Nostocales – *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*; Chroococcales – *Microcystis*; Oscillatoriales – *Planktothrix*) and also in the exudates of green algae, but the inhibitory potencies may substantially differ between the strains. This was most obvious between the extracts of *Aph. flos-aquae* PCC 7905 and *Aph. flos-aquae* SAG 31.87, or between extracts and exudates of *Aph. gracile* RCX 06 and *Aph. gracile* SAG 31.79. These observations correspond well to the fact that the main classes of cyanobacterial oligopeptides (aeruginosins, anabaenopeptins, cyanopeptolines, microcystins, microviridins, microginins and cyclamides) as well as other cyanotoxins (anatoxins, saxitoxins, cylindrospermopsins) can be found in phylogenetically distant cyanobacterial genera, yet the actual spectrum of bioactive metabolites produced by morphologically indistinguishable strains of the same species or morphotype can be different depending on the presence and activity of genes responsible for biosynthesis of these metabolites (Pearson et al., 2010; Welker and von Dohren, 2006). Similarly to the cyanotoxin production and toxicity of cyanobacterial samples, it seems that GJIC-inhibitory potencies also cannot be reliably predicted from morphological species identification.

Although the chemical(s) responsible for GJIC-inhibitory effects of cyanobacterial extracts in our *in vitro* model have not been characterized yet, they were most likely different from microcystins and cylindrospermopsin. Blaha et al. (2010a) reported no modulation of GJIC in WB-F344 cells after exposure to microcystin-LR (100 μM) or cylindrospermopsin (60 μM) and it has been proposed that eventual lack of expression of respective organic anion transport proteins required for microcystin uptake and toxicity OATPs could be responsible for the absence of microcystin effects in WB-F344 cells, since most cell lines *in vitro* do not express OATPs (Blaha et al., 2010a). These findings are strongly supported by our study, where GJIC-inhibitory potencies of the tested samples did not correlate with the content of microcystins or cylindrospermopsin in the studied samples and significant inhibitions of GJIC were elicited by the strains that did not produce these toxins in our study, such as *A. flos-aquae* UTEX 1444, *Aph. flos-aquae* SAG 31.87, *Aph. gracile* RCX 06 and SAG 31.79, *Aph. klebahnii* CCALA 009 or *C. raciborskii* SAG 1.97 (Table 1). Apparently, WB-F344 cells may be a target of GJIC-inhibiting chemicals different from microcystins and cylindrospermopsin. Inhibition of GJIC in WB-F344 cells might be of particular importance, since these normal liver epithelial cells possess characteristics of so-called oval cells, which have been recognized to have an important role in liver tumor promotion and hepatocarcinogenesis (Ruch and Trosko, 1999).

4. Conclusion

Although the research of carcinogenic and tumor promoting effects of cyanobacteria have mostly focused on

microcystins or cylindrospermopsin so far, cyanobacteria seem to produce a range of other but not-yet-identified metabolites with potential tumor promoting effects. We demonstrated that extracts as well as exudates of different cyanobacterial strains as well as extracts of natural water bloom samples inhibit GJIC in a model *in vitro* system. Inhibition of GJIC was caused by most of tested cyanobacteria with extracts of *A. flos-aquae* UTEX 1444, *Aph. flos-aquae* SAG 31.87, *Aph. gracile* RCX 06, *M. aeruginosa* PCC 7806, *C. raciborskii* SAG 1.97, *P. agardhii* CCALA 159 and SAG 32.79 being the most potent inhibitors, whereas green algal extracts did not alter GJIC. However, exudates of green algae downregulated GJIC to similar extent as several cyanobacterial exudates. Interestingly, inhibition of GJIC caused by both samples (extract and exudate) was reported only for few investigated strains, namely *Aph. flos-aquae* SAG 31.87, *Aph. klebahnii* CCALA 009, *C. raciborskii* SAG 1.97 and *M. aeruginosa* PCC 7806.

The data shows that the ability to inhibit GJIC is common among different cyanobacterial species and bloom samples, but may differ significantly between different strains. Identification and characterization of these bioactive compound(s) using approaches of the effect-directed analysis (EDA) is the subject of our ongoing research.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Ethical statement

The authors hereby declare that this manuscript has never been published/submitted for the publication elsewhere. All authors have approved the final version of the article.

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2.3. Towards identification and characterization of novel tumor promoting metabolites produced in *Cylindrospermopsis raciborskii*

Paper V

Nováková, K., Kohoutek, J., Adamovský, O., Babica, P., Jálová, V., Hilscherová, K., Bláha, L. Towards identification and characterization of novel tumor promoting metabolites produced in *Cylindrospermopsis raciborskii*. **Manuscript in preparation for Toxicological Sciences**

Introduction

Various cyanobacterial metabolites attract scientific attention because of their potential bioactivities. Some of the toxins such as Microcystin-LR were studied in detail. Microcystins are potent inhibitors of eukaryotic regulatory enzymes protein serine/threonine phosphatases PP1 and PP2A, and its tumor promoting activity has been demonstrated in both *in vitro* and in vivo experiments (Campos and Vasconcelos 2010; Humpage and Falconer 1999). Exposures of humans to drinking water contaminated with microcystins have been associated with increasing prevalence of liver or colorectal cancer (Fleming, et al. 2002; Svircev, et al. 2009; Yu 1995; Zhou, et al. 2002). Cylindrospermopsin is another cyanotoxin implicated in carcinogenesis. It was shown to irreversibly inhibits proteosynthesis and to induce genotoxic effects *in vitro* (Humpage, et al. 2000; Humpage, et al. 2005) and in vivo (Shen, et al. 2002), increase morphological transformation *in vitro* (Maire, et al. 2010), and initiate tumors in vivo (Falconer and Humpage 2001).

However, number of recent papers document toxic effects independently on cyanotoxins presence (Falconer 2007), which indicates existence of unknown metabolite/s playing the role in toxicity of complex cyanobacterial samples. We have recently reported on novel tumor promoting compound/s in intracellular content of cyanobacteria (Blaha, et al. 2010) but little evidences exist about extracellular mixtures (exudates) of cyanobacteria. It was shown that one cyanobacterial strain may produce various compounds with diverse bioactivity but their contributions to overall toxicity have not been explored in detail. For example, Microcystis aeruginosa PCC 7806 is known to produce cyanopeptolins A-D, cyanopeptolin 963A, [D-Asp3]microcystin-LR, microcystin-LR, as well as aerucyclamides A-D (Gademann, et al. 2010).

Extracellular metabolites of algae and cyanobacteria may play important roles such as communication between the cells of the same species (quorum sensing) or may affect other organisms (allelopathy). Several papers reported various effects of cyanobacterial exudates that included modulations of bacterial growth (Casamatta and Wickstrom 2000; Jaki, et al. 2000; Kaushik and Chauhan 2008; Seymour, et al. 2010); effects on green algae motility (Kearns and Hunter 2001), modulation of algal antioxidative markes (Bártová 2010), algal growth (Leao, et al. 2010; Suikkanen, et al. 2004), algal alkaline phosphatase (APase) activity ((Bar-Yosef, et al. 2010), growth in fungi (Zulpa, et al. 2003) and callus regeneration in rice (plant) (de Cano, et al. 2003) and growth in Lemna (Jang, et al. 2007). Nevertheless, biological activities and toxicities of the cyanobacterial exudates were explored much less than effects of whole cell extracts.

One of the key regulatory systems maintaining homeostatic balance in the tissues is direct communication between cells through gap junctions. This mechanism is known to control cell differentiation, proliferation, and deat, and downregulation of gap junctional intercellular communication (GJIC) is a common response to growth factors, oncogene activation or tumor promoters (Trosko 2007; Trosko and Upham 2005). Also environmental contaminants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls or organochlorine pesticides were shown to inhibit GJIC (Umannova, et al. 2008). Besides downregulation of GJIC, other epigenetic events are needed to remove a quiescent cell from the growth suppression and include for example mitogenic-signaling pathways (Trosko and Upham 2005). Indeed, tumor promoters and chemicals inhibiting GJIC often activate MAPKs (Machala, et al. 2003; Ruch, et al. 2001; Rummel, et al. 1999; Upham, et al. 2009) and other intracellular signaling mechanisms regulating cell proliferation, differentiation, survival and adhesion (Alberts, et al. 2002). Inhibition of GJIC and activation of MAPKs thus seem to be central signaling events in the cell proliferative phases of human disease, such as cancer, and they are considered as important *in vitro* biomarkers of tumor promoting potential (Upham, et al. 2009).

Another cellular mechanisms controlling cell growth, proliferation and differentiation are nuclear receptors, a family of intracellular transcription factors. Important and widely studied mechanism

of endocrine disruption is the interference of xenobiotics with estrogen receptor (ER) signaling. ERs are responsible for controlling the reproduction, cell proliferation and differentiation in various tissues and developmental process. Estrogens also control the bone formation, regulation of the overall organism homeostasis, cardiovascular system and behaviour. Toxic potencies of estrogens and xenoestrogens may be related to their activity towards binding and activation of the estrogen receptor, a transcription factor belonging to the steroid receptor family (Gillesby and Zacharewski 1998). A wide range of compounds including natural products, pharmaceuticals and industrial chemicals has been shown to be estrogen mimics. The aims of our study were to study toxic effects (potencies to inhibit GJIC and activate ER) of the extracellular metabolites produced by *C. raciborskii*. The material was selected with respect to several criteria. First, *C. raciborskii* is globally distributed cyanobacteria, and it forms dense blooms and produce toxic metabolites including known toxin cylindrospermopsin. Second, our preliminary experiments revealed high GJIC-inhibiting activities in both cellular extracts and extracellular metabolites. Third, we focused on the exudates because their importance is in general less explored than cellular metabolites, and they are of higher environmental relevance (release of compounds during the growth of cyanobacteria). Fourth, the exudates were considered less complex mixture in comparison to whole cellular extracts, which provided advantage during investigation of the causative tumor promoting agent/s. We have sequentially fractionated the extracellular matrix and studied biological activities of individual fractions by the assays for *in vitro* inhibition of GJIC and activation of ER (reporter gene assay with MVLN cell line). The most active compounds were analysed using the LC/MSMS to search for responsible bioactive compound, and possible mechanisms involved in GJIC inhibition were screened using the inhibitors of signaling pathways.

Material and methods

Chemicals and reagents

Methanol (LC/MS grade) was purchased from Biosolve (Valkenswaard, Netherlands). Deionized water was prepared with the Millipore Simplicity 185 system (Millipore, Bedford, USA). Standards of cylindrospermopsin and microcystin-LR were purchased from Enzo life Sciences (Lausen, Switzerland) and arachidonic acid, oleoyl-L-α-lysophophatidic acid sodium salt and 3-sn-phosphatidic acid sodium salt from egg yolk lecithin were obtained from Sigma-Aldrich (Prague, Czech Republic).

