

THE GENOTOXIC POTENTIAL OF
CYANOBACTERIAL TOXINS, BISPHENOL A,
ITS ANALOGUES, AND THEIR MIXTURES

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GENOTOKSIČNI POTENCIAL IZBRANIH
CIANOBAKTERIJSKIH TOKSINOV, BISFENOLA A IN
ANALOGOVI TER NJIHOVIH ZMESI
Doktorska disertacija

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To Life and Justice.

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Abstract

Exposure to environmental pollutants in everyday life is inevitable. Humans and other organisms are constantly exposed to a mixture of chemicals consisting of naturally present and anthropogenic substances. Lack of data on toxicological properties and biochemical interactions between different chemicals is the main limitation for efficient risk assessment. In this thesis, two groups of pollutants originating from distinct sources were selected and their cytotoxic and genotoxic potential was assessed – cyanotoxins as naturally produced and bisphenols as anthropogenic pollutants. Cyanotoxins are cyanobacterial metabolites present in waters, especially where the growth of cyanobacteria is expanded due to eutrophication and global warming. Adverse effects of certain known cyanotoxins are proven; however, numerous newly discovered cyanotoxins that are increasingly occurring in blooming water have been detected recently while their toxicological profiles have not been investigated yet. Contrary to cyanotoxins, bisphenol A and its analogues are produced by humans as important components in the production of plastic and epoxy materials. They are present in various food packagings and other products in everyday use, but due to the leakage from the production industry as well as plastic waste, they are nowadays ubiquitously present in the environment. Both groups of these pollutants include several substances known to be genotoxic. This thesis aims to assess genotoxic potential of cyanobacterial toxins and BPA congeners for which this information is missing and their genotoxic potential in combined exposure scenarios relevant for the environmental exposure.

We provided the first data on the genotoxicity of cyanotoxins L-BMAA and microginins. Results showed that L-BMAA is not causing mutations and DNA damage in the bacterial test system, while microginins have the potential to induce DNA strand breaks and genomic instability in the human-derived cell line HepG2.

Exposure of HepG2 cells to cyanobacterial extract containing different microginins and to the mixture of microcystin LR (MCLR) and cylindrospermopsin (CYN) revealed that combined genotoxic effects of these cyanotoxins are not equal to the sum of individual effects. The results indicate antagonistic interactions or prevalence of the effect of one substance. In the MCLR/CYN mixtures, the genotoxic effect CYN was shown to be predominant. MCLR is a well-studied cyanotoxin characterised as a possible human carcinogen; however, it was observed that CYN evinces even stronger genotoxic activity.

The comparison of genotoxic activity of bisphenol A and its most common analogues, bisphenols (BPs) S, F, and AF, revealed that BPAF has the highest genotoxic potential, despite the presumption that the analogues are safer than BPA itself. The transcriptomic analyses demonstrated that BPA and BPAF, as well as their combinations with other bisphenols, induced changes in gene expression also at low, for human exposure relevant concentrations. Combined exposure to bisphenols and cyanotoxin CYN confirmed the predominant DNA damaging effect of CYN, while transcriptomic analyses indicated additive or synergistic interactive effects in the CYN/BPs mixtures on the expression of several tested genes.

The need for reliable *in vitro* toxicological data for the risk assessment is rising with increasing production of new chemicals and the ethical issue of animal testing. The 3D experimental models that better represent *in vivo* conditions than conventional monolayer cell cultures are being developed to bridge the gap between *in vitro* and animal studies. In this study, we applied a novel 3D *in vitro* HepG2 model, which we developed for assessing the cytotoxicity and genotoxicity of CYN. CYN affected the growth of spheroids and induced cell cycle arrest. Gene transcription analyses revealed deregulation of genes involved in phase I and phase II metabolism, DNA damage response, cell proliferation and apoptosis.

The results of the thesis provided the missing toxicological data of emerging pollutants - cyanotoxins and bisphenols, and contributed to better understanding of chemical interactions in the co-exposure. It was again confirmed that the concentration addition concept in mixture risk assessment is unjustified, since the experimental data indicate more complex interactions. Moreover, we emphasise the importance of considering the co-exposure to chemicals of distinct sources and functions, which is often neglected in risk assessment.

Povzetek

Izpostavljenost okoljskim onesnažilom je v vsakdanjem življenju neizogibna. Ljudje in drugi organizmi smo nenehno izpostavljeni mešanici kemikalij, sestavljeni iz naravno prisotnih in antropogenih snovi. Pomanjkanje podatkov o toksikoloških lastnostih kemikalij in biokemijskih interakcijah med njimi je glavna omejitev za učinkovito oceno tveganja.

V doktorskem delu je ocenjen citotoksični in genotoksični potencial izbranih onesnažil različnega izvora, ki smo jim v okolju lahko sočasno izpostavljeni – cianotoksinov kot naravno proizvedenih in bisfenolov kot antropogenih onesnažil. Cianotoksini so cianobakterijski sekundarni metaboliti, prisotni v vodah, zlasti tam, kjer je razmnoževanje cianobakterij pospešeno zaradi evtrofikacije in vplivov globalnega segrevanja. Škodljivi učinki nekaterih znanih cianotoksinov so dokazani, v zadnjem času pa so bili odkriti številni prej nepoznani cianotoksini, ki se vse pogostejše pojavljajo v cianobakterijskem cvetu, njihove toksične lastnosti pa še niso raziskane. V nasprotju s cianotoksini so bisfenol A in njegovi analogi antropogenega izvora, saj gre za pomembne surovine pri proizvodnji plastičnih in epoksi materialov. V različnih vrstah embalaže za živila in drugih izdelkih so v človekovi vsakodnevni uporabi, preko izcednih voda iz proizvodne industrije in plastičnih odpadkov pa so široko prisotni tudi v okolju. V obeh izbranih skupinah onesnažil je več snovi, za katere je znano, da so genotoksične. Namen te disertacije pa je bil oceniti genotoksični potencial cianobakterijskih toksinov in analogov BPA, za katere te informacije manjkajo, ter genotoksične učinke ob izpostavljenosti njihovim mešanici, ki bolje odražajo izpostavljenost v okolju.

Disertacija podaja prve znanstvene podatke o genotoksičnosti cianotoksinov L-BMAA in mikrogininov. Rezultati so pokazali, da L-BMAA ne povzroča mutacij in poškodb DNK v bakterijskem testnem sistemu, medtem ko mikroginin povzroča prelome DNA in genomsko nestabilnost v celični liniji humanega hepatokarcinoma HepG2.

Izpostavljenost HepG2 celic cianobakterijskim ekstraktom, ki so vsebovali mešanico različnih mikrogininov oz. mešanici mikrocistina LR in cilindrospermopsina, je pokazala, da kombinirani genotoksični učinki niso enaki vsoti učinkov posameznih cianotoksinov. Rezultati kažejo na verjetnost antagonističnih interakcij med cianotoksini oz. prevladujoč učinek enega izmed toksinov. V kombiniranih izpostavitvah se je pokazal predvsem dominanten učinek cilindrospermopsina.

Kljub splošni domnevi, da so analogi varnejši od samega bisfenola A, je primerjava genotoksičnih učinkov BPA in njegovih najpogostejših analogov, bisfenolov S, F in AF, pokazala, da ima BPAF najvišji genotoksični potencial. V transkriptomskih analizah se je izkazalo, da BPA in BPAF ter njune kombinacije z drugimi bisfenoli povzročajo spremembe v izražanju izbranih genov tudi pri nizkih koncentracijah, relevantnih za človekovo izpostavljenost. Ob hkratni izpostavljenosti bisfenolom in cianotoksinu cilindrospermopsinu je bilo ponovno potrjeno, da ima cilindrospermopsin pri nastanku poškodb DNA prevladujoč učinek. Hkrati pa so se na podlagi transkriptomskih analiz več izbranih genov izkazali aditivni oz. sinergistični učinki interakcij cilindrospermopsina in bisfenolov v mešanicah.

S povečano proizvodnjo novih kemikalij in okrepljeno zavestjo o etični vprašljivosti uporabe laboratorijskih živali narašča potreba po zanesljivih *in vitro* toksikoloških podatkih za oceno tveganja. Kot odgovor na to situacijo intenzivno poteka razvoj 3D *in vitro* modelov, ki v primerjavi s klasičnimi celičnimi kulturami bolje ponazarjajo stanje v tkivih živih organizmov. Izboljšani modeli obetajo možnost za zanesljivejše *in vitro* raziskave in zmanjšanje potrebe po poskusih na živalih.

V raziskavi smo nov 3D *in vitro* model, razvit z uporabo HepG2 celic, uporabili za oceno citotoksičnosti in genotoksičnosti cilindrospermopsina. Rezultati so pokazali, da cilindrospermopsin vpliva na rast sferoidov in povzroči zaustavitev celičnega cikla. Transkriptomske analize so pokazale deregulacijo genov, vključenih v fazo I in fazo II metabolizma ksenobiotikov, odziv na poškodbe DNK, proliferacijo celic in apoptozo.

Rezultati disertacije podajajo nove toksikološke podatke o vse pogostejše prisotnih onesnažilih – cianotoksinih in bisfenolih – ter prispevajo k boljšemu razumevanju biokemijskih interakcij ob sočasni izpostavljenosti. Ponovno je bilo potrjeno, da posplošena uporaba modela seštevanja koncentracij ali modela neodvisnega delovanja za obravnavo mešanic kemikalij ni upravičena, saj eksperimentalni podatki kažejo na kompleksnejše interakcije. Poleg tega poudarjamo pomembnost obravnave mešanic kemikalij z različnim izvorom in potmi izpostavitve v oceni tveganja, kar je pogosto zanemarjeno.

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Abbreviations

Adha	... α -hydroxy- β -amino decanoic acid derivate
BPA	... Bisphenol A; 4,4'-(propane-2,2-diyl)diphenol
BPAF	... Bisphenol AF; 4-[1,1,1,3,3,3-Hexafluoro-2-(4-hydroxyphenyl)propan-2-yl]phenol
BPF	... Bisphenol F; 4,4'-Methylenediphenol
BPS	... Bisphenol S; 4,4'-sulfonyldiphenol
CYN	... Cyindrospermopsin
CYP 450	... Cytochromes P450
DAB	... 2,4-diaminobutyric acid
DNA	... Deoxyribonucleic acid
ECHA	... European Chemicals Agency
EFSA	... European Food Safety Authority
ERs	... Estrogen receptors
L-BMAA	... β -metilamino-L-alanin
LPS	... Lipopolysaccharides
MCLR	... Microcystin-LR
MGs	... Microginins
MCs	... Microcystins
MTS	... 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	... 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OATP	... organic anion transporting polypeptides
OECD	... The Organisation for Economic Co-operation and Development
REACH	... Registration, Evaluation, Authorisation and Restriction of Chemicals
ROS	... intracellular reactive oxygen species
US EPA	... United States Environmental Protection Agency
γ H2AX	... Histone H2A variant X phosphorylated at Serine 139
2D	... Two-dimensional
3D	... Three-dimensional

Chapter 1

Introduction

1.1 Pollution and Combined Exposure

Human activities result in the introduction of contaminants that provoke adverse change in the natural environment – pollution. Pollution may be in the form of energy (emission of light, heat, noise) or chemical substances. According to their source, chemical pollutants can be either naturally occurring contaminants or anthropogenic. However, also the production of natural pollutants can be indirectly influenced by anthropogenic activity. Therefore, freshwater environments (e.g. rivers, lakes and reservoirs) are the examples of chemical cocktails consisting of micropollutants from different sources. (Brusseau, Pepper, & Gerba, 2019)

In everyday life, people are in contact with dozens of different chemical substances that may affect their health through various biological pathways. In case of interference of those pathways, the combined final effects are difficult to predict based on the toxicological data of a single compound since synergistic, antagonistic or potentiating effects can emerge. From that perspective, the present work is focused on the geno/toxic effects of two groups of substances that could be simultaneously present in freshwater ecosystems as naturally produced toxins (cyanobacterial toxins) or a consequence of anthropogenic pollution (bisphenols). Freshwater is one of the crucial entities in human life; thus, people can be exposed to water pollutants through consumption of contaminated drinking water or food originating from it as well as by inhalation and dermal exposure during work or recreational activities, showering and other hygienic tasks. Therefore, the exposure to each individual or a combination of water pollutants is highly plausible. Nevertheless, the combined effect can also be a consequence of being exposed to chemicals through different routes since they interact in the organism regardless of their source.