Cyanobacterial strain and Culture Conditions

The cyanobacterial strain Cylindrospermopsis raciborskii was purchased from the Goettingen University Collection of Cyanobacteria - SAG (Goettingen, Germany) and was cultivated in the mixture of Zehnder medium (Schlosser 1994), Bristol (modified Bold) medium (Stein 1973) and distilled water in the ratio 1:1:2 (v/v/v). Organism was grown for 21 days at 22°C ± 2°C under continuous light (cool white fluorescent tubes, 3000 lux) and aeration with air filtered through 0.22 μm filter (Labicom, Olomouc, Czech Republic). The cultivations were started with the 20% (v/v) of the inoculum with optical density 0.3 at 680 nm.

Exudate preparation

Growth-spent media were separated from the cyanobacterial cells (biomass) by centrifugation (2880 x g, 10 min, 25°C) and filtered through 0.6 μm glass fiber filter (Fisher Scientific, Pardubice, Czech Republic). Organic compounds present in the media were concentrated by solid phase

extraction (SPE) using two columns connected in the tandem: Oasis HLB column (Waters, Milford, MA, USA) and Carbograff column (Alltech, Deerfield, Illinois USA). SPE columns were eluted by 100% methanol and eluates evaporated to dryness. Residual material was redissolved in 100% methanol to reach the final volume that corresponded to 2000-times concentrated original media and sonicated for 5 minutes.

Cyanotoxin analyses

Microcystins were analysed by HPLC Agilent 1100 Series coupled with PDA detector (Agilent Technologies, Waldbronn, Germany) using C18 Supelcosil ABZ+Plus column, 150 x 4.6 mm, 5 µm (Supelco, Bellefonte, PA, 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, -LW, -LF (Enzo Life Sciences, Lausen, Switzerland) and quantified using external calibrations (method limit of detection was 5 µg/g d.w. for microcystin). For cylindrospermopsin analyses, the exudate was reconstituted in Milli-Q water and analysed by the Agilent 1200 system coupled to 6410 Triple-Quad mass spectrometer with the electrospray (ESI) interface. Separation was achieved on C18 Supelcosil ABZ+Plus column at 35°C using a gradient elution with methanol acidified with 5 mM ammonium acetate. The mass spectrometer was operated in a multiple reaction monitoring mode (MRM) by monitoring the cylindrospermopsin transition ions m/z 416.2 (M+H+) to 194.2 and m/z 416.2 to 176.1 for 250 ms dwell time. Identification and quantification was achieved using the product ions (194.2 and 176.1 transition) based on external calibration of cylindrospermopsin standard (Enzo Life Sciences, Lausen, Switzerland). Method limit of detection was 10 ng/g d.w. (Blahova, et al. 2009).

Cell cultures

The WB-F344 rat liver epithelial cell line was isolated by Drs. J.W. Grisham and M.S. Tsao of the University of North Carolina (Chapel Hill, NC) (Tsao, et al. 1984), and cultured in DMEM/Ham´s F-12 supplemented with 5% fetal bovine serum (PAA, Pasching, Austria) on 24-well tissue culture plates (TPP, Trasadingen, Switzerland). The MVLN, human breast carcinoma cells transfected with a luciferase gene under control of estrogen receptor activation (Demirpence et al., 1993), was cultivated in medium DMEM/F12 (Sigma-Aldrich) supplemented with 10% fetal calf serum Mycoplex (PAA, Pasching, Austria). Both cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO2.

Gap-junctional intercellular communication (GJIC) assay

GJIC was assessed by scrape loading-dye transfer technique adapted from (Elfouly, et al. 1987) on WB-F344 rat liver epithelial cell line. The samples were added directly to the cell culture medium from the concentrated stock solutions. Appropriate solvent controls

(methanol) were tested in each experiment and did not induce any responses significantly different from the non-treated control. After the exposure (5, 15 or 30 minutes), cells were washed with phosphate-buffered saline (PBS) and Lucifer Yelow dye (1 mg/ml of PBS; Sigma-Aldrich) was added. This membrane non-permeable dye was introduced into the cells with ten scrapes through the monolayer of confluent cells using a surgical steel scalpel blade. The transfer of the dye through gap junction channels was allowed for three minutes, followed by a thorough rinse of the cells with PBS to remove extracellular dye. Cells were fixed using a 4% formaldehyde solution, and the dye transfer through the cell monolayer was observed at 100-times magnification using a Zeiss OBSERVER.Z1 epifluorescence microscope equipped with a Zeiss AxioCam HRc camera. The

images acquired by AxioVision software and quantified using the image analysis program created by Mr. Zdenek Kuna using MATLAB. Each experiment was performed three times independently. The results were expressed as a fraction of the dye spread in the solvent control (fraction of the control, FOC). Cytotoxicity of all studied samples was first tested by the Cytotoxicity Detection Kit Plus LDH (Roche Applied Sciences), and only those concentrations that had no adverse effects on the cellular viability were used for further assessment.

Bioluminiscence estrogenicity assay

MVLN cell line was exposed in DMEM/F12 (PAA, Pasching, Austria) supplemented with 5% dialyzed fetal calf serum (PAA, Pasching, Austria), which was additionally dextran/charcoal treated to further decrease background concentrations of estradiol. Approximately 24 h after plating, cells were exposed to the extracts dissolved in DMSO and calibration reference 17β estradiol (E2; Sigma-Aldrich; Czech Republic; dilution series 0–500 pM) for MVLN. Effects of extracts on MVLN cells were assessed either singly or in combination with competing endogenous ligand. The estrogenicity was assessed by simultaneous exposure of the sample extract and 17β estradiol (11pM). The final concentration of the solvent was less than 0.5% v/v in the exposure media and appropriate solvent controls were tested. Several dilutions of each extract were tested to provide dose–response curve for each sample. Every dilution was tested in triplicate. At least two independent assays were conducted for each sample in all test systems. After 24 h of exposure, the intensity of luciferase luminescence corresponding to the respective receptor activation was measured using the Promega Steady Glo Kit (Promega, Madison, USA) (Novak et al., 2009).

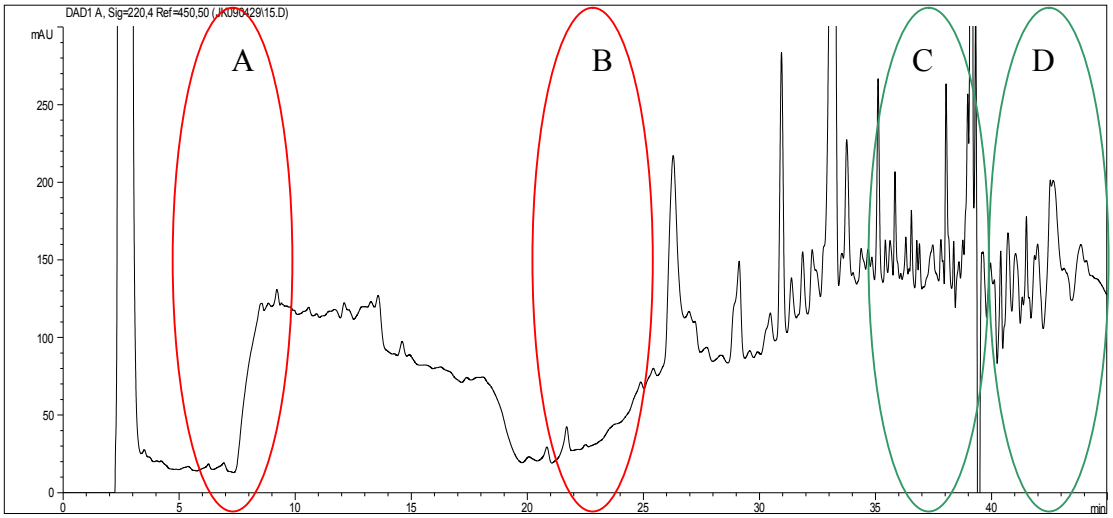
Liquid Chromatography Electrospray Ionization Mass Spectrometry Analyses

Analyses were performed with the HPLC apparatus Agilent 1100 series (Agilent Technologies, Waldbronn, Germany) consisting of a vacuum degasser, a binary pump, an autosampler, and a thermostatted column compartment kept at 30 deg.C. The column was a Phenomenex Synergi Fusion RP-C18, 100 x 2mm, 4┘ m, 80A (Phenomenex, Torrance, CA, USA). The mobile phase consisted of water (A) and methanol (B). The non-linear binary pump gradient was as follows (0 min - 15.00 min, 99% B; 15.01 min – 20.00 min, 100% B; 20.01 min – 25.00 min, 50% B); the flow rate was 0.25 mL/min (0 min – 20.00 min) or 0.5 mL/min (20.01 – 25 min), respectively. 10 ┘ L of individual sample was injected for the analyses. The mass spectrometer was an AB Sciex 5500 QTrap (AB Sciex, Concord, ON, Canada) with electrospray ionization (ESI). Ions were detected in the positive mode. The ionization parameters were as follows: capillary voltage, 5.5 kV; ion source temperature, 400 deg.C; curtain gas (CUR) 15 psi, gas 1 (GS1) 40 psi, gas 2 (GS2) 30 psi, declustering potential (DP) 100 V, entrance potential (EP) 10 V. Ions were monitored in full scan mode (Q1 MS, mass range 250 – 1250 m/z). Analyst 1.5 and LightSight® software were used for analysis of acquired spectra. Fragmentation pattern of unknown protonated molecule (m/z 321.2) was determined using the same instrument in Enhanced Product Ion (EPI) and MS3 modes. In EPI mode precursor ion (m/z 321.2) was fragmented (collision energy, CE; 25 – 55 V, collision gas; N2, setting HIGH) and fragments were monitored in mass range 50 – 1000 m/z. In MS3 mode selected fragments (m/z 303.1, 149.0 and 109.0) of unknown compound were further fragmented (linear ion trap fill time; 20.0 msec, excitation time; 50.0 msec) and second generation fragments were monitored in mass range 50 – 1000 m/z.

Results and Discussion

Originally tropical species cyanobacterium C. raciborskii has expanded to colder world regions (Briand, et al. 2004), which was followed also by detection of cyanotoxin cylindrospermopsin (CYN) in many countries. In our study, we investigated the presence of known toxins in C. raciborskii exudates, and studied biological activities of complex mixtures as well as individual fractions. The preliminary experiments (not presented here) demonstrated high potencies to inhibit GJIC, and we focused on detailed characterization of this effect. As a first step, concentrated exudates were fractionated using the HPLC C-18 chromatographic separation (Fig.1) in 5 min intervals of the retention time.

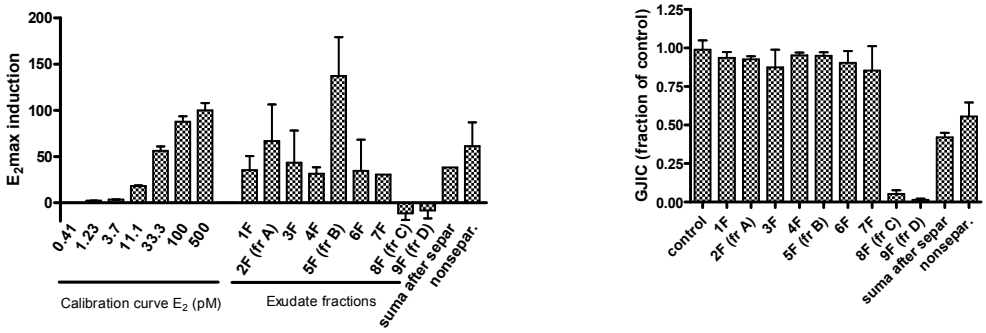
Figure 1 Chromatogram (220 nm) of complex C. raciborskii SAG 1.97 exudate, fractions were separated in 5 min intervals of retention time. Fractions (marked by circles) A and B showed estrogenic potential, fractions C and D strongly inhibited GJIC.