1.2 Cyanobacterial Toxins

Cyanobacteria are photosynthetic prokaryotes common in freshwater environments as well as in estuaries and marine waters. They play a significant role in the carbon, nitrogen, and oxygen biochemical cycle. In the last decades, global warming and water eutrophication led to the more abundant growth of cyanobacteria, spreading of tropical species to the temperate zone and stimulated the frequency, duration, and extent of cyanobacterial blooms (Huisman et al., 2018; Meriluoto, Spoof, & Codd, 2016). Some bloom-forming species of cyanobacteria produce toxic metabolites – cyanotoxins, which exhibit harmful effects on humans and other organisms. The number of reports on the occurrence of cyanotoxins and poisoning is arising worldwide (Svirčev et al., 2019) and emerging global concern is also recognized by health protection organizations US EPA

(<https://www.epa.gov/cyanohabs>) and EFSA (Testai et al., 2017). Moreover, toxicity testing of cyanobacterial cells or extracts in bioassays is continuously revealing the toxic effects that cannot be explained by the currently known cyanotoxins (Bernard et al., 2011; K. Nováková et al., 2013) and the discovery of novel cyanobacterial toxic products of environmental and health significance is still anticipated.

The main route of human exposure to cyanobacterial toxins is drinking contaminated water (raw or insufficiently treated) and consuming foods originating from waterbodies with mass populations of cyanobacteria (finfish, shellfish, and prawns) or contaminated dietary supplements (Buratti et al., 2017; Roy-Lachapelle, Sollic, Bouchard, & Sauvé, 2017). Cyanobacterial cells can also be consumed attached to plant leaves and vegetables that have been spray-irrigated with contaminated water (Meriluoto et al., 2016). Many drinking water treatment procedures are efficient in removing cyanobacteria and cyanotoxins; therefore, in developed countries, exposure via drinking raw water mostly occurs incidentally during recreational activities. In occupational and recreational situations, dermal and inhalation exposure routes may be predominant; however, their consequences are less understood. The most severe consequence of the exposure to cyanotoxins was reported in haemodialysis patients in which severe illnesses and deaths occurred due to the cyanobacteria-contaminated haemodialysis water (Jochimsen et al., 1998).

1.2.1 Classification of Cyanobacterial Toxins

Cyanotoxins are structurally diverse chemicals; therefore, also the molecular mechanisms behind their toxic effects are different. According to their chemical structure, cyanotoxins belong to four main groups: cyclic peptides (microcystins and nodularins), alkaloids (cylindrospermopsins, anatoxin-a, anatoxin-a(s), saxitoxins, lyngbyatoxins, aplysiatoxins), diamino acids (BMAA and DAB) and lipopolysaccharides (Table 1) (Meriluoto et al., 2016).

Table 1.1: Summary of chemical structures, examples of main producers, and modes of action of cyanotoxins. [Adapted from Meriluoto J, Spoof L, Codd GA (eds) (2016) Handbook of Cyanobacterial Monitoring and Cyanotoxin Analysis. John Wiley & Sons, Ltd, Chichester, UK (Meriluoto et al., 2016) and Huisman, J., Codd, G.A., Paerl, H.W., Ibelings, B.W., Verspagen, J.M.H., Visser, P.M. (2018) Cyanobacterial blooms. Nat. Rev. Microbiol. 16, 471–483 (Huisman et al., 2018)].

Cyanotoxin	Chemical structure	Cyanobacteria producers (genus)	Modes of action
Microcystins	peptides Cyclic heptapeptides	<i>Microcystis</i> , <i>Anabaena</i> , <i>Nostoc</i> , <i>Planktothrix</i> , <i>Phormidium</i> , <i>Oscillatoria</i> , <i>Radiocystis</i> , <i>Gloeotrichia</i> , <i>Anabaenopsis</i> , <i>Rivularia</i> , <i>Tolypothrix</i> , <i>Hapalosiphon</i> , <i>Plectonema</i>	Hepatotoxic, tumour promoting, inhibition of eukaryotic protein phosphatase PP1, PP2A, and phosphoprotein phosphatases PPP4, PPP5
Nodularins		<i>Nodularia spumigena</i> , <i>Nostoc (symbiotic)</i>	As for microcystins, plus weak carcinogenicity
Microginins		<i>Microcystis</i> , <i>Anabaena</i> , <i>Woronichinia</i> , <i>Planktothrix</i> , and <i>Nostoc</i>	Inhibition of zinc metalloprotease enzymes
Cylindrospermopsins	Tricyclic guanidine alkaloids	<i>Cylindrospermopsis</i> , <i>Umezakia</i> , <i>Anabaena</i> , <i>Oscillatoria</i> , <i>Raphidiopsis</i> , <i>Aphanizomenon</i>	Inhibition of protein synthesis, multiple organ toxicity, neurotoxic, genotoxic,
Anatoxin-a	alkaloids Bicyclic alkaloids	<i>Anabaena</i> , <i>Phormidium</i> , <i>Aphanizomenon</i>	Neurotoxic, binds competitively at acetylcholine receptors
Anatoxin-a(S)		Phosphorylated cyclic N-hydroxyguanine <i>Anabaena</i>	Neurotoxic, inhibits acetylcholine esterase
Saxitoxins		Tricyclic alkaloids <i>Aphanizomenon</i> , <i>Anabaena</i> , <i>Lyngbya</i> , <i>Cylindrospermopsis</i> , <i>Planktothrix</i>	Neurotoxic, blocks voltage-gated sodium channels
Lyngbyatoxins, Aplysiatoxins	Indole alkaloids	<i>Lyngbya</i> , <i>Oscillatoria</i> , <i>Schizothrix</i>	Tumour-promoting, binds to eukaryotic protein kinase C
BMAA, DAB	Diamino acids	Many genera	Neurotoxic, developmental toxin, erroneous insertion into proteins
LPS	Lipopolysaccharides	All genera	Inflammatory, promotion of cytokine secretion

In this thesis, we addressed cytotoxic and genotoxic properties of freshwater cyanotoxins microcystin-LR (MCLR), microginins (MGs), cylindrospermopsin (CYN), and beta-N-methyl-amino-L-alanine (L-BMAA). MCLR is one of the most studied cyanotoxins and it is among the most frequently detected cyanotoxins, while CYN has been identified as an emerging cyanotoxin in the last decade. On the other hand, the adverse toxic effects of L-BMAA and microginins are recently being studied and not much is known on their mechanisms of action.

The following description of cyanotoxins and bisphenols related to this thesis is a brief overview of their main chemical characteristics and known toxic and genotoxic properties, while the concentrations at which the toxic effects were observed are not stated, although we are aware of the significance of these data.

1.2.1.1 Microcystin-LR

Microcystins (MCs) are a diverse group of hepatotoxic cyclic heptapeptides (Carmichael et al., 1988). Their general structure is cyclo[-D-Ala-L-X-D-erythro- β -methyl-Asp-L-Z-Adda-D-Glu-Mdha] where Adda refers to unique amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. The L-amino acid residues X and Z in positions 2 and 4 denote the most variable part and are indicated in the name of the corresponding MC congener as a two-letter suffix. Over 248 MCs congeners have been identified so far (Meriluoto et al., 2016), among which microcystin-LR (MCLR), with leucine and arginine in positions 2 and 4, is one of the most frequently detected and well-studied.

MCs are mostly produced by freshwater cyanobacteria belonging to at least 47 species, which have been documented in 80 countries (Meriluoto et al., 2016). The studies of European water bodies are reporting detection of MCs in the concentration range between 0.1 and 10 $\mu\text{g/L}$, but it also reached 2800 $\mu\text{g/L}$ (Faassen & Lüring, 2013).

MC molecules pass cell membranes by active transport through organic anion transporting polypeptides (OATP) (Fischer et al., 2005); therefore, the main target organ of MC toxicity is liver, while also the effects on gastrointestinal tract, kidney and brain were confirmed. The primary mechanism of MC toxicity is the inhibition of serine/threonine-specific protein phosphatases, including PP1 and PP2A, which are ubiquitously expressed and they are major regulators of protein dephosphorylation (Shi, 2009). Therefore, irreversible inhibition of PP1 and PP2A by MCs can affect cell metabolic processes including glycogen synthesis, cell contractility, secretion and transmembrane transport, cellular division, and gene expression resulting MC intoxication symptoms such as gastroenteritis, irritation reactions, liver diseases (reviewed in De Figueiredo, Azeiteiro, Esteves, Gonçalves, & Pereira, 2004). Recently, also the evidence of the endocrine-disrupting potential of MCLR is accumulating (Z. Liu, Tang, Li, Hu, & Wang, 2015; Su et al., 2016).

International Agency for Research on Cancer classified MCLR into the 2B group of possible human carcinogens (IARC, 2010) since it was shown to act as a tumour-promoter (Humpage, Hardy, et al., 2000; Nishiwaki-Matsushima et al., 1992). MCLR has been recognised as genotoxic, causing DNA strand breaks *in vitro* and *in vivo* at non-cytotoxic concentrations (for review see Žegura, 2016). The molecular mechanisms involved in its genotoxicity are not entirely understood, but most of the research reports strong evidence that MCLR induces DNA damage indirectly through increasing the production of intracellular reactive oxygen species (ROS) and consequent oxidation of DNA bases (Bouaïcha, Maatouk, Plessis, & Périn, 2005; Žegura, Lah, & Filipić, 2004; Žegura, Lah, & Filipić, 2006). At the same time, it compromises DNA repair mechanisms (Lankoff et al., 2006a), which results in enhanced genomic instability. Additionally, MCLR induced expression of proto-oncogenes c-fos, c-jun, and c-myc in rat tissue, which might be a mechanism of its tumour-promoting activity (H. Li, Xie, Li, Hao, & Xiong, 2009).

1.2.1.2 Microginins

Microginins (MGs) are linear peptides composed of α -hydroxy- β -amino decanoic acid derivate (Ahda) at the N-terminal end extended by three to five additional amino acids at the C-terminal end (Ishida, Kato, Murakami, Watanabe, & Watanabe, 2000). The group consists of about 40 congeners and the number is rising with new identifications (Lodin-Friedman & Carmeli, 2018; Riba et al., 2019; Stewart, Ravindra, Van Wagoner, & Wright, 2018). Variants with chlorinated or methylated Ahda have been reported as well as

derivatives containing a modified octanoic acid moiety instead (Gesner-Apter & Carmeli, 2008; Ishida et al., 2000; Kodani, Suzuki, Ishida, & Murakami, 1999; Lifshits, Zafrir-Ilan, Raveh, & Carmeli, 2011; Lodin-Friedman & Carmeli, 2018).

MGs are predominantly produced by *Microcystis* spp. but also species from the genus *Anabaena*, *Woronichinia*, *Planktothrix*, and *Nostoc* were found to produce MG congeners (Meriluoto et al., 2016). There are several reports about the detection and identification of MGs, while only a few quantified the number of metabolites in samples (reviewed in Janssen, 2019). Up to date, the highest reported environmental concentration of MGs from cyanobacterial blooms in eutrophic lakes was 2.2 µg/L (Beverdors, Weirich, Bartlett, & Miller, 2017).

The biological activity of MGs is not well understood. Due to the binding of Ahda moiety to the active site of zinc metalloprotease enzymes, MGs act as their inhibitors at nanomolar or micromolar concentrations (Ishida et al., 2000; Kraft, Schleberger, Weckesser, & Schulz, 2006; Lodin-Friedman & Carmeli, 2018). Many of them exhibit inhibition of the angiotensin-converting enzyme (ACE). ACE inhibitors play a role in human medicine as blood pressure-lowering agents and thus, MGs may serve as lead compounds in the discovery of novel antihypertensive drugs (US7846686B2, 2010).

The data about the potential toxicity of MGs are scarce. Recently, three studies reported evidence demonstrating toxic activity towards model aquatic organisms; the crustaceans *Thamnocephalus platyurus* (Bober & Bialczyk, 2017), nematode *Caenorhabditis elegans* (Lenz, Miller, & Ma, 2019) and fish *Astyanax altiparanae* larvae (Fernandes et al., 2019). The first findings about the potential genotoxic effects of microginins are a part of this thesis.

1.2.1.3 Cylindrospermopsin

Cylindrospermopsin (CYN) is structurally a polyketide-derived alkaloid with a tricyclic carbon skeleton containing a guanidine functional moiety combined with hydroxymethyluracil attached to it (Ohtani, Moore, & Runnegar, 1992). Currently, five CYN analogues are known. It has been detected worldwide, produced by more than 12 species of cyanobacteria. The main CYN producers *Cylindrospermopsis raciborskii* and *Anabaena ovalisporum* are invading several subtropical and temperate water reservoirs and lakes, expanding its occurrence (Suklenik, Hadas, Kaplan, & Quesada, 2012). Usually, CYN is detected in concentrations between 1 and 10 µg/L, while up to 126 µg/L has been detected in an Italian lake (de la Cruz et al., 2013).