Fractions were tested for estrogenic and tumor promoting potential *in vitro*. The effects are shown in Figure 2.

Figure 2 Biological activity (estrogenicity and tumor promotion) of fractions of *C. raciborskii* exudate: **A)** The estrogenic activity (expressed as % of maximal ER-mediated induction caused by standard estradiol – E_2max) in MVLN cell line after 24h exposure (20x concentrated fractions). **B)** Gap-junctional intercellular communication (GJIC) in WB-F344 cells after 30min exposure (48x concentrated fractions) Terms: sum after separation – fraction joined together after separation; nonsepar – non separated exudate (original) Data are means ± standard deviations of three independent experiments.

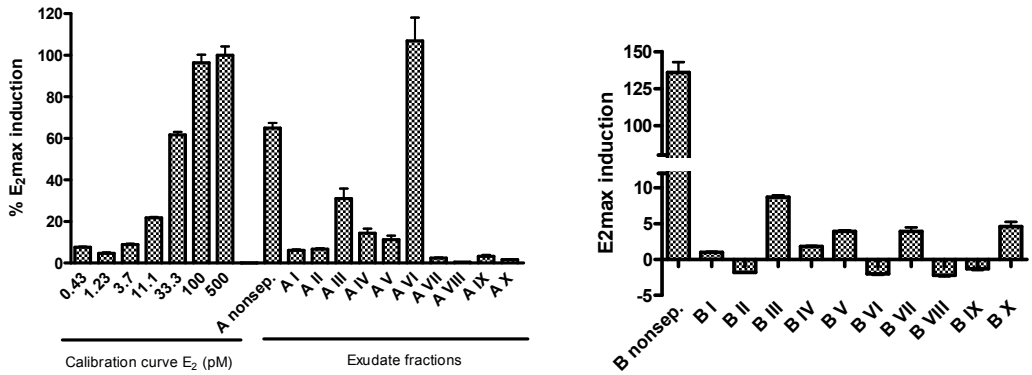
2A **2B**



As shown in Figure 1, estrogenic activity was induced by two hydrophilic fractions (fractions A and B in Fig.1), while inhibitions of GJIC were recorded in two highly hydrophobic fractions (fraction C and D in Fig 1). For detailed characterization, the bioactive (estrogenic and tumor promoting) fractions were further fractionated by collecting fractions at the 30s interval of retention. Figure 3 shows estrogenicity of individual subfractions. Interestingly, in the fraction A, the level of estrogenic potential remained preserved, while at the subfractions of the originally active B were not estrogenic (Fig 3A and 3B). Clear peak of the activity was observed especially at the fraction A-VI, which indicates presence of one (or few) structurally related estrogenic compounds. To our knowledge, this is one of the first reports on estrogenic metabolites in cyanobacteria, and their detailed characterization will require further attention.

Figure 3 The estrogenic activity (expressed as % of maximal ER-mediated induction caused by standard estradiol – E_2max) in MVLN cell line after 24h exposure **(A)** to fraction A and **(B)** fraction B of *C. raciborskii* exudate (20x concentrated). Data are means ± standard deviations of three independent experiments

3A **3B**

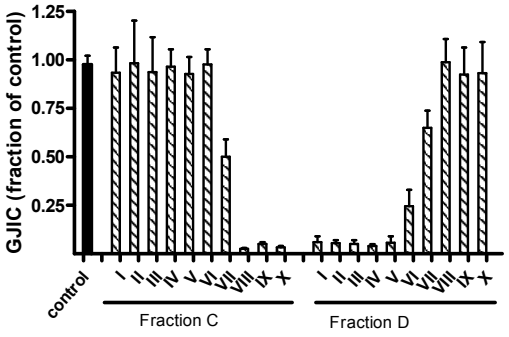


Interestingly, a recent study documented estrogenic activity of cyanotoxins microcystin-LR and nodularin using *in vitro* bioassays in stably transfected cell line with an estrogen-regulated luciferase gene (Oziol and Bouaicha 2010; Teneva, et al. 2003). Another cyanotoxin cylidrospermopsin was shown to inhibit progesterone production in granullosa cells *in vitro* (Young, et al. 2008). However, analyses of these toxins in our C. raciborskii strain did not reveal presence of known toxins, and thus other compounds are most probable responsible for the estrogenicity observed.

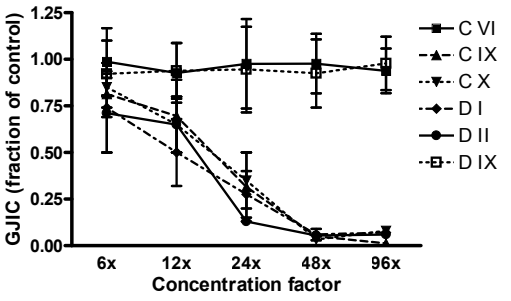
Besides estrogenicity, our previous work (Blaha, et al. 2010) as well as inhibitions of GJIC present here, indicate presence of unknown tumor promoting metabolite/s, and our further research focused on their detailed chemical characterization and investigation of possible mechanism of action. The fractions C and D inhibiting GJIC were further fractionated on the RP-HPLC, and as shown in Figure 4, strong biological activities were recorded in the individual subfraction (Figure 4A, the most potent inhibitions at subfractions CVIII to DV). Individual bioactive subfractions also induced dose-response effects (Fig. 4B).

Figure 4 Gap-junctional intercellular communication (GJIC) in WB-F344 cells after 30min exposure to: **A)** Separated fractions from *C. raciborskii* exudate (48x concentrated), **B)** Dose-response GJIC effects after exposure to chosen fractions. Data are means ± standard deviations of three independent experiments

4A

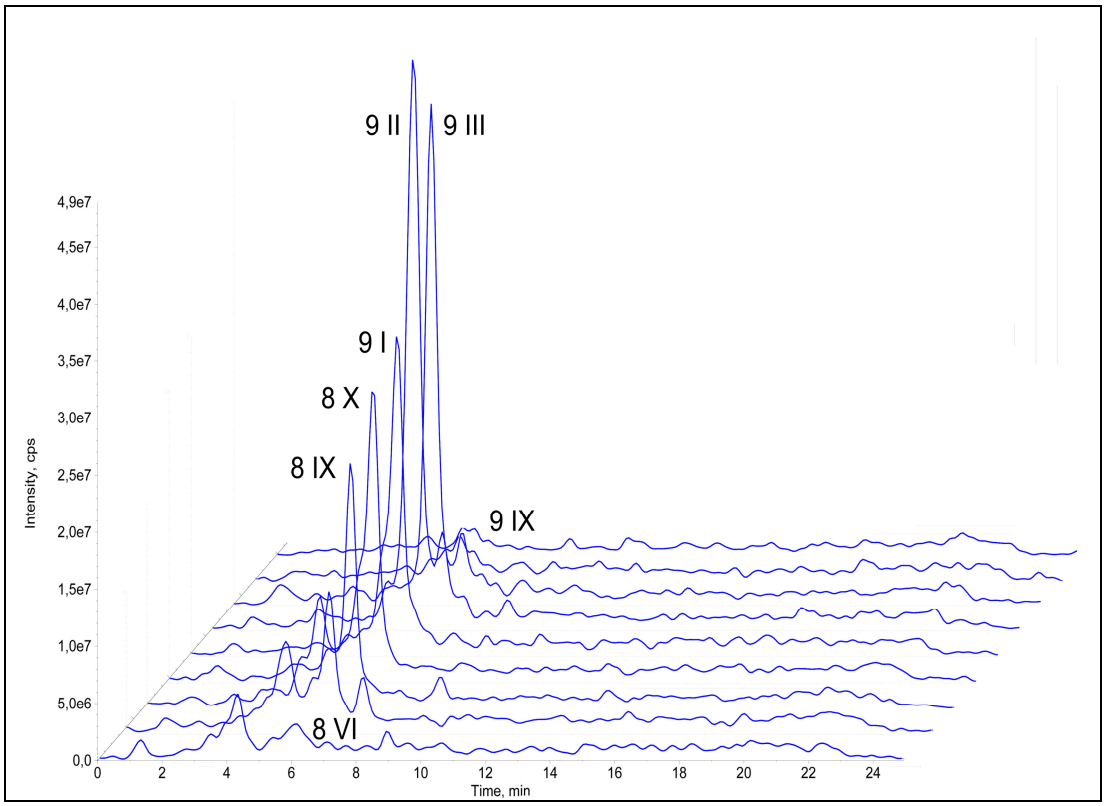


4B



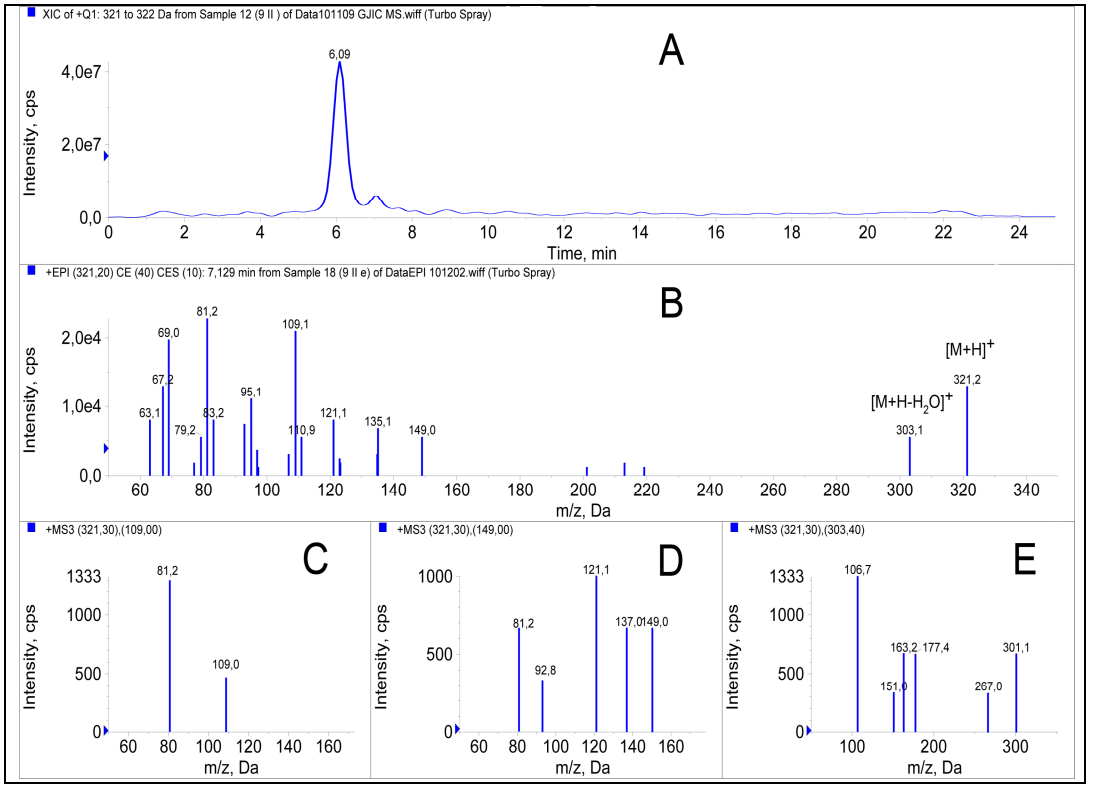
To search for potential bioactive agent present in the GJIC-inhibiting fractions, the full scan LC/MS was performed on all studied fractions. The data were then deconvoluted and a complex batch of MS spectra was analyzed. Finally, one compound with the m/z 321.2 was found to be present in the bioactive fractions (Figure 5). More interestingly, the respective molecular ion was absent in the inactive fractions, which seem to indicate that this compound may be responsible for the observed effect, i.e. inhibition of GJIC. This hypothesis is further supported by the "peak"-shape of the ion intensity in individual subfractions with maxima in the fractions 9II and 9III (Figure 5).

Figure 5: LC-MS determined relative abundance of unknown compound (m/z 321.2) in active (8IX – 9III) and inactive (8VI, 9IX) fractions (in GJIC inhibition test) from *Cylindrospermopsis raciborskii* SAG extract.



In next step the selected ion was fragmented to obtain its MS/MS spectra. Product ion spectra (Fig. 6 - B) contain numerous fragments, including the molecular ion [M+H]+ and ion indicating neutral loss of one molecule of water [M+H-H2O]+. Unfortunately, most of the fragments have low m/z and consecutive determination of structure of unknown molecule will require more precise analysis. As a final step of MS characterization we have performed MS3 experiments (MS/MS/MS) with selected ions (m/z 303.1, 149.0 and 109.0) from product ion spectra (Fig. 6 – C,D,E). Ion [M+H-H2O]+ with m/z 303.1 provided fragments with m/z 301.1 (double bond formation) and 267.0 (neutral loss of 2 H2O). Other fragments are difficult to interpret. Ions with m/z 149.0 and 109.0 provided fragments that occur also in the product ion spectra of precursor ion [M+H]+ (m/z 321.2), which proves that they are parts of the unknown compound but their detailed interpretation will require TOF-MS analyses. The obtained fragmentation pattern target characterization of the unknown bioactive compound by LC-MS/MS using particular MRM transitions (321.2→303.1, 321.2→149.0, 321.2→109.1, 321.2→81.2).

Figure 6: Fragmentation patern of unknown compound (m/z 321.2) from *Cylindrospermopsis raciborskii* SAG extract: **A)** extracted ion chromatogram, **B)** fragmentation spectrum of unknown compound m/z 321.2 (peaks of molecular ion $[M+H]^+$ and neutral loss of water molecule $[M+H-H_2O]^+$ are labelled), **C)** MS3 of precursor m/z 109.0, **D**) MS3 of precursor m/z 149.0, E – MS3 of precursor m/z 303.1 $[M+H-H_2O]^+$



Because our tentative compounds had high hydrophobicity, we speculated that possible bioactive compound might belong among the derivatives of lipids of phospholipids. Further line of evidence is relatively close MW of these compounds (ranging around MW=300-350). Some model commercially available phospholipids (not of the cyanobacterial origin) were 10 tested for their potencies to inhibit GJIC, and two of the studied compounds (the 3-sn-phosphatidic acid and arachidonic acid) down regulated GJIC (Figure 7). These results are in line with previous investigation that showed the importance of phospholipases, signaling enzymes, which may produce some of the phospho-acids tested (Machala, et al. 2003).

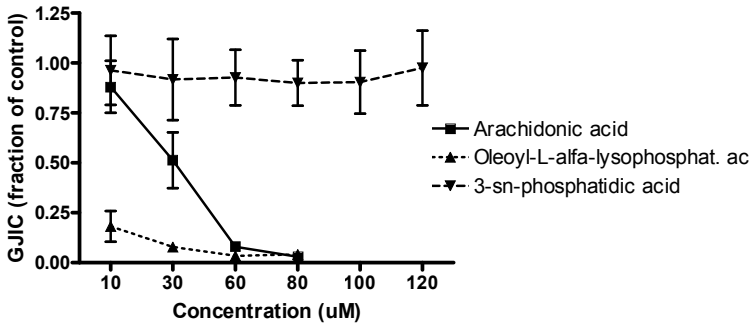


Figure 7 Dose – response curve of gap-junctional intercellular communication (GJIC) in WB-F344 cells after 30min exposure to various types of phospholipids

Nevertheless, GJIC inhibitions observed at the studied phophatidic and arachidonic acid are only one of the indirect lines of evidences, and our further research will aim to isolate the compound with m/z 321.2 and to test its activity and study the chemical composition. To provide further insights into the potential mechanisms behind the GJIC inhibition, we studied the role of several signaling pathways (Figure 8). The cells were pre-treated by the inhibitors of selected signaling pathways (D609, U0126, genistein and H59), and then exposed to cyanobacterial sample (cyn. extract) as well as to model compounds (epidermal growth factor - EGF, PC153, 1-methylanthracene). As it is clear, only U0126, an inhibitor of Mek, prevented the effect of cyanobacterial extracts on GJIC, while inhibitions of the other pathways had no effect.

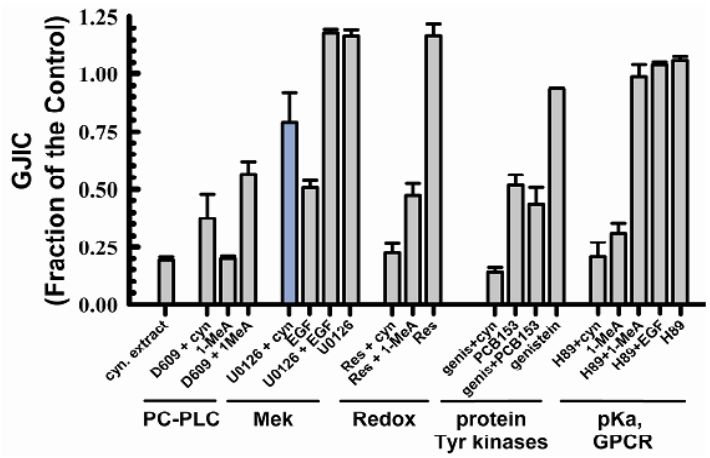


Figure 8: Screening of the biochemical mechanisms involved in the GJIC inhibition caused by the exudates of C. raciborskii.

These results suggest that cyanobacterial sample act on the GJIC via different mechanism than other tumor promoting compounds such as PAHs or PCBs, and the effects seem to be related to the mechanism of EGF (see Figure 8). Exudates of cyanobacteria are an interesting material, and they were shown to contain various proteins, polysaccharides, enzymes (hydrolytic enzymes, alkaline phosphatase, leucine aminopeptidase and beta-glucosidase) (Dziga, et al. 2009; Myklestad 1995; Prasanna, et al. 2008) or odor causing substances (geosmins) (Huang, et al. 2007). Most of the previous studies investigated effects of intracellular extracts but only few described and characterized biologically active metabolites in exudates (Leao, et al. 2010). Thus, our results extend the evidence on importance of extracellulary produced bioactive metabolites of cyanobacteria and bring new information about their tumor promoting and estrogenic properties.

Acknowledgements

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2.4 Potentiation effects in mixtures of toxic cyanobacteria and anthropogenic contaminants fluorathene and PCB 153 on an *in vitro* marker of tumor promotion - gap junctional intercellular communication

Paper VI

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Introduction

Many external and environmental factors are contributing to carcinogenesis such as diet, cigarette smoking and betel chewing, alcohol drinking, radiation or infection (Wogan, et al. 2004). Contamination of the air, food or water by toxic compounds may also increase a risk of developing cancer (Loeb and Harris 2008; Oliveira, et al. 2007), and various anthropogenic contaminants were shown to be carcinogens, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzodioxins and polychlorinated dibenzofurans (PCDD/Fs) and polychlorinated biphenyls (PCBs) (Brody, et al. 2007).

Besides the man-made chemicals, massive developments of toxic cyanobacteria resulting from nutrient pollution of surface and coastal marine waters represent another global environmental problem, since cyanobacteria produce a wide range of bioactive and toxic chemicals (Pearson, et al. 2010; van Apeldoorn, et al. 2007). Toxic cyanobacteria have been associated with increased prevalence of cancer, and the most prevalent and studied cyanotoxin, microcystin-LR (MC-LR), is currently classified as a possible human carcinogen IARC Group 2B (Svircev, et al. 2010). MC-LR, an inhibitor of eukaryotic protein serine/threonine phosphatases PP1 and PP2A, is known to alter processes of cellular differentiation, proliferation, and death which may induce tumor promotion *in vivo* (Dietrich 2005). Furthermore, heterocyclic cyanotoxin cylindrospermopsin (CYN) irreversibly inhibits proteosynthesis and causes other effects possibly leading to tumor initiation (Falconer and Humpage 2006).

Processes and mechanisms underlying the chemical carcinogenesis are still not completely understood but it is now well accepted that development of cancer is a multistep process (Loeb and Harris 2008; Vineis, et al. 2010). Traditionally, mutations and genotoxicity of chemicals were extensively studied but various non-genotoxic mechanisms were shown to play a crucial role in the process of cancer development (Hernandez, et al. 2009; Ziech, et al. 2010). Disruption of the tissue homeostasis mediated by gap junctional intercellular communication (GJIC) represents an important non-genotoxic mechanism in tumor promotion (Naus and Laird 2010; Trosko 2007; Trosko, et al. 1990). Downregulation of GJIC caused by chemicals *in vitro* can serve as a potent biomarker of tumor promotion and its assessment provides good predictions of *in vivo* tumor promoting potencies of chemicals (Rosenkranz, et al. 2000; Upham, et al. 2009).

Number of studies demonstrated inhibition of GJIC caused by various classes of chemicals including known tumor promoters and carcinogens such as phorbol ester (Madhukar, et al. 1996), polycyclic aromatic hydrocarbons (Blaha, et al. 2002; Sharovskaya, et al. 2006) or organochlorine compounds such as polychlorinated biphenyls (Machala, et al. 2003). More recently, we have shown that also cyanobacterial extracts inhibit GJIC (Blaha, et al. 2010) but the effects could not be related to known cyanotoxins such as MC-LR and CYN. Rather, other unknown toxic metabolite(s) produced by cyanobacteria are responsible for the observed toxicity *in vitro* (Blaha, et al. 2010).

Since organisms including humans are typically exposed to multi-component chemical mixtures from the environment and food, the issue of mixture toxicity is being intensively addressed in toxicological and ecotoxicological research (McCarty and Borgert 2006a; McCarty and Borgert 2006b; Reffstrup, et al. 2010; Syberg, et al. 2009)). Toxic effects of cyanotoxins have been often examined in mixtures due to frequent use of complex cyanobacterial extracts in many studies, but information on cyanotoxin effects in combination with other environmental contaminants is limited. Interestingly, there is a recent evidence that exposures to toxic cyanobacteria may enhance effects of other non-cyanobacterial toxicants and stressors, such as heavy metals or viral infection (Pikula, et al. 2010), or pesticides (Cerbin, et al. 2010) .