CYN is a highly water-soluble zwitterionic molecule that can be transported to cells by simple passive diffusion through membranes; however, the transport through the bile acid transport system is more efficient (Chong, Wong, Lam, Shaw, & Seawright, 2002). Toxic effects of CYN depend on its metabolic activation, but the exact metabolic pathway remains unclear. A lot of indirect evidence shows that various isoforms of cytochrome P450 (CYP450) family are involved in its metabolic activation (Bazin, Mourrot, Humpage, & Fessard, 2010; Suzanne M. Froscio, Humpage, Burcham, & Falconer, 2003; Hercog et al., 2017; Humpage, Fontaine, Froscio, Burcham, & Falconer, 2005; Kittler, Hurtaud-Pessel, Maul, Kolrep, & Fessard, 2016; Štraser, Filipič, Gorenc, & Žegura, 2013; Štraser, Filipič, & Žegura, 2011, 2013; Žegura, Gajski, Štraser, & Garaj-Vrhovac, 2011), while the research on the role of phase II enzymes in metabolism of CYN is scarce. Based on its metabolism, primary target organs of CYN are the liver and kidney; however, also spleen, thymus, lung, heart, and lymphocytes might be affected (Falconer & Humpage, 2006). Its primary mechanism of toxicity is irreversible inhibition of protein synthesis (S M Froscio, Humpage, Burcham, & Falconer, 2001; M. T. Runnegar et al., 2002; Terao et al., 1994). The molecular

mechanism of CYN toxicity is not fully understood yet, but it appears an interplay between the uracil, hydroxyl and guanidine groups might be the key (Evans et al., 2019). Toxic effects of CYN are mainly observed in liver; however, developmentally toxic (Berry, Gibbs, Schmale, & Saker, 2009), immunotoxic (Moosova et al., 2019; Poniedzialek, Rzymiski, & Wiktorowicz, 2014), neurotoxic (Hinojosa et al., 2019), endocrine disrupting (J. Liu, Hernández, Swift, & Singhal, 2018; Young, Micklem, & Humpage, 2008) and genotoxic activities have also been identified.

The exposure to CYN is causing the inhibition of glutathione synthesis (Maria Runnegar, Berndt, & Kaplowitz, 1995; MT Runnegar, Kong, & Zhong, 1994) and lipid peroxidation (Evans et al., 2019; Liebel et al., 2011; Poniedzialek, Rzymiski, & Karczewski, 2015) which results in oxidative stress. In metabolically competent cells, it is causing genotoxic effects in the form of DNA strand breaks (Humpage et al., 2005; Sieroslawska & Rymuszka, 2015; Štraser, Filipič, Gorenc, et al., 2013; Štraser, Filipič, Novak, & Žegura, 2013; Žegura, Gajski, et al., 2011) and chromosomal damage (Bazin et al., 2010; Hercog et al., 2017; Humpage, Fenech, Thomas, & Falconer, 2000; Sieroslawska & Rymuszka, 2015; Štraser et al., 2011; Žegura, Gajski, et al., 2011). There are some preliminary indices of its tumour-initiating activities (Falconer and Humpage, 2000).

1.2.1.4 -N-methylamino-L-alanine

β -N-methylamino-L-alanine (L-BMAA) is a non-protein amino acid with three natural isomers (L-DAB, AEG, and BAMA). It has been identified first in the seeds of a cycad tree (*Cycas micronesica*) (Vega & Bell, 1967) and thought to be its product; however, it has later been discovered that L-BMAA is produced by symbiotic cyanobacteria (*Nostoc* sp.) in roots of cycad trees, transferred to the plant and accumulated in the seeds (Cox et al., 2005). Since the cycad fruits and seeds are an important source of food, the toxin spreads through the food chain. Chamorro residents of Guam island were exposed via flour made from the seeds and consuming meat of animals which fed on cycad fruits and accumulate BMAA in the tissues (especially flying foxes), as well as through the use of cycad in traditional medicine (Spencer et al., 1987). Looking for the cause of extremely high incidence of a neurodegenerative disorder called Lou Gehrig's disease (amyotrophic lateral sclerosis/Parkinsonism dementia complex (ALS/PDC)) among inhabitants of Marianas islands Guam and Rota (Murch, Cox, & Banack, 2004) led to the discovery of BMAA in the first place.

Nowadays, it is known that BMAA is produced by a broad spectrum of different cyanobacteria (Cox et al., 2005) as well as some diatoms (Jiang et al., 2014) all around the world. Since the two groups of organisms play a significant role of primary producers in water ecosystems, BMAA is widely spread and biomagnified in higher trophic levels (Cox, Banack, & Murch, 2003) providing a route for potential poisoning of humans consuming contaminated organisms such as molluscs, crustaceans and fish (Brand, Pablo, Compton, Hammerschlag, & Mash, 2010). The presence of the toxin in water used for agriculture represents another concern for food safety (Main et al., 2018).

The neurotoxicity of BMAA has been confirmed in numerous studies reviewed in Karamyan & Speth, 2008. It is a significant environmental risk in the development of neurodegenerative disorders (for review see Cox, Kostrzewa, & Guillemin, 2018) such as Alzheimer's disease (Pablo et al., 2009), amyotrophic lateral sclerosis (Bradley & Mash, 2009), and the amyotrophic lateral sclerosis/ parkinsonism-dementia complex (ALS-PDC) (Holtcamp, 2012). The mechanism underlying the pathology of neurodegenerative disorders is still not fully understood and there are various hypotheses discussing the involvement of different pathways that may also coexist (reviewed in Delcourt, Claudepierre, Maignien, Arnich, & Mattei, 2018). The chemical structure of L-BMAA is very similar to glutamate

(Cucchiaroni et al., 2010); therefore, BMAA binds to its receptors at a glutamatergic synapse, which is causing an over-stimulation of postsynaptic neurons leading to ER stress and cell apoptosis, a phenomenon termed excitotoxicity (Van Den Bosch, Van Damme, Bogaert, & Robberecht, 2006). Another BMAA toxicity mechanism is related to its ability to bind to the amino acid tRNA synthetase, which leads to a misincorporation of BMAA into the protein in place of L-serine (Dunlop, Cox, Banack, & Rodgers, 2013). However, BMAA can also inhibit enzyme activity and strongly interfere with protein folding in the absence of de novo protein synthesis, which may constitute another mechanism of toxicity (van Onselen & Downing, 2018).

The current state of knowledge recognizes L-BMAA as neurotoxic, while the first data about the potential genotoxicity of BMAA was a part of this thesis. The results of the OECD standardised Ames test showed that L-BMAA is not mutagenic for bacteria (Novak, Hercog, & Žegura, 2016). Afterwards, nitrosated BMAA (N-BMAA) has been shown to act as an alkylating agent and cause DNA strand breaks *in vitro* (Potjewyd, Day, Shangula, Margison, & Povey, 2017) and the induction of DNA strand breaks was observed in immune cells of freshwater mussels after exposure to BMAA (Lepoutre et al., 2018). Recent study on human peripheral blood cells reported the formation of micronuclei and nucleoplasmic bridges corresponding to clastogenic potential, while markers of oxidative damage were not affected (Gerić et al., 2019).

1.3 Bisphenols

Bisphenols (BPs) are a group of synthetic compounds, among which bisphenol-A (BPA) is the most abundant. Chemically they are diphenylmethanes, which contain two benzene rings, separated by bridge element, usually with the 4-OH substituent on both benzene rings. Bisphenols are used as a primary raw material for the production of polymers, especially polycarbonate plastic and epoxy resins – materials that form a wide variety of products for consumers' everyday use, such as beverage bottles, food packaging, metal can linings, baby bottles and toys, water pipes, medical tubings, dental sealants, eyeglass lenses, thermal paper and other paper products, lacquers, etc. Due to their massive production and a wide variety of applications, consequential constant human exposure and disposal of bisphenols into the environment are inevitable.

Due to the enormous manufacture of BPs, they are ubiquitous in the environment. The origin of environmental pollution is of i) preconsumer sources related to effluents from manufacturing BPs and BPs-products, their transport and processing; and ii) postconsumer sources associated with disposal of waste including effluent discharge from municipal wastewater treatment plants, leaching from landfills, combustion of domestic waste, and degradation of plastics in the environment (Corrales et al., 2015). The average concentrations of BPA measured in surface waters are usually around 100 ng/L, but they depend importantly on location. In Asian industrial regions, concentrations of up to 44 µg/L were recorded (Corrales et al., 2015). The data about the presence of BPA analogues in the environment are more scarce, but the existing measurements are mostly in the range between 1 and 100 ng/L. Again, in regions near industrial effluents, even 100-fold higher concentrations were measured, and different BPs were simultaneously detected in the same samples (Z. Huang et al., 2020; Yamazaki et al., 2015).

Several studies reported detection of BPA in more than 90% of tested samples of human urine with concentrations ranging between 0.5-20 ng/mL (reviewed in R. ping Huang et al., 2018; Vandenberg, Hauser, Marcus, Olea, & Welshons, 2007), while its uptake is estimated to 50-60 ng/kg bw/day (R. ping Huang et al., 2018). The main route of human

exposure to bisphenols is the consumption of food and drinks contaminated via leaching of BPs from their plastic containers or cans. Dermal exposure to thermal paper and recycled paper products, inhalation of indoor dust and dental sealants are also important sources of human exposure to BPs (Björnsdotter, de Boer, & Ballesteros-Gómez, 2017; Vandenberg et al., 2007). BPA analogues are detected in urine less frequently and in lower concentrations (below 1 ng/mL); however, they occur simultaneously in the same samples (Chen et al., 2016; R. ping Huang et al., 2018; Yang, Lu, et al., 2014; Ye et al., 2015).

1.3.1 Bisphenol -A

Bisphenol-A (BPA, 2,2-bis(4-hydroxydiphenyl)propane) was the first bisphenol widely used in the industry. The first commercial epoxy resin was synthesized from BPA in the 1940s and in 1957, a hard plastic called polycarbonate was discovered by polymerization of BPA (Vogel, 2009), although its estrogenic activity had been known before (Dodds & Lawson, 1936). Since then, the global yearly BPA production increased to around 8 million metric tons in 2016 and is projected to exceed 10.5 million tons by 2022 (Lehmle, Liu, Gadogbe, & Bao, 2018). During that time, it was revealed that BPA is leaching out of polycarbonate products (Krishnan, Stathis, Permuth, Tokes, & Feldman, 1993) and an enormous amount of scientific reports evidencing its harmful effects on human health and other organisms have accumulated, especially emphasizing its endocrine disrupting activity.

BPA has been considered as a very weak environmental estrogen as its structure enables it to bind to the estrogen receptors (ERs), but the binding affinity of BPA to ER α and ER β is 10,000 times lower than 17 β -estradiol (Kuiper et al., 1998; Matthews, Twomey, & Zacharewski, 2001). Early research suggested that BPA would have little to no effect in the human body due to its rapid metabolism and thus, low bioavailability (Völkel, Colnot, Csanády, Filser, & Dekant, 2002). However, more recent studies of molecular mechanisms of BPA action have revealed a variety of pathways through which BPA can stimulate cellular responses as high as estradiol at very low (pico and nanomolar) concentrations (Gould et al., 1998; Wozniak, Bulayeva, & Watson, 2005; reviewed in Wetherill et al., 2007). BPA binds to many other nuclear endocrine receptors such as androgen receptor, estrogen-related receptor γ , thyroid hormone receptor, peroxisome proliferator-activated receptors, and human pregnane X receptor, and activates them at concentrations lower than those needed to activate ER α and ER β (reviewed in Acconcia, Pallottini, & Marino, 2015; Wetherill et al., 2007). BPA also binds to the membrane-bound form of ER α and transmembrane ER (G protein-coupled receptor 30) and induces non-genomic changes (Mileva, Baker, Konkole, & Bielajew, 2014). Moreover, BPA induces changes in hormone metabolism and epigenetic changes through DNA methylation (reviewed in Acconcia et al., 2015). Taken together, BPA is an endocrine-disrupting compound in the broad sense of the definition (Wetherill et al., 2007).

The literature data are evidencing BPA genotoxic effects while the underlying mechanisms have not been fully explained. BPA does not exhibit mutagenic properties in bacterial (Anja Fic, Žegura, Gramec, & Mašič, 2014; Schweikl, Schmalz, & Rackebbrandt, 1998; Tiwari et al., 2012; L. Xin et al., 2015) or mammalian cell models *in vitro* (Schweikl et al., 1998; Tsutsui et al., 1998), while the induction of DNA damage and reduced chromosomal integrity have been reported. BPA as well as its metabolites have been shown to induce DNA strand breaks *in vitro* (A. Fic, Žegura, Sollner Dolenc, Filipič, & Peterlin Mašič, 2013; García-Córcoles et al., 2018; X. Li, Yin, & Zhao, 2017; Ramos et al., 2019; F. Xin et al., 2014; L. Xin et al., 2015) and *in vivo* (Tiwari et al., 2012), induce DNA adducts formation *in vitro* (Kolšek, Mavri, & Sollner Dolenc, 2012; Tsutsui et al., 1998; Wu et al., 2017; L. Xin et al., 2015) and *in vivo* (Izzotti, Kanitz, D'Agostini, Camoirano, & De Flora,

2009), and lead to structural and numerical chromosomal aberrations *in vitro* (Aghajanjpour-Mir et al., 2016; Ramos et al., 2019; Santovito, Cannarsa, Schleicherova, & Cervella, 2018; L. Xin et al., 2015) and *in vivo* (Tiwari et al., 2012).

BPA clearly represents a threat to human health; therefore, its use is being gradually limited and restricted by international agencies and different government organizations starting with the ban in child care and feeding products and thermal paper (Buka, Osornio-Vargas, & Walker, 2009; EC, 2011; Lehmler et al., 2018; US FDA, 2014). Based on new scientific data EFSA lowered the tolerable daily intake from previous 50 $\mu\text{g}/\text{kg}$ bw/day to 4 $\mu\text{g}/\text{kg}$ bw/day in 2015 and consequentially the specific migration limit for BPA in food contact materials was lowered to 0.05 mg/kg of food (EFSA, 2015). Since 2017, BPA is also included on the Candidate List of substances of very high concern by the European Chemical Agency (ECHA). However, the US Food and Drug Administration continues to support the safety of BPA for the currently approved uses in food containers and packaging and the existing NOAEL for oral exposure (5 mg/kg bw/day) (US FDA, 2014).

The restrictions provided an inducement for replacing BPA in the industry with its presumably safer analogues. That resulted in an increased presence of various bisphenols in the environment and increased human exposure. Due to their structural similarity with BPA, the safety of these analogues is questionable and research data have shown them to possess very similar or even higher endocrine-disrupting and toxic potential as BPA (Rochester & Bolden, 2015; Siracusa, Yin, Measel, Liang, & Yu, 2018; Usman & Ahmad, 2016). Nowadays, there are sixteen BPA analogues used in the industry, among which bisphenols S, F and AF are the most abundant (Chen et al., 2016).

1.3.2 Bisphenol-A Analogues

In this thesis, we focused on adverse cytotoxic and genotoxic effects of BPA and its three analogues: bisphenol-S (BPS), bisphenol-F (BPF) and bisphenol-AF (BPAF).

BPA analogues are less studied than BPA; however, up to date many biological activities comparable to BPA were confirmed (Table 2) (Chen et al., 2016; Rochester & Bolden, 2015; Usman & Ahmad, 2016).

Table 1.2: Toxic effects of bisphenol A analogues BPS, BPF and BPAF confirmed in previous studies. [Adopted from Chen, D., Kannan, K., Tan, H., Zheng, Z., Feng, Y. L., Wu, Y., & Widelka, M. (2016). Bisphenol Analogues Other Than BPA: Environmental Occurrence, Human Exposure, and Toxicity - A Review. Environmental Science and Technology, 50(11), 5438–5453 (Chen et al., 2016) and updated.]

	BPS	BPF	BPAF
Estrogenic	+	+	+
Androgenic	+	+	/
Anti-estrogenic	+	+	+
Anti-androgenic	+	+	+
Cytotoxicity	+	+	+
Genotoxicity	+	+	+
Reproductive toxicity	+	+	+ ^c
Teratogenicity ^b	+	+	+
Neurotoxicity	+	+	+ ^d
Oxidative toxicity ^a	+	+	+
Acute toxicity	+	+	+ ^b

^a(Maćczak, Cyrkler, Bukowska, & Michałowicz, 2017), ^b(Moreman et al., 2017), ^c(Siracusa et al., 2018), ^d(Lee, Kim, Shin, & Kim, 2013), /- no data

1.3.2.1 Bisphenol-S

BPS (bis(4-hydroxyphenyl)sulfone) possesses two phenol rings linked through the sulfonyl group. It is one of the most frequent substitutes for BPA in a variety of industrial applications due to its heat and light resistance comparable to BPA (Qiu et al., 2019). Therefore, it is found as an anticorrosive agents in epoxy resins used in food containers inside linings, it is present in all thermal paper samples and finally, it is the main substitute of BPA in baby bottles, child equipment, and other BPA-free consumer products. BPS occurrence in the environment is ubiquitous. The highest concentrations in surface waters and sediments have been detected in China, India, and Japan, corresponding to locations of the primary manufacturing plants (Qiu et al., 2019). Due to its presence in thermal paper, dermal contact and indoor dust inhalation are among important routes of human exposure to BPS (Björnsdotter et al., 2017). However, contaminated food remains to be the main source of human intake. BPS has been detected in canned food and beverages, cereal products, meat, seafood, milk, and other food products from all over the world (Qiu et al., 2019).

There is evidence of BPS acute toxicity, endocrine disrupting activity, neurotoxicity and immunotoxicity (Qiu et al., 2019). BPS has been shown to cause developmental and reproductive toxic effects in rats, zebrafish and chicken models *in vivo* (Crump, Chiu, & Williams, 2016; Ji, Hong, Kho, & Choi, 2013; Naderi, Wong, & Gholami, 2014; Yamasaki, Noda, Imatanaka, & Yakabe, 2004). Its estrogenic activity has been proven in several studies (reviewed in Qiu et al., 2019; Rochester & Bolden, 2015). Additionally, weak antiandrogenic (Kitamura et al., 2005), antiestrogenic (Stossi et al., 2014) and androgenic (Molina-Molina et al., 2013) effects have been reported. BPS poses comparable or lower endocrine disruption potential than BPA.

The data on the genotoxicity of BPS is very limited. BPS does not cause mutations in the bacteria-based assay (Anja Fic, Žegura, Sollner Dolenc, Filipič, & Peterlin Mašič, 2013). It is inducing ROS formation followed by oxidation of purines (Mokra, Woźniak,

Bukowska, Sicińska, & Michałowicz, 2018). Increased formation of DNA strand breaks after exposure to BPS has been detected in HepG2 cells (A. Fic et al., 2013) as well as in blood monocytes (Mokra, Kuźmińska-Surowaniec, Woźniak, & Michałowicz, 2017). However, in experiments testing different bisphenol analogues, BPS usually exhibited the lowest genotoxic potency.

1.3.2.2 Bisphenol-F

BPF (bis(4-hydroxyphenyl) methane), possesses two phenol rings which are linked through methylene. The main technical advantages of BPF are its low viscosity and better resistance of its epoxy resins to solvents. It is increasingly used as a BPA replacement in the production of epoxy resins and polycarbonates found in food packaging, water pipes linings, dental sealants, industrial floors, grouts, electrical equipment, coatings, lacquers (Rochester & Bolden, 2015). BPF is a predominant bisphenol contaminant in prepacked meat and personal care products (Lu et al., 2018). It was also detected in canned drinks and food, paper products, commercial milk, etc. (Usman, Ikhlas, & Ahmad, 2019).

BPF causes liver toxicity (Higashihara et al., 2007), developmental toxicity and oxidative stress (reviewed in Usman et al., 2019). The endocrine-disrupting potential of BPF is comparable to BPA (reviewed in Rochester & Bolden, 2015; Usman et al., 2019). BPF has estrogenic (Goldinger et al., 2015; Lei, Huang, et al., 2018), progesteronic (Feng et al., 2016) and anti-androgenic (Roelofs, Berg, Bovee, Piersma, & Duursen, 2015) properties. The data about the interaction with the glucocorticoid receptor are not conclusive, but the endocrine disturbing potential is confirmed (Kolšek, Gobec, Mlinarič Raščan, & Sollner Dolenc, 2015; Roelofs et al., 2015). BPF also acts as a potent disruptor of thyroid hormonal regulation (Y. F. Zhang et al., 2018). It has been demonstrated that BPF poses the risk of estrogen-dependant cancer progression to a similar extent as BPA (Kim et al., 2017).

BPF does not act as a bacterial mutagen (Cabaton et al., 2009; A. Fic et al., 2013), but it exhibits genotoxic potential in eukaryotic cells. In different human *in vitro* models, BPF and its metabolites have been shown to cause DNA strand breaks (Audebert, Dolo, Perdu, Cravedi, & Zalko, 2011; Cabaton et al., 2009; Hercog et al., 2019; Mokra et al., 2017) most probably related to oxidative stress (Mokra et al., 2018), while no micronucleus formation corresponding to chromosomal aberrations has been observed (Cabaton et al., 2009).

1.3.2.3 Bisphenol-AF

BPAF (4,4-(hexa fluoroisopropylidene)diphenol) is a structural analogue of BPA in which the two methyl groups on the central carbon are replaced by trifluoromethyl groups. BPAF has especially good technical characteristics and is used as a high-performance monomer in specialty polymers as well as a cross-linker in fluoropolymers and fluoroelastomers. Therefore, it is found in optical fibres, waveguides, and electronic devices, but is also present in food contact packaging and other plastic applications. BPAF has been detected in indoor dust, surface water as well as solid sediment and sludge (Yang, Lu, et al., 2014), generally in low concentrations, but also elevated up to 15.3 µg/L near the industrial plants in Asia (Chen et al., 2016). It is strongly persistent in the environment, with half time approximately three times longer than the majority of bisphenols (Chen et al., 2016). Human exposure to BPAF is lower compared to BPA, however, together with its metabolic products, it has been detected in urine and bile samples (Yang, Guan, Yin, Shao, & Li, 2014; Ye et al., 2015).

Toxicity data on BPAF is even more limited than on BPS and BPF. It significantly decreases cell viability and increases ROS levels and lipid peroxidation to a higher extent than any other compared bisphenol compound (Lei, Sun, et al., 2018; Maćczak et al., 2017; Michałowicz, Mokra, & Bak, 2015; Mokra et al., 2018). The endocrine-disrupting activity of BPAF is estimated to be 2.81 to 12.6-fold higher compared to BPA (Chen et al., 2016). It exhibits high potential for binding to estrogenic receptors with preferred affinity to ER (Kitamura et al., 2005). Its antagonistic potency against 17-estradiol activity is 47.6 times higher than of BPA (Matsushima, Liu, Okada, Shimohigashi, & Shimohigashi, 2010). Besides anti/estrogenic, it exhibits antiandrogenic potency (Kitamura et al., 2005). It has been reported that BPAF is highly developmentally toxic to zebrafish (Moreman et al., 2017). BPAF was even shown to induce an increase of multinucleated germ cells in testis associated with testicular germ cell cancer in humans (Ferrara et al., 2006). A recent review on the reproductive and developmental toxicity of common BPA analogues recognised BPAF as the highest concern (Siracusa et al., 2018).

The studies so far show that BPAF possesses higher genotoxic potential than BPA and other analogues. Similarly as described for other BPs, it is not a bacterial mutagen (A. Fic et al., 2013; Tsutsui et al., 2000), while it is a potent inducer of DNA strand breaks, particularly those associated with oxidative stress in human hepatoma cells (A. Fic et al., 2013), peripheral blood mononuclear cells (Mokra et al., 2017, 2018) and breast cancer cells (Lei, Sun, et al., 2018). Furthermore, BPAF caused numerical chromosomal abnormality (Tsutsui et al., 2000), disruption of microtubule complex and micronuclei formation (Pfeiffer, Rosenberg, Deuschel, & Metzler, 1997) in hamster cells.

1.4 Combined Exposure to Different Chemicals

Organisms in the environment are exposed to a mixture of chemicals of different sources rather than to individual entities. A series of challenges for human and environmental risk assessment arise from this fact, starting with the identification of contaminants and their concentrations in each exposure scenario, assessing their individual bioactive properties and risks and finally assessing the effects of combined exposure. Currently, risk assessment required for regulatory purposes mostly relies on the assessment of an individual chemical or the exposure to multiple chemicals is considered within one legislative framework (e.g. plant protection products and chemicals) excluding all other plausible combined exposure scenarios. Recently, a lot of effort is devoted to establishing a consistent harmonised approach for mixture's risk assessment and management across different regulatory sectors (Bopp et al., 2018; More et al., 2019).

1.4.1 Mixture Risk Assessment Approaches

A series of theoretical methods to anticipate the combined effects based on the chemical structure and biological effects of individual compounds have been proposed (reviewed in Kienzler et al., 2014). The basic mathematical models assume that the components of the mixture either possess the same mode of action (concentration addition concept) or their modes of action are independent (response addition concept). Thus, the discrepancy between the calculated assessments and the adverse effects observed in organisms or cell models occurs regularly resulting from either neglected contaminants in the field or interactions of compounds in mixtures. Biological interactions may occur i) at the toxicokinetic level, as chemicals might affect the uptake, distribution, metabolism and excretion pathways of each other; or ii) at the toxicodynamic level, through interactions

with receptors or cellular target or organs. Interference results in the final effect less than additive (antagonistic, inhibitive, masking) or greater than additive (synergistic, potentiating) (Klaassen, 1991). If there is evidence of biological interaction of different chemicals, the case of coexposure should be addressed differently, considering the interactions between chemicals. Development of mathematical models which would describe and predict the mixture effect as well as possible is the focus of numerous recent studies (More et al., 2019). Since the data on bioactive and toxic properties of individual substances is missing, which is especially often in the case of recently emerging or discovered chemicals, it is impossible to predict the effect of combined exposure. To fill that knowledge gap, there is a strong need for research on toxic effects and the underlined molecular mechanisms as well as the identification of chemical and biological interactions of chemicals to which humans and other organisms are simultaneously exposed.