The objective of the present study was to evaluate *in vitro* tumor promoting potencies of mixtures containing of two known inhibitors of GJIC, anthropogenic environmental contaminants fluoranthene (FLU) and hexachlorobiphenyl PCB 153, in mixtures with cyanobacterial extracts of *Aphanizomenon gracile* or *Cylindrospermopsis raciborskii* or pure cyanotoxins, MC-LR and CYN.

Materials and methods

Chemicals

2,2´,4,4´,5,5´-hexachlorobiphenyl (PCB 153) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Fluoranthene (FLU), cylindrospermopsin (CYN) and lucifer yellow were supplied by Sigma-Aldrich (Prague, Czech Republic). Cyanotoxin microcystin-LR (MC-LR) was provided by Enzo Life Sciences (Lausen, Switzerland).

Cyanobacterial strains and Culture Conditions

Cyanobacterial culture of *Aphanizomenon gracile* RCX 06 was obtained from the RECETOX Culture Collection of Cyanobacteria and Algae, Czech Republic. This species originates from the Culture Collection of Autotrophic Organisms, Institute of Botany, Academy of Sciences of the Czech Republic (CCALA strain 008), but has been long-term cultivated at RECETOX laboratories and its properties differ from the original strain. Cyanobacterium *Cylindrospermopsis raciborskii* SAG 1.97 was purchased from the Goettingen University Collection of Cyanobacteria (Goettingen, Germany). Both microorganisms were cultured in the mixture of Zehnder medium (Schlosser 1994), Bristol (modified Bold) medium (Stein 1973) and distilled water in the ratio 1:1:2 (v/v/v). Organisms were grown at 22°C ± 2°C under continuous light (cool white fluorescent tubes, 3000 lux) and aerated with sterilized air by passing through 0.22 um filter (Labicom, Olomouc, Czech Republic).

Biomass extract preparation

Biomasses produced by cyanobacterial cultures were collected by the centrifugation (2880 x g, 10 min, 25°C) and freeze-dried. 500 mg of lyophilized biomasses were reconstituted in 100 ml of 50% methanol and sonicated on ice (Bandelin Sonopuls HD2070, Berlin, Germany). After sonication samples were centrifuged (2880 x g, 10 min, and 25°C), supernatants were collected, freeze-dried reconstituted in 0.5 ml of 50% methanol to reach concentration 1000 mg d.w./ml and sonicated on ice.

Microcystin analysis

Samples with 50% methanol were analysed for MCs content by HPLC Agilent 1100 Series coupled with PDA detector (Agilent Technologies, Waldbronn, Germany) on Supelcosil ABZ + Plus column, 150 x 4.6 mm, 5 um (Supelco, Bellefonte, PA, USA) using gradient elution with acetonitrile (Babica, et al. 2006). Microcystins were identified by comparing the UV spectra and retention times with microcystins standards (-RR, -YR, -LR, -LW, -LF) and quantified using external calibrations and limit of detection was 5 μg / g d.w. for microcystin determined by LC-DAD.

Cylindrospermopsin (CYN) analysis

Cyanobacterial extracts were transferred to Milli-Q water acidified with trifluoroacetic acid (TFA, 0.1% v/v). The extracts were analysed by LC/MS system Agilent 1200 coupled to 6410 Triple-Quad MS (Agilent, USA) with electrospray (ESI) interface. Separation was achieved on C18 Supelcosil ABZ+Plus column at 35°C, 150x4.6mm I.D., 5μm (Supelco, Bellefonte, PA, USA) with a flow rate 0.4 ml/min and gradient elution with mobile phases containing methanol and water acidified with 5 mM ammonium acetate). The mass spectrometer was operated in a multiple reaction monitoring mode (MRM) with collision energy 40 eV. The capillary voltage and fragmentation energy were 4000V and 140V, respectively. The cylindrospermopsin transition m/z 416.2 (M+H+) to 194.2 and m/z 416.2 to 176.1 were monitored for 250 ms dwell time. Quantification of CYN was achieved using product ions (194.2 and 176.1 transition) in retention time 8.25 min and based on external calibration of CYN standard (Sigma-Aldrich, Prague, Czech Republic). Limit of detection of LC/MS was 10 ng/g d.w. (Blahova, et al. 2009).

Cell culture

The WB-F344 rat liver epithelial cell line was originally isolated by Drs. J. W. Grisham and M. S. Tsao of the University of North Carolina (Chapel Hill, NC) (Tsao, et al. 1984). Cells were grown in D-medium/Ham´s F-12 with L-Glutamine supplemented with 5% fetal bovine serum (PAA, Pasching, Austria). Cells were cultured in 24-well microplates (TPP, Trasadingen, Switzerland) at 37 ºC in a humidified atmosphere containing 5% CO2 and 95% air. Bioassays were conducted with confluent cultures obtained after two to three days of growth.

Experimental design

Interactions of mixtures cyanobacterial samples or cyanotoxins with model anthropogenic contaminants (FLU and PCB 153) were evaluated by using GJIC *in vitro* assay in several experimental set-ups in order to characterize:

1) Concentration-dependent effects of individual chemicals (PCB 153, FLU, MC-LR or CYN) or cyanobacterial extracts (*A. gracile* or *C. raciborskii*) on GJIC after 30 min exposure.
2) Effects of binary mixtures of PCB 153 (5, 10, 30 [mu]M) or FLU (20, 30, 40 [mu]M) and pure cyanotoxins (100 [mu]M MC-LR or 60 [mu]M CYN) on GJIC after 30 min exposure.
3) Effects of binary mixtures of PCB 153 (10, 20 and 30 [mu]M) or FLU (30, 40 and 60 [mu]M) with cyanobacterial extracts of *A. gracile* or *C. raciborskii* (1, 2 and 3 mg d.w./ml) on GJIC after 30 min exposure
4) Effects of single compounds, cyanobacterial extract or their binary mixtures (FLU or PCB 153 mixed with *A. gracile* extract) on GJIC after prolonged exposures (1 h, 6 h and 24 h).

In order to recognize eventual additive, synergistic or antagonistic effects, concentrations of chemicals or extracts inducing none or only partial inhibition of GJIC (to 20-80% of the solvent control) were included in the design of binary mixture experiments.

Gap-junctional intercellular communication (GJIC) assay

The scrape loading-dye transfer (SL-DT) technique was adapted after the method of (Elfouly, et al. 1987). The test samples were added directly to the cell culture medium from concentrated stock solutions. Appropriate solvent controls (0.25% methanol, v/v, in the case of cyanobacterial extracts) were run in each experiment and did not induce responses significantly different from non-treated control. After the exposure, cells were washed with phosphate buffered saline (PBS) followed by the addition of 1 mg/ml of Lucifer-Yellow dissolved in PBS. The dye was introduced into the cells with five scrapes through the monolayer of confluent cells using a surgical steel scalpel blade. The transfer of dye through gap junction channels was allowed for three minutes, followed by a thorough rinse of cells with PBS to remove extracellular dye, and then fixation with a 4% formaldehyde solution in PBS. Migration of the dye in the cells was observed at 200-times magnification using a Zeiss OBSERVER.Z1 epifluorescence microscope equipped with an AxioCam HRc camera (Zeiss) and the images were acquired by AxioVision image analysis software. The fluorescence area of the dye migration from the scrape line was quantified using image analysis program created by Zdeněk Kuna and based on MATLAB software. Each SL-DT experiment was performed three times independently.

Data analysis and statistics

The results were expressed as fraction of the solvent control. Effects of binary mixtures were predicted from the effect of individual compounds or extracts using the simple effect-additive model (Andersen, et al. 2009). Predicted and observed effects were then compared by Student's t-test.

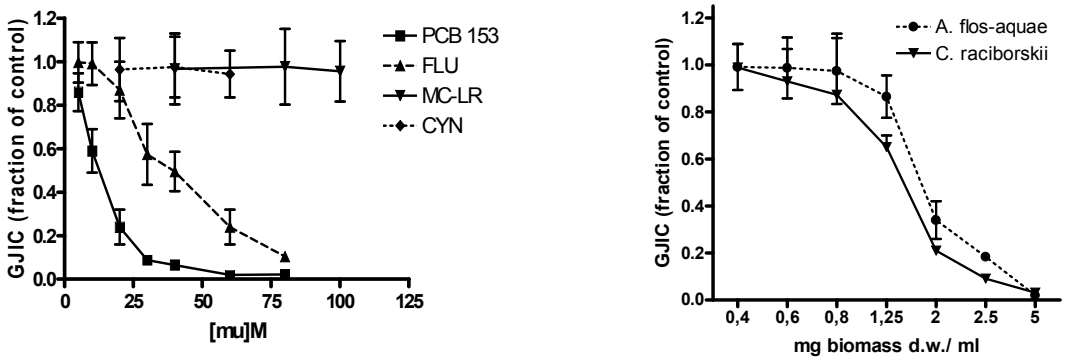
Results

Concentration-response curves for 30 min exposure were obtained for individual organic contaminants and cyanobacterial extracts in SL-DT assay (**Fig. 1**). GJIC was completely or almost completely inhibited after 30 min exposure to > 25 [mu]M PCB153, >75 [mu]M FLU, >2.5 mg d.w./ml of *A. gracile* or *C. raciborskii* extract. The tested concentration range of MC-LR (≤100 [mu]M) or CYN (≤60 [mu]M) did not affect GJIC after 30 min exposure.

Figure 1

Gap-junctional intercellular communication (GJIC) in WB-F344 cells after 30 min exposure to individual chemicals or extracts: **A)** fluoranthene (FLU), hexachlorobiphenyl PCB 153, microcystin-LR (MC-LR) and cylindrospermopsin (CYN) **B)** cyanobacterial extracts of *A. gracile* and *C. raciborskii* (0.4-5 mg d.w./ml). Data are means ± standard deviations of three independent experiments.

1A **1B**

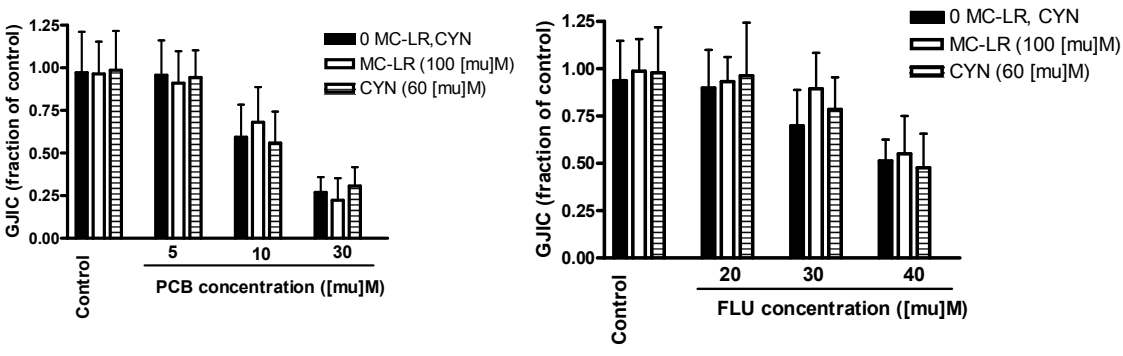


The effects of tested chemicals and extracts on GJIC were further studied in binary mixture experiments. Neither pure MC-LR (100 [mu]M) nor CYN (60 [mu]M) modulated inhibition of GJIC caused by 30 min exposure to PCB 153 (5-30 [mu]M) or FLU (20-40 [mu]M) (**Fig. 2**).