1.4.2 Combined Exposure to Cyanotoxins

Cyanobacterial blooms generally consist of different toxin-producing and non-toxic species; therefore, alterations in species composition can induce important changes in toxin occurrence and concentrations. The mixture of different cyanotoxins in a bloom is more probable than a single one. Moreover, the production of toxins alters among the strains of the same species and is influenced by environmental factors. Thus, it is not possible to predict the composition of toxins in cyanobacterial blooms based on species abundance (reviewed in Huisman et al., 2018). Many studies evaluated the toxic effects of whole cyanobacterial extracts from the environment since the studies of cyanotoxin mixtures with the known composition are rare. However, there are indications that combined effects might exceed the additivity concept (Martin, Stallrich, & Bereman, 2019).

The number of publications reporting simultaneous occurrence of MCLR and CYN in cyanobacterial blooms (Brient, Lengronne, Bormans, & Fastner, 2009; Jančula, Straková, Sadílek, Maršálek, & Babica, 2014; Oehrle, Southwell, & Westrick, 2010), as well as co-occurrence of their producers (Bláhová et al., 2009; Kokociński et al., 2009; Miller & McMahon, 2011) is arising. Therefore, the risk of co-exposure to both cyanotoxins is highly plausible. They are both hepatotoxins inducing DNA damage (Buratti et al., 2017; Žegura, Štraser, & Filipič, 2011), while the underlying molecular mechanisms are not clear, which makes the usage of computational modelling for assessment of their combined effects unreliable. Recently discovered cyanotoxins like microginins with only scarce toxicological profiles can also be present in the same bloom affecting the final risk of toxic consequences. Thus, there is a colossal need for identification of bloom composition and research on their individual as well as combined toxicity.

There are only few studies addressing interactions between cyanotoxins with other chemicals in coexposure and they differ markedly. Combined exposure to cyanobacteria, lead and immunological challenge has been studied as an oxidative stress inducer in birds (Paskova et al., 2011). Co-exposure to cyanobacterial extracts has been shown to enhance biomarker affiliated with tumour promotion process of known carcinogens *in vitro*, while pure CYN and MCLR did not induce any change (Kateřina Nováková, Bláha, & Babica, 2012). Another group reported a compound named CAEP, which in co-exposure attenuated the toxicity of microcystin-LR in OATP1B-expressing cells (Komatsu et al., 2013). There was also a study on combined genotoxic effects of MCLR and a drinking-water disinfection by-product 3-chloro-4-dichloro-methyl-5-hydroxy-2(5H)-furanone describing enhanced mutagenicity and oxidative stress-induced DNA damage to an extent greater than those expected for additivity (Wang et al., 2013).

1.4.3 Combined Exposure to Bisphenols

Since the expansion of reports evidencing the adverse effects of BPA and the following restrictions, the production of structural analogues increased. In some products, they are the replacements of BPA but for their specific properties, they are also used in other applications. Different bisphenols have been simultaneously detected in numerous environmental as well as human blood and urine samples. The concentration of an individual congener depends on its applications and the time since it has been introduced to the industry. Together with the still-increasing production of BPA and its analogues, the probability of combined exposure is ascending. The products containing bisphenols are associated with everyday life in modern human civilization, thus combined exposure is inevitable. A recent study on endocrine-disrupting chemicals, including bisphenols, found in eutrophic lake water showed higher estrogenic potency of the environmental extract than predicted from individual estrogenic potencies of detected chemicals (Jia et al., 2019).

1.4.4 Cyanotoxins and Bisphenols in Combined Exposure

Recently, the importance of studies on BPA co-exposure with other chemicals and environmental stressors that appear together in real-life situations has been emphasized for assessment of combined effects on human health (Sonavane & Gassman, 2019). The amount of plastic waste released into the environment is enormous. According to estimations, it will exceed 12.000 million metric tons by 2050 (Geyer, Jambeck, & Law, 2017). Its degradation is causing the constituents of plastic to become ubiquitously present pollutants of water environments (Yamazaki et al., 2015). Freshwater lakes function as assembly points for various pollutants leaching from the catchment area and resulting in a mixture of nutrients and waste chemicals. The consequential eutrophic conditions support blooming of cyanobacteria and potential production of cyanotoxins, while at the same time, chemical pollution remains leading to inevitable co-exposure. A recent study suggested that cyanobacterial blooms could act as a sink for organic pollutants including bisphenols via cellular uptake and biomagnification through phytoplankton (Jia et al., 2019).

1.5 *In vitro* Models in Genetic Toxicology

Testing of chemicals and products for potentially harmful side effects is crucial for human health and environment protection. Current legislation dictates that genotoxicity testing is obligatory for all new chemicals and products such as drugs, cosmetics, food and feed additives, pesticides etc. According to international regulations and guidelines, a battery of *in vitro* tests with bacteria and mammalian cells is required as the first step; however, in the cases of positive results, further follow-up *in vivo* experiments with laboratory rodents are needed to assess the human health risk, which raise ethical concerns. In the last decades, the initiative for the reduction of animal testing has been transferred into international legislation (Directive on the protection of animals used for scientific purposes (2010/63) (EU, 2010)). However, aiming to fulfil the requirements of the European chemical safety legislation REACH program, there is an increasing need for toxicity testing, which enhances the problem of reduction of laboratory animals. The main strategy to improve the safety assessment of chemicals and to obtain less false positive results proposed

by the European Union Reference Laboratory for Alternatives to Animal Testing is to enhance the performance of the *in vitro* testing battery and to develop improved *in vitro* experimental models so that fewer *in vivo* follow-up studies are necessary (Corvi & Madia, 2017).

The development of alternative three-dimensional (3D) cell models represent a very promising tool in achieving physiologically more relevant *in vitro* models to reduce the use of animals in toxicity testing. Compared to the traditional two dimensional (2D) cell cultures, cell line-derived hepatospheres more closely represent *in vivo* cellular functions due to their improved cell-cell and cell-matrix interactions, resulting in higher cell differentiation as well as cell morphology and cytoskeleton architecture that more closely resemble the *in vivo* situation (Fey & Wrzesinski, 2012; Godoy et al., 2013; Y. Zhang et al., 2016). Primary human cells are considered to be the best approximation to live human tissue situation. However, their source is limited, the donor-to-donor variability is high, they are hard to maintain and they do not proliferate in laboratory conditions, which limit their use in routine toxicity testing. Spheroids constructed from immortal human hepatocytes appear equally useful for the determination of cytotoxic effects *in vitro* as primary human hepatocytes, while the classical 2D culture techniques show significantly lower correlation (Fey & Wrzesinski, 2012). Another advantage of 3D cell models is that they allow prolonged exposures due to their higher stability as they retain cell viability, morphology, and functionality for several weeks (Bell et al., 2016). Misleading toxicology data from *in vitro* testing due to false-positive results cause either a ban of potentially promising chemicals or unnecessary follow-up animal testing. Therefore, the development and improvement of *in vitro* systems for more reliable toxicological data as well as their application are crucial future steps in pursuing the two goals – the reduction of animal testing and efficient risk assessment.

1.6 Research Aims and Hypothesis of this Thesis

The overall goal of (genetic)toxicology is to support appropriate risk assessment by providing toxicological data – by identification of potentially harmful chemicals, testing their toxic effects and elucidating the underlying mechanisms. Additionally, in order to better approximate the theoretical estimations to the real-life situation of human and environmental health threat, the questions of interactions and co-exposure must be addressed. Moreover, development of new *in vitro* methods and experimental models in toxicology is of great importance in the movement towards the reduction of animal testing and improvement of the reliability of the results.

Microginins and L-BMAA are recently identified cyanobacterial metabolites for which the mutagenic and genotoxic potential has been completely unknown at the time of our research. After their identification, their prevalence was intensively studied, which revealed their widespread occurrence and high probability of human exposure. Missing data on their adverse effects preclude the risk assessment and safety measures. Mutagenic potential in bacteria is one of the basic characteristics required for safety valuation of chemicals. Therefore, **the first aim** of this thesis was to provide the data on mutagenicity and genotoxicity of L-BMAA with bacterial *Salmonella*/microsomal reverse mutation assay (Ames test) performed according to OECD guidelines and SOS/umuC assay performed according to standard ISO/CD 13829.

Most of the analysed cyanotoxins, including microcystins, exhibit no mutagenic or genotoxic potential in bacterial models but they cause DNA damage in eukaryotic cells (Žegura, Štraser, et al., 2011). Microginins are a group of cyanotoxins consisting of about 40 congeners which are structurally very similar to microcystins. Since the data of their toxicity is very limited, **the second aim** of the thesis was to test the genotoxic potential of four different microginins, which were identified in the cyanobacteria extract prepared from the Lake Balaton bloom sample (Hungary) and the whole extract representing the naturally occurring mixture of microginins. The induction of DNA damage was tested in a human hepatocellular carcinoma cell line HepG2 at the level of single strand breaks with alkaline version of comet assay, double strand breaks with γ H2AX assay, and chromosomal instability with cytokinesis-block micronucleus assay. To cover immediate and prolonged effects, several exposure times were included.

Using up-to-date analytical techniques, recent studies are more and more frequently confirming the mixture of cyanotoxins present in natural blooms. There are data of genotoxic potential of individual most researched toxins while their possible interactions were not investigated at the time of our research. Therefore, **the third aim** of this thesis was to elucidate the combined genotoxic effects of microcystin-LR and cylindrospermopsin, which can simultaneously be present in several freshwater lakes, and to compare the obtained effects to those of single compounds. DNA damage was evaluated with comet assay and cytokinesis-block micronucleus assay after short and prolonged treatment time. Additionally, the changes in the expression of genes involved in the metabolism of xenobiotics, early response signalling and DNA damage response were analysed on the transcriptomic level with the qPCR method.

The composition of chemical mixtures to which humans are exposed is influenced by natural as well as anthropogenic pollutants. In the time of massive use of plastics, the production, leakage and degradation of plastic waste is an important source of bisphenols in the environment and human food (Usman & Ahmad, 2016). Increased use of BPA analogues, which are not well characterized in terms of (geno)toxicity, led us to provide data on individual and combined genotoxic potential of BPA and its most abundant analogues which are simultaneously detected in the environment and human body samples

as **the fourth aim** of this thesis. To explore the genotoxic activity and mechanisms of action, the studies were performed at concentrations that did not significantly reduce cell viability as well as at concentrations recently reported in the environment, which are more relevant for human exposure. The genotoxic activity was determined using γ H2AX assay that detects the induction of DNA double strand breaks and qPCR technique was applied to analyse the alterations in the amount of transcripts of selected genes involved in the metabolism of xenobiotics or response to oxidative stress and DNA damage.

From the existing literature it is evident that combined exposure to chemicals from various sources and legislative frameworks is almost completely missing. Since the scenario of simultaneous exposure to bisphenols and cyanotoxins is highly plausible in the cases of eutrophic water bodies, **the fifth aim** of this thesis was to investigate the potential (geno)toxic effects of combined exposure to the most abundant bisphenols and cyanobacterial toxin cylindrospermopsin, which all cause DNA damage by itself. Also for this study, we used γ H2AX assay to detect the induction of DNA double strand breaks and qPCR technique to analyse the alterations in the amount of transcripts of selected genes involved in the metabolism of xenobiotics or response to oxidative stress and DNA damage.

To improve the quality and reliability of *in vitro* toxicological results and reduce the number of experimental animals, the use of new models that better represent the *in vivo* physiological processes is crucial. Pro-genotoxins, among which also cyanotoxin cylindrospermopsin, are chemicals that exhibit their genotoxic effect only after being modified in metabolic processes. Thus, it is of great importance that *in vitro* cell models mimic the expression and inducibility of metabolic enzymes as much as possible to liver organ *in vivo*. Therefore, **the sixth aim** of the thesis was to investigate the (geno)toxic effects of cylindrospermopsin in 3D spheroids obtained from HepG2 cells – *in vitro* model that was shown to better express the genes encoding enzymes involved in the metabolism of xenobiotics compared to the HepG2 2D cell model (Štampar, Tomc, Filipič, & Žegura, 2019). At the same time, it was the first application of the newly established method on a 3D cell model. Additionally, a broad list of genes involved in xenobiotic metabolism, DNA damage response, apoptosis and oxidative stress was studied for the alterations in their transcriptomes to elucidate the potential mechanisms of CYN biological activity.

To achieve the main aims of the thesis, the following hypotheses are proposed in the doctoral dissertation:

1. Cyanobacterial extracts usually exert higher genotoxic potential than expected from the genotoxic potential of single cyanobacterial toxins. Five different microginin congeners were identified in the examined cyanobacterial extract for which genotoxicity data is not available. We hypothesize that:

- a) Microginins are genotoxic and contribute to the genotoxicity of the cyanobacterial extract.
- b) The cyanobacterial extract containing a mixture of microginins exhibits stronger genotoxic potential than individual pure microginins.

2. MCLR and CYN are both hepatotoxic and genotoxic, but their mechanisms of toxicity differ. We hypothesize that:

- a) Combined genotoxic effects MCLR and CYN in HepG2 cells are more pronounced than the effects of a single toxin and the analyses of the transcriptomic profiles of exposure

to single cyanotoxins and combination will enable identification of the mechanism of their interactions.

3. Recently, BPA analogues have been shown to exhibit similar or even stronger toxic effects than BPA. We hypothesize that:

a) BPA and its analogues BPS, BPF and BPAF have different potentials to induce DNA strand break and the effects of combined exposure are more pronounced than the effects expected based on the potential of single compounds.

b) The changes at the transcriptional level present the most sensitive marker to observe the adverse effects of combined exposure to BPA and its analogues at environmentally relevant concentrations.

4. CYN and bisphenols are expected to be simultaneously present in many areas of polluted aquatic environment. We hypothesize that:

Combined exposure to CYN and bisphenols induces DNA damaging effects that are more pronounced than induced by exposure to individual compounds and the transcriptomic analyses will unravel the pathways involved in the interactions of the studied pollutants.

5. The 3D cell models exhibit higher physiological relevance compared to 2D cell models and better express the enzymes involved in the metabolism of xenobiotics. We hypothesize that:

a) CYN negatively influences the viability of cells and causes genotoxic effects in HepG2 spheroids.

b) The transcriptomic study will elucidate the involvement of phase I and phase II enzymes in the metabolism of CYN.

c) 3D models are suitable for the evaluation of (geno)toxic effects *in vitro*.

1.7 Publications Included and Candidate' s Contributions

The first manuscript *‘Assessment of the mutagenic and genotoxic activity of cyanobacterial toxin beta-N-methyl-amino-L-alanine in Salmonella typhimurium’* provides the data on L-BMAA bacterial mutagenicity and genotoxicity. It is the first published study of L-BMAA genotoxic activity and represents important information for the preparation of appropriate risk assessment documentation. The PhD candidate is a co-author of this publication. Together with the other authors, she designed and performed the experiments for testing bacterial mutagenicity with the Ames tests and analysed and interpreted the data.

For the second manuscript *‘The cyanobacterial oligopeptides microginins induce DNA damage in the human hepatocellular carcinoma (HepG2) cell line’* the preparation and characterization of the environmental sample was done by Hungarian co-authors, while the testing of cytotoxicity and genotoxicity of four microginins and the cyanobacterial extract was performed at NIB. This paper is the first published evidence of genotoxic potential of microginins. The PhD candidate is the co-author of this publication. She introduced the flow cytometry methods to the first author, collaborated in experimental

design and supported the first author at experimental work as well as analysis and interpretation of the data.

The third manuscript '*Genotoxic potential of the binary mixture of cyanotoxins microcystin-LR and cylindrospermopsin*' reports the combined effects of two common cyanotoxins on DNA single strand breaks formation and genomic instability in HepG2 cells. Furthermore, the potential interactions were studied at transcriptomic level via the changes in the expression of biomarker genes. The PhD candidate is the first author of this publication. She designed and performed all the *in vitro* experiments using well-established techniques – MTS assay, alkaline comet assay, cytokinesis-block micronucleus assay and qPCR. She also analysed and interpreted the data and prepared the manuscript.

In **the fourth manuscript** '*Genotoxic activity of bisphenol A and its analogues bisphenol S, bisphenol F and bisphenol AF and their mixtures in human hepatocellular carcinoma (HepG2) cells*' cytotoxic and genotoxic potential of individual bisphenols is assessed after two exposure times. Moreover, the combined effects of bisphenol A and the three common analogues are studied at concentrations relevant for human exposure. The PhD candidate is the first author of this publication. She designed and prepared all the experiments and supervised the work of MSc student, a co-author who performed MTT assays and prepared the cells for flow cytometry. The candidate performed the qPCR experiments, analysed and interpreted all the data, and drafted the manuscript.

The fifth manuscript '*Plastics in Cyanobacterial Blooms - Genotoxic Effects of Binary Mixtures of Cylindrospermopsin and Bisphenols in HepG2 Cells*' describes genotoxic effects induced by co-exposure to the emerging cyanotoxin cylindrospermopsin and four most abundant bisphenols in HepG2 cells after 24 and 72h of exposure. The cytotoxicity was evaluated with the MTS assay, genotoxicity with the γ H2AX assay and deregulation of selected genes was assessed using q-PCR. The PhD candidate designed and performed all the experiments as well as analysed the results and prepared them for the manuscript.

The sixth manuscript '*Application of advanced HepG2 3D cell model for studying genotoxicity of cylindrospermopsin*' presents the influence of exposure to cylindrospermopsin on HepG2 cells in 3D spheroids. Cell viability was evaluated with MTS assay and life-dead staining and imaging of whole spheroids. Proliferation, cell cycle progression, γ H2AX formation was simultaneously studied using flow cytometry, while gene expression was appraised by qPCR on microarrays. The PhD candidate designed the study, prepared the spheroids and performed all the *in vitro* experiments except confocal microscopy. She also collaborated in design and validation of the applied flow cytometry method for simultaneous analysis of proliferation, cell cycle progression, γ H2AX formation. The candidate analysed and interpreted the obtained results, and prepared the manuscript.

Chapter 2

Scientific Publications

2.1 Assessment of the Mutagenic and Genotoxic Activity of Cyanobacterial Toxin Beta-N-methyl-amino-L-alanine in *Salmonella Typhimurium*

Matjaž Novak, Klara Hercog, Bojana Žegura

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Assessment of the mutagenic and genotoxic activity of cyanobacterial toxin beta-N-methyl-amino-L-alanine in *Salmonella typhimurium*

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ABSTRACT

A neurotoxin β-N-methylamino-L-alanine (L-BMAA) is a non-protein amino acid produced by most cyanobacteria ubiquitously present in aquatic and terrestrial environments. Due to its global presence in surface waters, a widespread human exposure is possible and therefore this toxin represents a health risk for humans and animals. L-BMAA has been linked to the development of a variety of neurodegenerative diseases. Its neurotoxic activity has been extensively studied, while nothing is known on its genotoxic properties. In the present study we evaluated for the first time L-BMAA mutagenic potential using Ames assay on several *Salmonella typhimurium* strains (TA97a, TA98, TA100, TA102 and TA1535). The results showed that the toxin (up to 0.9 mg/plate) did not induce mutations without or with S9 metabolic activation. Its genotoxic activity was further studied with the SOS/umuC assay on *S. typhimurium* TA1535/pSK1002 and the results showed that it was not cytotoxic nor genotoxic for bacteria. The present study represents the first evidence that L-BMAA is not mutagenic nor genotoxic for bacteria even at concentrations much higher than those typically found in the environment. However, as most of the cyanobacterial toxins are not bacterial mutagens it is very important to further elucidate its genotoxic activity in eukaryotic cells.

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1. Introduction

β-N-methylamino-L-alanine (L-BMAA), a non-protein amino acid, has been described as a potent neurotoxin (Cox et al., 2003, 2005; Banack et al., 2007; Lobner et al., 2007; Johnson et al., 2008; Metcalf et al., 2008; Karlsson et al., 2009b; Nunn, 2009; Jonasson et al., 2010). It has been implicated in the etiology of human diseases as a significant environmental factor leading to neurologic damage in the brain and central nervous system of humans and animals, potentially contributing to one of the several neurodegenerative disorders of aging such as Alzheimer's disease (Cox et al., 2003; Pablo et al., 2009), amyotrophic lateral sclerosis (Bradley and Mash, 2009; Caller et al., 2009; Cox et al., 2009; Banack et al., 2015), and the amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS-PDC) (Borenstein et al., 2007; Holtkamp, 2012). The neurotoxicity of L-BMAA and associated mechanisms of action have been extensively studied in

neurons and various neural cell lines *in vitro* (Chiu et al., 2012; Lee and McGeer, 2012; Dunlop et al., 2013; Munoz-Saez et al., 2013) as well as animal test systems (Karamyan and Speth, 2008; Karlsson et al., 2009b, 2014b; de Munck et al., 2015; Munoz-Saez et al., 2015; Tian et al., 2016).

L-BMAA has been originally identified in seeds from the false sago palm (*Cycas micronesica* Hill.) (Vega and Bell, 1967) and has been discovered in the traditional diets of the Chamorro people of Guam (in Western Pacific Ocean); such as flour made from seeds of cycads as well as in animals feeding on cycad seeds (e.g. flying foxes, deer, and feral pigs) (Spencer et al., 1987, 2010). Later on, Cox et al. (Cox et al., 2005) have discovered that L-BMAA is actually produced by cyanobacteria of *Nostoc* genus that live symbiotically in roots of cycad trees. In recent years L-BMAA has gained increased attention as several studies have demonstrated that it is produced by most of the globally widespread cyanobacterial species (Cox et al., 2005; Esterhuizen and Downing, 2008). Moreover, it has been detected also in planktonic diatoms (Jiang et al., 2014a; Reveillon et al., 2016) and dinoflagellates (Lage et al., 2014). As cyanobacteria are found ubiquitously in water bodies they are considered a growing problem in aquatic ecosystems worldwide

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due to the variety of secondary metabolites including toxins they produce.

Although L-BMAA is water soluble its bioaccumulation in higher aquatic organisms such as shellfish and fish (Banack and Cox, 2003; Cox et al., 2003; Brand et al., 2010; Jonasson et al., 2010; Jiang et al., 2014b; Reveillon et al., 2015; Reveillon et al., 2016) has been documented and thus it can biomagnify in food web. Eventually it can enter the human diet, and thereby lead to human exposure and consequently trigger neurodegenerative diseases. Other possible routes of exposure are direct contact during recreational activities, consumption of contaminated water, and even inhalation of aerosols and dusts (Banack et al., 2010; Stommel et al., 2013). Hence, the presence of L-BMAA in the environment may represent a public health concern. Furthermore, in mice model BMAA can be maternally transferred, either directly (Karlsson et al., 2009a) or through milk during lactation (Andersson et al., 2013), which means that it is likely that exposure to L-BMAA can have transgenerational effects.

Since the discovery of L-BMAA it has been a challenge to clarify its mechanism of action and the link with the pathology of neurodegenerative disorders. Recently, it has been demonstrated that free L-BMAA can bind to the amino acid tRNA synthetase and can be mistakenly incorporated into the protein structure in place of L-serine during *de novo* synthesis (Dunlop et al., 2013). These findings support the proposed mechanism of L-BMAA bioaccumulation (Murch et al., 2004) despite its non-lipophilic and non-protein nature. Moreover, L-BMAA incorporated in proteins can serve as an endogenous toxin reservoir in humans or animals, from which L-BMAA is slowly released back to free form through acid hydrolysis (Murch et al., 2004) and proteolytic cleavage (Dunlop et al., 2013) even for years or decades after its consumption. This fact may explain the long latency period presented by neurodegenerative disorders like ALS-PDC presumably caused by a chronic L-BMAA exposure. Thus L-BMAA by misincorporation into the proteins may represent an environmental factor that can lead to alterations in protein structures, may trigger protein misfolding, misfolding and/or aggregation (Dunlop et al., 2013; Karlsson et al., 2014a) and induce apoptosis (Dunlop et al., 2013; Chiu et al., 2015; Tian et al., 2016) and endoplasmic reticulum stress (Okle et al., 2013) that may lead to neurodegenerative diseases (Walker and Atkin, 2011; Dunlop et al., 2013; Karlsson et al., 2014a; Tian et al., 2016). In addition L-BMAA is structurally very similar to glutamate (Cucchiaroni et al., 2010). It has been shown that the main mechanism by which L-BMAA causes neurotoxicity is by acting as an excitotoxin on glutamate receptors (L-BMAA is mixed glutamate receptor agonist) such as N-methyl-D-aspartic acid (NMDA) and calcium dependent α -amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptors (Chiu et al., 2012), which is believed to induce activation of calcium-dependent enzyme pathways (Bae et al., 2013) and oxidative stress by depletion of glutathione (Chiu et al., 2012; Engskog et al., 2013; Okle et al., 2013) as well as mitochondrial dysfunction (de Munck et al., 2013). Nevertheless, the exact impact of non-protein amino acid L-BMAA on protein structure and function remains unknown.