Figure 2

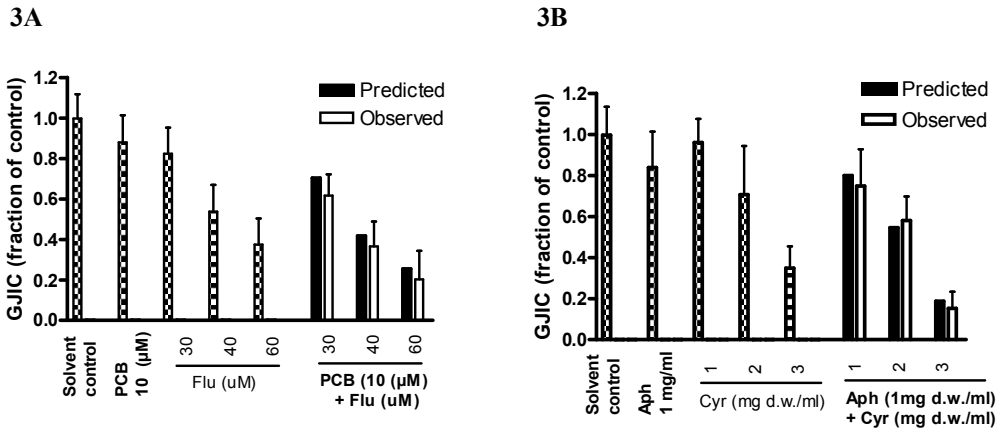
Gap-junctional intercellular communication (GJIC) in WB-F344 cells after exposure to binary mixtures of anthropogenic contaminants and cyanotoxins: **A)** PCB 153 (PCB) (10-30 [mu]M) and microcystin-LR (MC-LR) or cylindrospermopsin (CYN). **B)** fluoranthene (FLU) (20-40 [mu]M) and microcystin-LR (MC-LR) or cylindrospermopsin (CYN). Data are means ± standard deviations of three independent experiments.

2A **2B**



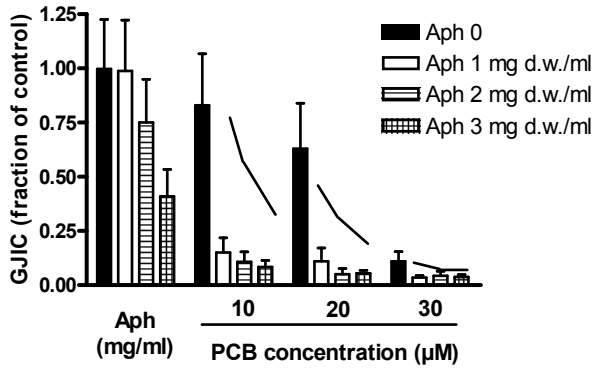
Simultaneous exposure to PCB 153 (10 [mu]M) and FLU (30-60 [mu]M) induced clearly additive response (**Fig. 3A**), as indicated by no statistical difference between observed GJIC inhibition and values predicted by the effect-addition model. Similarly, binary mixtures of *A. gracile* (1 mg d.w./L) and *C. raciborskii* extract (1-3 mg d.w./L) caused additive inhibition of GJIC (**Fig. 3B**).

Figure 3
Gap-junctional intercellular communication (GJIC) in WB-F344 cells after 30 min exposure to binary mixtures of anthropogenic contaminants and cyanobacterial extracts: **A)** binary mixture of fluoranthene (FLU) (30-60 uM) and hexachlorobiphenyl PCB 153 (PCB) (10 [mu]M). **B)** binary mixture of cyanobacterial extracts of *A. gracile* (1 mg d.w./ml) and *C. raciborskii* (1-3mg d.w./ml). "Predicted"- predicted additive effects of binary mixtures calculated by simple effect–additive model; "Observed"- observed effects of binary mixtures. Data are means ± standard deviations of three independent experiments.



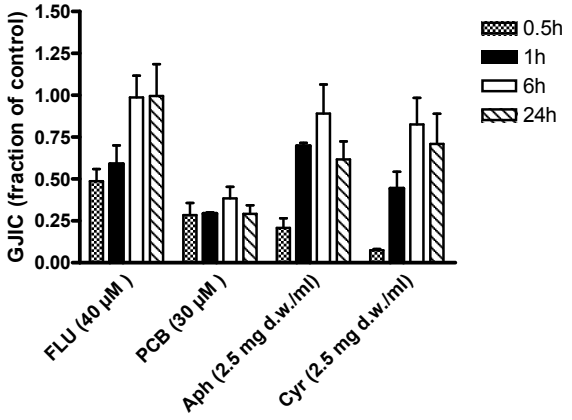
However, exposure of WB-F344 cells to PCB 153 or FLU in mixture with cyanobacterial extract of *A. gracile* or *C. raciborskii* resulted in a significant synergistic effect on GJIC. The observed levels of GJIC communication were significantly lower than values predicted from the effect-addition model (**Fig. 4**) in all tested combinations, i.e. PCB 153+*A. gracile*, PCB 153+*C. raciborskii*, FLU+*A. gracile* and FLU+*C. raciborskii* (**Table 1**). The strong synergism is illustrated by the fact that concentrations of PCB 153, FLU or cyanobacterial extracts not inhibiting GJIC when added to the cells separately (10 [mu]M, 30 [mu]M or 1 mg/ml d.w., respectively) induced complete or nearly complete inhibition in the cells exposed to these concentrations of anthropogenic contaminants and cyanobacterial extracts in binary mixtures.

Figure 4
Gap-junctional intercellular communication (GJIC) in WB-F344 cells after 30 min exposure to binary mixture of PCB153 (PCB) (10-30 [mu]M) with *A. gracile* extract (Aph) (1-3 mg d.w./ml) "Lines"express predicted effects calculated according to effect–additive model. Data are means ± standard deviations of three independent experiments.



Inhibition of GJIC induced by PCB 153, FLU, extract of *A. gracile* and *C. raciborskii*, and binary mixtures of anthropogenic contaminants and cyanobacterial extracts was examined also after prolonged exposures (1, 6 and 24 h). Whereas GJIC remained inhibited by PCB 153 (30 [mu]M) throughout 24 h of exposure, the inhibitory effects of FLU (40 [mu]M) and both cyanobacterial extracts (2.5 mg d.w./ml) decreased with the increasing exposure time (**Fig. 5**), which indicates a transient character of GJIC inhibition induced by FLU and cyanobacterial extracts. After 6 h incubation with FLU or cyanobacterial extracts, the extent of GJIC was similar to the GJIC level in the control, although a partial downregulation of GJIC was observed in the cells exposed to cyanobacterial extracts for 24 h.

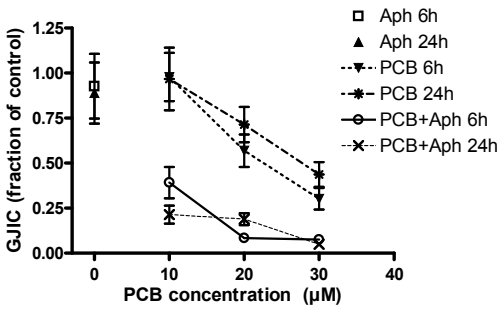
Figure 5
Effects on gap-junctional intercellular communication (GJIC) in WB-F344 cells of individual chemicals or extracts in time-course experiment (0.5, 1, 6 and 24 h). FLU = fluoranthene, Aph = *A. gracile* extract, Cyr = *C. raciborskii* extract. Data are means ± standard deviations of three independent experiments.



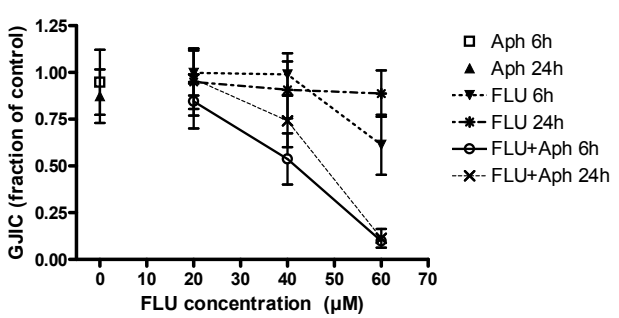
Prolonged exposure to mixtures of *A. gracile* extract (2 mg d.w./ml) and PCB 153 (10-30 [mu]M) or FLU (20-60 [mu]M) also resulted in strong synergistic effects (**Fig. 6**). Most notably, neither cyanobacterial extract (2 mg d.w./ml) nor PCB 153 (10 [mu]M) downregulated GJIC after 6 or 24 h when tested separately, whereas their binary mixture caused significant inhibition of GJIC (**Fig. 6A**). Similarly, rapid inhibition of GJIC induced by FLU (60 [mu]M) or *A. gracile* extract (2 mg d.w./ml) was observed (**Fig. 1**), but the communication restored when the exposure to FLU or cyanobacterial extract continued for 6 or 24 h (**Fig. 6B**). However, no such recovery of GJIC was observed when the cells were exposed to 60 [mu]M Flu and 2 mg d.w./ml *A. gracile* simultaneously (**Fig. 6B**).

Figure 6
Gap-junctional intercellular communication (GJIC) in WB-F344 cells after 6 h and 24 h exposure to binary mixtures of anthropogenic contaminants and cyanobacterial extracts: **A)** binary mixture PCB 153 (PCB) (10-30 [mu]M) and *A. gracile* extract (2 mg d.w./ml). **B)** binary mixture of fluoranthene (FLU) (20 -60 [mu]M) and *A. gracile* extract (Aph) (2 mg d.w./ml) Data are means ± standard deviations of three independent experiments.

6A



6B



The cyanobacterial extracts of *A. gracile* and *C. raciborskii* contained neither microcystins as revealed by HPLC-DAD nor CYN as revealed by LC-MS analyses (method limit of detection was 5 ug/g d.w. for MC-LR and 10 ng/g d.w. for CYN).

Table 1
Gap-junctional intercellular communication (GJIC) in WB-F344 cells after 30 min exposure to binary mixtures of anthropogenic contaminants and cyanobacterial extracts: PCB 153 (10-30 [mu]M) with *A. gracile* (1-3 mg d.w./ml); PCB153 (10-30 [mu]M) with *C. raciborskii* (1-3 mg d.w./ml); Fluoranthene (30-60 [mu]M) with *A. gracile* (1-3 mg d.w./ml); Fluoranthene (30-60 [mu]M) with *C. raciborskii* (1-3 mg d.w./ml). Data are means ± standard deviations of fraction of control, repeated in three independent experiments. Asterisks indicate statistically significant synergistic effects. The values of fraction of control predicted from effect-additive model are listed in parentheses.