The current state of knowledge recognizes that L-BMAA is neurotoxic and most of the research is focused on its potential neurodegenerative effects, while no research has addressed the question whether L-BMAA can induce damage to DNA. Therefore the aim of our study was to investigate the mutagenic and genotoxic potential of this small non-protein amino acid. The mutagenic activity was evaluated with the *Salmonella*/microsomal reverse mutation assay on five different strains of *Salmonella typhimurium* (TA97a, TA98, TA100, TA102 and TA1535) in the absence and presence of metabolic activation. Its genotoxic activity was further studied with the SOS/umuC assay on *S. typhimurium* TA1535/

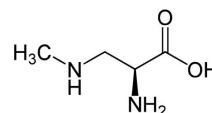


Fig. 1. Structural formula of β -N-methylamino-L-alanine.

SK1002 in the absence and presence of metabolic activation.

2. Materials and methods

2.1. Chemicals

Beta-N-methyl-amino-L-alanine (Fig. 1) (L-BMAA; C4H10N2O2; 154.6 g/mol; CAS number 15920-93-1) was obtained from Sigma-Aldrich (St Louis, MO, USA). A 9 mg/mL (58.21 μ M) stock solution of L-BMAA was prepared in sterile bidistilled water, aliquoted and stored at -20°C . Lyophilized Aroclor 1254 induced male rat liver post-mitochondrial fraction (S9) was obtained from Moltox (Boone, USA). NADPH-Na2 was from Merck (Darmstadt, Germany) and G-6-P was from Sigma-Aldrich (St Louis, MO, USA). All other chemical reagents were of the purest grade available and all solutions were made using Milli-Q water.

2.2. Determination of mutagenicity with *Salmonella*/microsomal reverse mutation assay (Ames) assay

Mutagenicity of L-BMAA was tested using the plate incorporation assay with *S. typhimurium* strains TA97a, TA98, TA100, TA102 and TA1535 without and with external metabolic system (Maron and Ames, 1983) according to the Organisation for Economic Cooperation and Development guideline 471 (OECD TG 471, 1997). L-BMAA is soluble at the concentration of 9 mg/mL; therefore, this concentration was used as the highest tested concentration. The bacterial strains were obtained from Trinova Biochem GmbH (Giessen, Germany). Strains TA97a and TA98 were used for detection of frame-shift mutation and strains TA100, TA102 and TA1535 for base pair substitution. Briefly, 100 μ L of L-BMAA (0.11, 0.33, 1, 3 and 9 mg/mL corresponding to the final concentration of 0.011, 0.033, 0.1, 0.3 and 0.9 mg/plate, respectively), 100 μ L bacterial culture of *S. typhimurium* (overnight culture at 37°C) and 500 μ L of phosphate buffer (for assays without metabolic activation) or 4% S9 mix (for assays with metabolic activation) were added to 2 mL of molten top agar containing a limited amount of histidine/biotin. Afterwards these were mixed gently and poured onto minimal agar plates. The plates were incubated for 48 h (TA97a, TA100, TA102 and TA1535) and 72 h (TA98) at 37°C . Subsequently the numbers of spontaneous and L-BMAA induced His⁺ revertants were counted. The plates were checked for possible toxic effect of L-BMAA. Three plates were used per experimental point. The number of spontaneous and induced revertant colonies was counted and the Induction Factor (IF) over the control was calculated. IF was determined as the ratio of the mean value from induced revertants for each tested concentration divided by the mean value from spontaneous revertants (control). IF ≥ 2 was taken as the threshold at which the sample was considered as mutagenic. Statistical significance between exposed groups and the control was determined by One-way analysis of variance (ANOVA) and Dunnett's Multiple Comparison Test using GraphPad Prism 6 (GraphPadSoftware). * $p < 0.05$ was considered as statistically significant; however it has to be pointed out that the statistical significance is not the determining factor for positive result. In addition to IF (Table 1; in

Table 1
Mutagenic activity of beta-N-methyl-amino-L-alanine [L-BMAA] in *Salmonella typhimurium* TA97a, TA98, TA100, TA102 and TA1535 without and with S9 (41) metabolic activation determined with the Salmonella Mutagenicity Test (Ames).

Average number of revertant colonies (Mean ± SD) and (mutagenicity index)										
µg/plate	TA97a		TA98		TA100		TA102		TA1535	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
NC	172.67 ± 8.08	151.67 ± 9.07	32.33 ± 7.57	35.33 ± 2.52	104.67 ± 8.96	111.33 ± 16.01	425.67 ± 28.94	465.33 ± 30.99	12.00 ± 4.58	9.67 ± 1.16
PC	512.67 ± 10.26 ^a	668.33 ± 52.56 ^a	255.33 ± 15.04 ^b	260.67 ± 18.04 ^c	399.67 ± 37.29 ^b	854.33 ± 35.02 ^c	2035.00 ± 100.42 ^c	2120.00 ± 85.43 ^c	161.67 ± 8.33 ^d	46.33 ± 11.68 ^e
11	(2.97) ^f	(4.41) ^f	(7.90) ^f	(7.38) ^f	(3.82) ^f	(7.67) ^f	(4.78) ^f	(4.56) ^f	(13.47) ^f	(4.79) ^f
	162.00 ± 4.36	171.00 ± 7.81	27.33 ± 3.51	45.67 ± 4.93	95.00 ± 9.64	106.33 ± 6.81	414.00 ± 16.64	457.33 ± 31.98	14.33 ± 2.082	8.67 ± 2.08
	(0.94)	(1.13)	(0.85)	(1.29)	(0.95)	(0.98)	(0.94)	(0.98)	(1.19)	(0.90)
33	158.00 ± 13.89	161.00 ± 18.19	30.33 ± 5.03	40.33 ± 1.53	93.67 ± 19.40	111.67 ± 10.21	415.67 ± 23.54	454.33 ± 23.44	10.67 ± 3.06	9.33 ± 0.58
	(0.92)	(1.06)	(0.94)	(1.142)	(0.90)	(1.00)	(0.98)	(0.98)	(0.89)	(0.97)
100	158.67 ± 11.93	153.67 ± 13.05	37.00 ± 2.65	40.33 ± 9.29	106.00 ± 9.54	104.00 ± 17.06	416.67 ± 14.19	453.33 ± 10.79	13.33 ± 2.08	11.33 ± 1.53
	(0.92)	(1.01)	(1.14)	(1.142)	(1.01)	(0.93)	(0.98)	(0.97)	(1.11)	(1.17)
300	162.00 ± 6.25	156.33 ± 7.02	28.00 ± 8.72	38.33 ± 4.16	92.67 ± 10.60	102.00 ± 11.00	425.00 ± 25.71	460.33 ± 14.74	15.67 ± 4.04	11.33 ± 4.04
	(0.94)	(1.03)	(0.87)	(1.09)	(0.89)	(0.92)	(1)	(0.99)	(1.31)	(1.17)
900	166.00 ± 6.08	139.67 ± 1.53	25.33 ± 6.51	30.00 ± 1.73	102.67 ± 12.06	101.67 ± 13.65	423.00 ± 19.16	441.00 ± 15.72	18.33 ± 5.51	14.67 ± 3.06
	(0.96)	(0.92)	(0.78)	(0.85)	(0.98)	(0.91)	(0.99)	(0.95)	(1.528)	(1.52)

NC, negative control; PC, positive control; p < 0.05 (*).
^a 4-Nitroquinoline oxide (4-NQO); 0.25 µg/plate).
^b Sodium azide (NaN₃; 0.25 µg/plate).
^c Mitomycin C 0.5 µg/plate.
^d Sodium azide (NaN₃; 0.125 µg/plate).
^e Benzo(a)pyrene (BaP; 5 µg/plate).
^f Benzo(a)pyrene (BaP; 2.5 µg/plate).
^g 2-aminoanthracene (2-AA; 10 µg/plate).
^h 2-aminoanthracene (2-AA; 5 µg/plate).

brackets) raw data are presented as the number of revertants/plate along with mean values and standard deviation (STD).

The positive controls of direct mutagenicity (–S9) were 2.5 µg/mL 4-nitroquinoline-N-oxide (4-NQNO) for TA97a and TA98, 0.5 µg/mL mitomycin C for TA102, and 2.5 µg/mL and 1.25 µg/mL sodium azide (NaN₃) for TA100 and TA1535, respectively. The positive control of indirect mutagenicity (+S9) was 50 µg/mL benzo(a)pyrene (BaP) for TA97a and 25 µg/mL for TA98, TA100, TA102 and TA1535.

2.3. SOS/umuC assay

The SOS/umuC assay was performed according to Reifferscheid et al. (Reifferscheid et al., 1991), with modifications described in the ISO standard ISO/CD 13829 (ISO/CD, 2000). L-BMAA was tested at concentrations of 0.32, 1.6, 8, 40, 200 and 1000 µg/mL. Briefly, the overnight bacterial culture was diluted 10 times with fresh TGA medium and incubated at 37 °C for 1.5 h with shaking until the bacteria reached the exponential growth phase. The test was performed on a microtiter plate in triplicate. The incubation mixture consisted of 180 µL toxin dilution, 20 µL 10x TGA and 70 µL of bacterial culture or when tested in the presence of metabolic activation it consisted of 180 µL toxin dilution, 20 µL 10x TGA with cofactors and 70 µL of S9 bacterial culture mixture prepared as described in the ISO standards. The bacteria were incubated at 37 °C for 2 h with shaking. Subsequently the incubation mixture was diluted with fresh TGA medium (1:10) and incubated for further 2 h. The bacterial growth rate was determined by measuring absorbance at 600 nm β-Galactosidase activity was determined using ortho-Nitrophenyl-β-galactoside (ONPG) as a substrate after 30 min of incubation at 25 °C with shaking. Absorption was measured at 420 nm using a reference solution without bacteria. The bacterial growth rate was calculated using the following formula: $G = [(sample\ OD600 - blank\ OD600)/(control\ OD600 - blank\ OD600)]$. A growth ratio less than 0.75 that represents 25% inhibition of biomass growth was considered to be indicative of samples being cytotoxic.

β-Galactosidase activity in relative units was calculated following the formula: $U = [(sample\ OD420 - blank\ OD420)/(sample\ OD600 - blank\ OD600)]$ and induction ratio (IR) by the formula: $(1/G) * [(sample\ OD420 - blank\ OD420)/(control\ OD420 - blank\ OD420)]$. An induction ratio ≥ 2 was taken as the threshold at which the sample was considered as genotoxic (ISO/CD, 2000). The standard deviation (SD) was calculated by error multiplication according to (Gellert et al., 1968). Distilled water was used as a negative control, while 4-nitroquinoline-N-oxide (4-NQNO; 2.5 µg/mL) was used as a positive control without and aflatoxin B1 (AFB1; 5 µg/mL) with metabolic activation.

3. Results

3.1. Mutagenicity of L-BMAA in *S. typhimurium*

The mutagenicity of L-BMAA was determined using the *Salmonella*/microsomal reverse mutation assay with five *S. typhimurium* strains: TA97a, TA98, TA100, TA102 and TA1535. The obtained results showed that at concentrations tested (up to 0.9 mg/plate) L-BMAA did not increase the number of revertants in any of the bacterial strain used, either in the absence (–S9) or presence (+S9) of metabolic activation system (Table 1). The influence of L-BMAA on the bacterial growth was evaluated microscopically by determining the background lawn and no signs of cytotoxicity or toxin precipitation were observed in any of the tested bacterial strain.

3.2. Cytotoxicity and genotoxicity of L-BMAA in *S. typhimurium*

The cytotoxic and genotoxic activities of L-BMAA were assessed using the SOS/umuC assay with *S. typhimurium* TA1535/pSK1002 in the absence (–S9) and presence (+S9) of metabolic activation (Table 2). Within the tested concentration range (0.32, 1.6, 8, 40, 200 and 1000 µg/mL), L-BMAA did not affect the growth of bacteria. The β-galactosidase activity was not increased showing no genotoxic activity of L-BMAA at experimental conditions used in the study.

4. Discussion

Cyanobacteria are ubiquitously present in terrestrial and aquatic environments worldwide. Due to the increasing appearance of massive cyanobacterial blooms as a consequence of water eutrophication and global warming they are of growing environment and human health concern, especially as they produce a variety of toxins, ranging from hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and irritant toxins (lipopolysaccharides) (Zegura et al., 2011). Nowadays it is well known that most of the cyanobacteria produce also neurotoxic non-protein amino acid L-BMAA (Cox et al., 2005; Esterhuizen and Downing, 2008). Due to the global presence of L-BMAA in the aquatic environment a widespread human exposure to this neurotoxin is possible. Most of the published studies are focused on its neurodegeneration and neurotoxicity, while in the scientific literature no data on its adverse effects on DNA exist. Therefore, to the best of our knowledge our study is the first report where mutagenic and genotoxic activity of cyanobacterial toxin L-BMAA has been studied with the Ames assay (plate incorporation method) and SOS/umuC assay, respectively.