			A. gracile **(mg d.w./ml)**			***C. raciborskii*** **(mg d.w./ml)**		
		0	**1**	**2**	**3**	**1**	**2**	**3**
Non-treated control		1.0						
Solvent control		1.0						
	0	1.0	0.988	0.750	0.410	0.998	0.867	0.473
PCB 153 ([mu]M)	**10**	0.997	0.150* (0.98)	0.180* (0.72)	0.084* (0.41)	0.345* (0.99)	0.180* (0.72)	0.055* (0.41)
	20	0.630	0.110* (0.61)	0.050* (0.28)	0.055 (0.04)	0.044* (0.62)	0.061* (0.49)	0.023 (0.1)
	30	0.110	0.035 (0)	0.043 (0)	0.038 (0)	0.055 (0.11)	0.023 (0)	0.018 (0)
Fluoranthene ([mu]M)	**30**	0.91	0.250* (0.875)	0.118* (0.637)	0.074* (0.702)	0.305* (0.885)	0.128* (0.754)	0.094* (0.361)
	40	0.65	0.160* (0.527)	0.085* (0.289)	0.055 (0.05)	0.120* (0.637)	0.185* (0.506)	0.055 (0.094)
	60	0.28	0.085 (0.298)	0.053 (0)	0.047 (0)	0.108* (0.308)	0.085* (0.177)	0.067 (0)

Discussion

Many environmental contaminants have been implicated in chemical carcinogenesis and associated with cancer in epidemiological studies, including PCBs (Brown, et al. 2007; Silberhorn, et al. 1990) or PAHs (Baird, et al. 2005). Considerable research effort focused on specific mechanisms of carcinogenic or tumor promoting properties of these groups of environmental contaminants and their individual representatives. The intensive research of aryl hydrocarbon receptor (AhR) mediated toxicity of coplanar PCBs (such as PCB 126) (Van den Berg, et al. 2006) and genotoxicity of some PAHs (Ohnishi, et al. 2002; Platt, et al. 2008) contributed to recognition of these individual chemicals as carcinogenic to human, or probable or possible human carcinogens (IARC Groups 1, 2A, 2B). However, there is reasonable evidence that mechanisms other than "dioxin-like" toxicity or genotoxicity may be involved in cancer development induced by PCBs or PAHs. Toxicity and tumor promoting activity have been reported for non-coplanar PCBs with no AhR binding activity, such as PCB 153 (Brown, et al. 2007; Glauert, et al. 2008; Knerr and Schrenk 2006; Kopec, et al. 2010; Lavoie, et al. 1994). Also tumorgenicity of FLU was demonstrated (Busby, et al. 1984; Lavoie, et al. 1994; Wang and Busby 1993), although FLU was not genotoxic in rodents *in vivo* (Stocker, et al. 1994) or in human cell line (Durant, et al. 1996). Interestingly, both PCB 153 and FLU have been shown to induce rapid inhibition of GJIC (Blaha, et al. 2002; Machala, et al. 2003; Weis, et al. 1998), which could be a mechanism involved in

tumor promoting activity and toxicity of these chemicals. These findings were confirmed in our study, which also provided novel information on the additivity of their inhibitory effects. The additive character of the effects may indicate that both PCB 153 and FLU downregulate GJIC via similar mechanism, by affecting the same molecular targets. This hypothesis is further supported by similarities in the response of WB-F344 cells to PCB 153 and 1-methylanthracene, another PAH which shares with FLU specific structural features (presence of the bay/bay-like region) and most likely also a mechanism of GJIC inhibition (Upham, et al. 1998; Weis, et al. 1998). Both PCB 153 and 1-methylanthracene not only inhibit GJIC, but also activate MAPKs ERK1/2 and induce release of arachidonic acid (Machala, et al. 2003; Umannova, et al. 2008; Upham, et al. 2008). Moreover, inhibition of GJIC induced by PCB 153 and 1-methylanthracene is prevented by D609, an inhibitor of phosphatidylcholine-specific phospholipase C (PC-PLC), and by H89, a protein kinase A (PKA) inhibitor (Machala, et al. 2003; Upham, et al. 2008). These data, supported by the additivity of effects observed in the present study, suggest that PCB 153 and PAHs might downregulate GJIC by common mechanism involving activation of PC-PLC and/or PKA. However, PCB 153 and FLU differed in the time-course of GJIC inhibition. Although GJIC remained inhibited in the presence of PCB 153 throughout the 24 h exposure, the inhibitory effect of FLU occurring after 30 min exposure became less pronounced with increasing exposure time and nearly normal communication was restored after 6 h. Differences in biotransformation kinetic of PCB 153 and FLU or different toxicities of biotransformation products of the two chemicals, are possible explanations which should be addressed by future research.

Consumption of drinking water contaminated with cyanobacterial toxins has been linked to increased prevalence of liver and colorectal cancer (Svircev, et al. 2010; Zhou, et al. 2002). While MC-LR has been identified as a tumor promoter (Dietrich 2005) and carcinogenicity of CYN is also being discussed (Falconer and Humpage 2006), a number of studies clearly demonstrated that toxicity of cyanobacterial extracts cannot be always attributed to the known cyanotoxins and that other bioactive compounds from cyanobacteria may be responsible for some observed effects (Falconer 2007). These effects include rapid inhibition of GJIC and activation of MAPK ERK1/2 in WB-F344 cells induced by various cyanobacterial extracts regardless microcystin or cylindrospermopsin content (Blaha, et al. 2010). In the present study, neither MC-LR nor CYN inhibited GJIC in WB-F344 after 30 min exposure, and the lack of effects was further demonstrated in experiments with binary mixtures, where toxins did not modulate inhibitory effects of anthropogenic contaminants PCB 153 or FLU. No observable effects on GJIC attributable to MC-LR and CYN might be simply a consequence of GJIC not being a cellular and molecular target for these cyanotoxins. However, the absence of effects in WB-F344 cells could be a result of the limited cellular uptake of microcystin, which strongly depends on the expression of respective organic anion transport proteins (Boaru, et al. 2006; Komatsu, et al. 2007; Monks, et al. 2007). Similarly, recent study reported that also cylindrospermopsin uptake might be limited in some cell lines resulting in their lower sensitivity to the toxin (Froscio, et al. 2009).

Although pure cyanotoxins did not affect GJIC, the extracts of *A. gracile* and *C. raciborskii* inhibited GJIC in concentration-dependent manner, with effective concentrations similar to those previously reported for laboratory cultures of *A. flos-aquae* and *M. aeruginosa* (Blaha, et al. 2010). The absence of detectable amounts of MC-LR or CYN in the extracts suggests that these cyanotoxins are less important toxic agent with respect to GJIC as a mechanism of tumor promotion, and that unknown metabolites might be contributing to tumor promotion.

The effects of cyanobacteria on GJIC were additive when the cells were exposed to the extracts of *A. gracile* and *C. raciborskii* simultaneously, similarly to the binary mixtures of PCB 153 and FLU. It may indicate that both extracts inhibited GJIC via the same mechanism and possibly that the same compound(s) were responsible for the inhibitory activity of both extracts. Isolation and

characterization of these compound(s) as well as identification of their mode of action need to be addressed by future research.

The most remarkable result of our study is the effect of anthropogenic contaminants in binary mixtures with cyanobacterial extracts, where strong synergistic effects on GJIC were observed in all tested combinations and exposure times. Mixture toxicity of various environmental contaminants is being intensively studied, but most studies are dealing with mixtures of chemicals sharing some general similarity or mechanism of action, whereas experiments with mixtures containing widely divergent components (e.g., organics, metals, inorganics) or chemicals in combination with non-chemical stressors are much less common (McCarty and Borgert 2006a). Interestingly, synergistic effects have been reported for mixtures of PAHs and metals (Feng, et al. 2003; Fleeger, et al. 2007; Lau and Chiu 2006; Silva, et al. 2009), PAHs and PCBs (Wassenberg and Di Giulio 2004), PCBs and organometals (Andersen, et al. 2009; Jensen, et al. 2000) or PCBs and various endocrine disruptors (Benachour, et al. 2007). Several recent studies also demonstrated synergistic effects of various cyanobacterial metabolites such as portoamide A and B (Leao, et al. 2010), lobocyclamides A and B (MacMillan, et al. 2002) or laxaphycins A and B (Bonnard, et al. 2007), but mixture effects of toxic cyanobacteria or cyanotoxins and other environmental contaminants have been so far only rarely investigated. A recent *in vivo* study with japanese quails showed synergistic toxicity between toxic cyanobacterium *Microcystis*, heavy metal – lead, and viral infection (Pikula, et al. 2010). Potentiation of effects of toxic cyanobacterium *Microcystis* and pesticide carbaryl in daphnids has been also reported (Cerbin, et al. 2010). Our study thus provides additional evidence on specific toxic interactions between cyanobacterial metabolites and environmental anthropogenic contaminants. It should be critically noted that both cyanobaterial extracts were complex mixtures of cyanobacterial metabolites and we cannot neglect potential interferences and interactions among the components of these original samples. Nevertheless, clear potentiation of GJIC inhibition observed in mixtures of anthropogenic contaminants (PCB 153 or FLU) and toxic cyanobacteria highlight the importance of studying effects of complex mixtures, which correspond to naturally occuring situation. The inhibition of GJIC independent of the content of MC-LR and CYN further suggest an existence of unknown cyanobacterial metabolites with tumor promoting activity, which may act synergistically with recognized environmental tumor promoters.

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Chapter 3

GENERAL DISCUSSION AND CONCLUSIONS

Massive development of cyanobacteria resulting from anthropogenic eutrophication of water reservoirs is a global problem especially because of human health risks and drinking water degradation due to production of various secondary cyanobacterial metabolites (Sivonen and Jones 1999). Despite intensive scientific attention only part of cyanobacterial biologically active substances has been identified and characterized. Their structural variety and different effects as well as modes of action offer opportunities for further research as novel biocide or novel cancer drugs (reviewed in Berry, et al. 2008; Sainis, et al. 2010). However, enlarging knowledge about novel cyanobacterial metabolites or new information about known metabolites can help to improve assessment of human health risks or prediction of release/fate of these metabolites in the environment.

Investigation of toxicity - special focus on mixtures

Present dissertation combines and follow development of several experimental approaches how to determine toxicity of complex cyanobacteria. The most common approaches investigate purified compounds-microcystins and toxicity of different structural congeners in wide range of concentrations. However, these types of studies do not fully reflect real situation, where cyanobacterial metabolites occurred in mixture of other oligopeptides and other components, with which they can interact

Another approach how to study toxicity of cyanobacteria used partially fractionated extract of real water bloom with high occurrence of microcystins. This study may better describe real situation and also reserve option of possible interactions among metabolites.

Further approaches can distinguish between intracellular and extracellular complex mixtures. This can refer to situation before cell lyses - bloom collapses and following intracellular content is released in the environment.

Characterization of possible interactions may also be studied in well defined experimental mixtures (in our studies between cyanobacterial extracts and anthropogenic pollutants, **Paper VI**, paragraph 2.4). Finally, EDA (Effect Directed

Analyses) has been suggested to characterize causative agents responsible for toxicity in contaminated environmental samples, and it has been successfully used

Among considered/discussed methods for toxicity assessment of cyanobacterial toxin mixtures belongs procedure based on toxicity equivalent factors (TEF) obtained from toxicological data presented for cyanobacterial toxins in the literature, where the most often detected cyanotoxins in Czech republic are MC-LR (50% from total amount of microcystins), MC-RR and MC-YR (Blahova, et al. 2008). The toxicity of mixture of microcystins can be expressed as toxicity of one congener, e.g. MC-LR has TEF=1 and TEF=0.1 belongs to other congener MC-RR. Multiplying of concentrations with corresponding TEF results in toxicity equivalents (TEQ) linked to MC-LR (Wolf and Frank 2002).

Effects of microcystins on growth of algae and cyanobacteria

First study presented in this dissertation (Paper I) investigated and compared toxicity of different purified structural congeners of microcystins (MC-LR and MC-RR) using growth inhibition assay on five algal species and one cyanobacterial species. These organisms were exposed to a wide concentration range of dissolved microcystins including low, environmentally relevant concentrations up to extremely high levels (concentrations range 1 - 25000 $\mu g\ l^{-1}$) for exposure 11 days. In our study we have found no significant growth changes after exposure to environmentally relevant concentrations (1 - 10 $\mu g\ l^{-1}$) for both congeners, whereas most effective level was the highest concentration (25000 $\mu g\ l^{-1}$). Our study seems to support evidence, that toxicity of microcystins is highly species-specific and also congener-specific. In spite of various hypotheses (Babica, et al. 2006), no signs of allelopathic effects were observed as microcystins did not affect growth of studied organisms. In general, growth is a complex process affected by various particular mechanisms. Thus, this parameter may seem to be less sensitive than some biochemical or molecular markers (Waterfield and Timbrell 1999). However, it is a key and integrating biological property, and its modulations indicate serious damage to the population (Klaassen 1995)

Effects of microcystins on cellular differentiation in cyanobacteria

The second study (Paper II) studied allelopathic properties/signalling role of microcystins on other cyanobacterium *Trichormus variabilis*. Although microcystins have been intensively studied before, the paper for the first time reported effects of microcystins on cell differentiation in cyanobacteria *Trichormus variabilis*. This pilot study examined semipurified *Microcystis* extract with microcystins at low concentrations 0.2-20 nM corresponding to 0.2-20 µg/l, which are commonly detected in the water with cyanobacterial water blooms (Blahova, et al. 2008). Results showed significant deceleration of cell differentiation, especially into akinetes after exposure to microcystins or other bioactive peptides, which may lead to changes in conservation and overwintering of *T. variabilis*. This study seems to support the hypotheses about allelopathy of MCs as well as their potential signalling role in cyanobacteria. Several studies reported presence of protein phosphatases in cyanobacterial cells, which are likely to be inhibited by microcystins but their role in cyanobacterial cell differentiation has been poorly studied (Shi, et al. 1999). To our knowledge this is the first study indicating effects of microcystins on cyanobacterial cell differentiation and further research is needed to fully explore this field.

Oxidative stress induced by toxic cyanobacteria in green algae

Oxidative stress is one of the biochemical mechanisms involved in toxicity of cyanobacterial toxins microcystins (MC), but its role in the effects of complex water blooms is elusive. The aim of our studies described in Paper III was to investigate effects of pure MCs and different complex mixtures (intra- and extra-cellular) of *Microcystis aerigunosa* on the growth and biochemical markers of oxidative stress and detoxification in green alga *Pseudokirchneriella subcapitata*. All tested exposures were adapted to contain similar MCs concentration 300 µg/L for better effect comparison. No growth modulation caused by pure MCs or intracellular mixture was observed. Extracellular mixture weakly stimulated algal growth. From biochemical markers, only glutathione reductase (GR) was stimulated by pure MC, while crude extract (intracellular content) had no effects on algal growth or any of the biomarkers. In contrast, extracellular mixture of the *M. aeruginosa* culture inhibited activities of glutathione-S-transferase (GST). All

observed effects seemed to be weak in comparison with the effects of oxidative stressor herbicide paraquat that influenced both growth and biomarkers. Our results as well as other studies (Pflugmacher, et al. 2006) demonstrate high variability in the biochemical responses to the oxidative stress, which complicates interpretation of the results. Taken together, biochemical changes may be used as exploratory tools in controlled laboratory experiments with pure chemicals but their use as biomarkers or early warnings of toxicity under natural conditions is fairly complicated.

Effects of cyanobacteria on markers of tumor promotion

Besides interactions between cyanobacteria and algae, we have also investigated other toxicity mechanisms of cyanobacterial metabolites related to tumor promotion, specifically to the potency to inhibit gap junctional intercellular communication (GJIC).

In Paper IV, we have demonstrated GJIC-inhibitory potencies of 10 laboratory strains representing common water bloom-forming cyanobacteria (*Anabaena, Aphanizomenon, Cylindrospermopsis, Microcystis* and *Planktothrix*) and six natural water bloom samples (dominated by *Aphanizomenon sp*. or *Microcystis*). The most expressive down-regulations of GJIC were caused by methanolic extracts (intracellular content) of 7 cyanobacterial strains but at two other strains, no effects were observed. Exudates (metabolites produced extracellularly) also induced species-specific inhibitory effects, but they did not necessarily correlate with the potencies of cellular extracts. Surprisingly, two exudates from green algae also inhibited GJIC, although their extracts caused no effects. This observation indicates that GJIC-inhibiting compounds may be produced in both prokaryotic and eukaryotic autotrophic microorganisms, and it should require further research attention. As we have observed no correlations with MC or CYN presence, GJIC inhibition is likely to be caused by unknown metabolite(s), which are produced in different species or strains, and they may be of variable chemical composition as suggested by highly variable chemotypes within cyanobacterial species or genera (Welker, et al. 2004). Our results are one of the first that clearly demonstrate production of BAC in cyanobacteria affecting an important tumor promotional mechanism - i.e. GJIC (Blaha, et al. 2010; Trosko and Goodman 1994).

Identification of tumor promoting agent in *C. raciborskii*

Our findings discussed in the previous paragraph motivated further studies described in Paper VI, where we tried to elucidate identity and chemical composition of the GJIC-inhibiting agent in the extracellular mixture (exudate) of cyanobacterium *Cylindrospermopsis raciborskii* (SAG 1.97). This cyanobacterial strain does not produce any of known toxins such as microcystin or cylindrospermopsin but its exudates strongly inhibited GJIC. Mixtures of extracellular metabolites produced during cyanobacterial growth were fractionated by semi-preparative HPLC using EDA approach (Leao, et al. 2010), and the fractions were repeatedly assessed for their potencies to inhibit GJIC. Fractions, which contained highly hydrophobic compounds and significantly inhibited GJIC were further fractionated and analyzed with LC-MS/MS/MS, and a single compound with m/z 321.2 was successfully identified as a potential agent inhibiting GJIC. This chemical was solely present only in bioactive, i.e. GJIC-inhibiting, fractions. Screening of biochemical mechanisms affecting GJIC by the use of specific inhibitors of signalling pathways suggested that effects of cyanobacteria are controlled by mechanisms different from those of other tumor promoting xenobiotics such as PAHs or PCBs (Machala, et al. 2003). Although well studied toxins such as MCs and CYN were shown to have possible carcinogenic potencies (Falconer and Humpage 2006; Svircev, et al. 2010), our study brings further evidences on novel tumor promoting agents produced by cyanobacteria..

Mixture effects of cyanobacteria with fluoranthene and PCB 153

Some recent studies demonstrated interesting interactive toxic effects in mixtures of cyanobacteria with other agents (Pikula, et al. 2010), and the aim of our final study was to investigate combined effects of anthropogenic environmental contaminants (2,2 ',4,4 ',5,5 '-hexachlorobiphenyl (PCB 153) and fluoranthene) with cyanobacterial samples on the GJIC. Investigated samples included cyanotoxins (microcystin-LR and cylindrospermopsin) and also extracts of laboratory cultures of cyanobacteria (*Aphanizomenon gracile* and *Cylindrospermopsis raciborskii*) that were shown to affect GJIC. Binary mixtures of PCB 153 with fluoranthene and mixtures of the two studied cyanobacterial

strains elicited simple additive effects in the *in vitro* GJIC assay, whereas microcystin-LR and cylindrospermopsin neither inhibited GJIC nor potentiated effects of PCB 153 or fluoranthene. Interestingly, synergistic (potentiation) effects were observed in the cells exposed to binary mixtures of anthropogenic contaminants with cyanobacterial extracts- These findings thus further indicate importance to study effects of complex environmental mixtures as also recently reported in other investigations addressing biochemical and immunological balance and life cycle of non-target species (Cerbin, et al. 2010; Pikula, et al. 2010). Understanding to mixture toxicity in the real environment is currently a major challenge to both science and regulators as highlighted by several activities of international agencies and societies such as World Health Organization (WHO), Society of Toxicology (SOT) or Society for Environmental Toxicology and Chemistry (SETAC).

Summary and conclusion

In summary, although toxic cyanobacteria have been studied for a long time, number of issues related to the mechanisms of their toxicity and effects in various organisms have not been fully elucidated. The present dissertation contributed to general understanding of interactions between cyanobacteria and algae studying both known toxins and complex mixtures including extracts of cellular content as well as extracellular exudates. Our further studies for the first time demonstrate presence of unknown hazardous tumor promoting agents in cyanobacteria affecting GJIC, and indicate need to further study less explored cyanobacterial metabolites with potential human health risks. Our studies also provide several evidences on the importance and needs to study toxicity of chemical mixtures, especially under realistic and ecologically relevant scenarios.

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ANNEXES

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EDUCATION AND TRAINING	
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Name and type of organization providing education and training	Faculty of Science, Masaryk University, Brno, Czech Republic
Principal subjects/occupational skills covered	Five years Masters program Ecotoxicology, finished by diploma work: *„Evaluation of effects of cyanotoxins and cyanocides on authotrophic organisms using biochemical markers"* (graduate cum laude)
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Dates	09/2001 to 06/2006

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Cooperation on research projects	2003 - 2005 GACR 206/03/1215 (Czech Science Foundation) - Overwintering strategy of Microcystis sp. - has microcystin a signal function? 2005 - 2009 MSMT 1M6798593901 (Ministry of Education, Youth and Sports, Czech Republic) - Bioindication and revitalization of toxic and anthropogenic substrates and water sources: application of cyanobacteria, algae, soil bacteria and symbiotic fungi 2008 - 2010 GAČR 524/08/0496 (Czech Science Foundation) Mechanisms of tumor promotion in toxic cyanobacteria 2009 FRVŠ 1789/2009 (Fond rozvoje vysokých škol - University development fund) – Introduction of cyanobacterial problems into the education of current ecotoxicology.
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Kateřina Nováková - Publications

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Oral presentations at international and national conferences

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