Salmonella/microsomal reverse mutation assay (also called Ames test), is widely used for initial screening to determine the mutagenic potential of various compounds (McCann and Ames, 1975) including cyanobacterial toxins (Zegura et al., 2011; Sieroslawska, 2013). The Ames test uses several strains of *Salmonella typhimurium* containing a specific mutation in the histidine operon. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. Mutagenicity is determined by comparing the frequency of spontaneous to chemical-induced point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs, which are manifested through increased number of induced revertant colonies (Maron and Ames, 1983). According to the OECD guidelines 471 (OECD TG 471, 1997) all chemicals, if soluble, should be tested at concentrations of 5 mg/plate. Due to the limiting solubility of L-BMAA the highest tested concentrations in the present study was 0.9 mg/plate. Our results showed that L-BMAA at tested concentrations did not increase the number of revertants in any of the bacterial strain used, either in the presence or the absence of the S9 metabolic activation system. Thus, it can be concluded that L-BMAA is not mutagenic for bacteria, even at concentrations much higher than those typically found in the environment.

Cytotoxic and genotoxic activity was further evaluated with the SOS/umuC assay with *S. typhimurium* TA1535/pSK1002. The SOS/umuC system is based on the ability of genotoxins to induce expression of the umuC gene, one of the SOS genes responsible for “error-prone repair” that is involved in mutagenesis (Oda et al., 1985). The tester strain *S. typhimurium* TA1535/pSK1002 is carrying a fused umuC–lacZ gene, which allows the monitoring of umuC expression by measuring β-galactosidase activity. It is a validated test system (ISO13829) for detection of environmental toxicants with genotoxic and carcinogenic properties (Oda et al., 1995; Reifferscheid and Heil, 1996; Hamer et al., 2000). There is a good correlation between Ames and SOS/umuC assay results, with a

Table 2

The cytotoxic and genotoxic activity of beta-N-methyl-amino-L-alanine (L-BMAA) in *Salmonella typhimurium* TA 1535/pSK1002 without (-S9) and with (+S9) metabolic activation determined with the SOS/umuC assay.

Concentration ($\mu\text{g mL}^{-1}$)		-S9		+S9	
		$G^a \pm \text{SD}$	$\text{IR}^b \pm \text{SD}$	$G^a \pm \text{SD}$	$\text{IR}^b \pm \text{SD}$
PC	AFB1	/	/	0.63 ± 0.08	$3.04 \pm 0.62^*$
BMAA	4-NQNO	0.80 ± 0.08	$8.37 \pm 0.45^*$	/	/
	0	1.00 ± 0.06	1.00 ± 0.02	1.00 ± 0.28	1.04 ± 0.19
	0.32	0.99 ± 0.05	0.96 ± 0.09	1.10 ± 0.41	1.13 ± 0.10
	1.6	1.00 ± 0.04	0.95 ± 0.05	1.04 ± 0.37	0.98 ± 0.18
	8	0.98 ± 0.02	0.95 ± 0.12	0.86 ± 0.18	1.57 ± 0.97
	40	0.92 ± 0.02	0.92 ± 0.08	0.78 ± 0.03	0.94 ± 0.19
	200	0.93 ± 0.03	0.95 ± 0.14	0.84 ± 0.34	0.97 ± 0.06
	1000	0.89 ± 0.05	0.94 ± 0.02	0.74 ± 0.16	0.92 ± 0.08

$p < 0.05$ (*).

^a G = bacterial growth rate; $G < 0.75$ is considered as toxic for bacteria.

^b IR = induction ratio; $\text{IR} > 2$ is considered as genotoxic response.

concordance of about 90% (Reifferscheid and Heil, 1996). Our results similarly to Ames assay showed that L-BMAA was not cytotoxic nor genotoxic, either in the presence or the absence of the S9 metabolic activation system. A variety of pure cyanobacterial toxins have been previously tested in the Ames assay [for details see (Zegura et al., 2011)] and the majority of the studies showed negative response in bacterial test systems. For example the most studied cyanobacterial toxin, microcystin-LR, is not mutagenic in bacteria [for details see (Zegura, 2016)] and similar negative results have been reported also for cylindrospermopsin and anatoxin-a (Zegura et al., 2011; Sieroslawski, 2013). These studies indicate that pure cyanotoxins are not mutagenic for bacteria, which is in line with the results of the present study showing no mutagenic activity of L-BMAA in *S. typhimurium*. Despite negative results obtained in bacterial mutagenicity assays, there is a substantial evidence describing that cyanobacterial toxins are mutagenic for eukaryotic cells [for details see (Zegura et al., 2011)]. One of the drawbacks of bacterial assays represents the absence of the metabolic activation system such as cytochrome P450, which is responsible for metabolizing of a large number of chemicals (Guengerich, 2006). Therefore, the addition of exogenous metabolic activation system (S9 fraction of rat liver) is used. However, S9 fraction contains only phase I metabolic enzymes (cytochrome P450), which are not the only enzymes involved in the metabolism of drugs and other xenobiotic chemicals. The enzymes involved in phase II reactions that are also responsible for bioactivation and detoxification of drugs (Guengerich, 2006) are not present in S9 mixture. Until now there are only a few publications on metabolism of cyanobacterial toxins mostly microcystins and cylindrospermopsin (Hudder et al., 2007; Straser et al., 2013; Zhang et al., 2015), while not much information is available concerning the metabolic activation of L-BMAA. Nunn and Ponnusamy (Nunn and Ponnusamy, 2009) have described that in rat liver and kidney but not in brain L-BMAA is metabolised to methylamine. It is known that methylamine in a reaction that is catalysed by semicarbazide-sensitive amine oxidase is metabolised to yield formaldehyde, hydrogen peroxide and ammonium (Yu, 1990). Later on it has been shown that in rodents L-BMAA is taken up by brain tissue and is enzymatically metabolized to compounds with genotoxic properties including the carcinogen formaldehyde (Kisby et al., 1992; Kisby and Spencer, 2011). Moreover hydrogen peroxide formed in the reaction may be degraded by catalase and thereby reactive oxygen species are formed. As a consequence oxidative stress is increased, which eventually can lead to DNA damage. All these facts point out that L-BMAA has the potential to be genotoxic for eukaryotic cells, which has to be further elucidated.

The answer to the question whether the L-BMAA has entered

the bacterial cell of *S. typhimurium* used in the Ames assay can be found in the specific mutation, *rfa* that is present in the strains. The *rfa* mutation causes partial loss of lipopolysaccharide barrier that coats the surface of the bacteria and allows increased cell permeability and greater penetration of chemical mutagens or large chemical molecules into the bacterial cell (Maron and Ames, 1983). Moreover, recently it has been reported that L-BMAA has not induced physiological and oxidative stress in microorganisms such as *Escherichia coli*, *Staphylococcus epidermidis*, *Lactobacillus casei*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Micrococcus luteus* and has not been misincorporated into cellular proteins of bacteria (van Onselen et al., 2015). However, it has been detected in both the free cellular and protein associated fractions of microorganisms included in the study meaning that bacteria have been able to take up the L-BMAA. The authors reported that the protein-associated L-BMAA concentrations were considerably lower than the free cellular BMAA concentrations (van Onselen et al., 2015).

The reported L-BMAA concentrations in the aquatic environment vary widely between studies ranging from ng/g dry weight to low $\mu\text{g/g}$ dry weight range in phytoplankton (Jonasson et al., 2010; Faassen, 2014). Concentrations similar to those for phytoplankton were reported also in animals used for human consumption, such as crabs, oysters, mussels, fish and algae food supplements (Scott et al., 2009; Jonasson et al., 2010; Niedzwiedek et al., 2012; Jiang et al., 2014b; Reveillon et al., 2015; Salomonsson et al., 2015). Considering that L-BMAA biosynthesized by symbiotic or/and free-living cyanobacteria can accumulate in organisms and can biomagnify in the food web, both in free amino acid and protein-bound forms, concentrations in organisms can increase significantly. This suggests that after consumption of food or water contaminated with L-BMAA humans can be exposed to tens or even some hundreds of μg of L-BMAA and therefore this toxin represents concern for human health. In addition although to minor extend also recreational activities, such as swimming, boating and water-skiing, are a potential source of exposure to L-BMAA in water bodies containing cyanobacterial blooms.

5. Conclusions

The present study has shown that L-BMAA is not mutagenic for bacteria; however, there are some indications that in addition to being neurotoxic, L-BMAA has the potential to act as genotoxin. Therefore for proper human risk-assessment further research on eukaryotic test systems is needed to elucidate whether L-BMAA can induce DNA damage especially in non-target cells and to understand possible initial effects and mechanisms involved in the adverse L-BMAA genotoxic effects.

Declaration of interest

The authors report no conflict of interest.

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Transparency document

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2.2 The Cyanobacterial Oligopeptides Microginins Induce DNA Damage in the Human Hepatocellular Carcinoma (HepG2) Cell Line

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The cyanobacterial oligopeptides microginins induce DNA damage in the human hepatocellular carcinoma (HepG2) cell line

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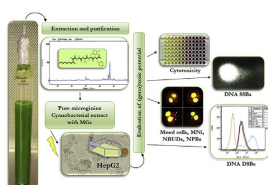
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HIGHLIGHTS

- B-14-01 extract reduced viability and proliferation of HepG2 cells.
- High concentrations of MGs decreased HepG2 cell proliferation.
- B-14-01 extract and MG congeners induced DNA single strand break formation.
- B-14-01 extract and MG congeners did not induce DNA double strand breaks.
- B-14-01 extract, MG GH787 and cyanostatin B induced genomic instability.

GRAPHICAL ABSTRACT



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ABSTRACT

Microginins (MGs) are bioactive metabolites mainly produced by *Microcystis* spp., (Cyanobacteria) commonly found in eutrophic environments. In this study, the cytotoxic and genotoxic activities of four MG congeners (MG FR3, MG GH787, cyanostatin B, MGL 402) and a well characterized cyanobacterial extract B-14-01 containing these metabolites were evaluated in the human hepatocellular carcinoma (HepG2) cell line. The cytotoxicity was measured with the MTT assay, while genotoxicity was studied with the comet, γ H2AX and cytokinesis block (CBMN) micronucleus assays. The viability of cells after 24 h was significantly affected only by the extract, whereas after 72 h a concentration dependent

Abbreviations: ACE, angiotensin-converting enzyme; Ahda, 3-amino-2-hydroxy-decanoic acid; Ahoa, 3-amino-2-hydroxy-octanoic acid; APM, aminopeptidase M; BaP, benzo[a]pyrene; BNC, binucleated cell; CBMN, cytokinesis block micronucleus assay; CYN, cylindrospermopsin; DMSO, dimethyl sulphoxide; DSB, double strand break; LAP, leucine aminopeptidase; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; MCLR, microcystin-LR; MG, microginin; MNI, micronuclei; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBUD, nuclear bud; NDI, nuclear division index; NPB, nuclear bridge; PBS, phosphate buffered saline; ROS, reactive oxygen species; SSB, single strand break.

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decrease in cell proliferation was observed for the extract and tested microginins, with MGL 402 being the most potent and MG FR3 the least potent congener. The extract and all tested congeners induced DNA strand breaks after 4 and 24 h exposure. The most potent was the extract, which induced concentration and time dependent increase in DNA damage at concentrations $\geq 0.01 \mu\text{g mL}^{-1}$. Among microginins the most potent was MGL 402 (increase in DNA strand breaks at $\geq 0.01 \mu\text{g mL}^{-1}$) and MG FR3 was the least potent (increase in DNA strand breaks at $\geq 1 \mu\text{g mL}^{-1}$). However, no induction of DNA double strand breaks was observed after 24 and 72-h exposure to the cyanobacterial extract or MGs. Induction of genomic instability was observed in cells exposed to MG GH787, cyanostatin B and the extract B-14-01. This study is the first to provide the evidence that microginins exert genotoxic activity.

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1. Introduction

Cyanobacteria produce various biologically active secondary metabolites, which can negatively affect the ecosystem and water quality as well as human health (Huisman et al., 2018). These metabolites among others include lipopolysaccharides, neurotoxic anatoxins, saxitoxins and β -N-methylamino-L-alanine, hepatotoxic microcystins and nodularins and/or cytotoxic cylindrospermopsin (Buratti et al., 2017; Huisman et al., 2018; Pearson et al., 2010; Stewart et al., 2006; Violi et al., 2019). The most diverse groups are cyanopeptides with more than 600 metabolites described from various taxa and they are categorised into classes with the conserved structural characteristics (Janssen, 2019). The seven main classes of oligopeptides are aeruginosins, microginins, anabaenopeptins, cyclamides (cyanobactins), cyanopeptolins, microcystins and microviridins. The best known and the most studied are microcystins (MCs) (Agha and Quesada, 2014; Janssen, 2019).

Microginins (MGs) are linear peptides consisting of a decanoic acid derivative, 3-amino-2-hydroxy-decanoic acid (Ahda) and three to five additional amino acids at the C-terminal end (Ishida et al., 1998), though variants with three or four amino acids were also reported (Sano and Kaya, 1997; Stewart et al., 2018). N-methylation can occur at positions 1, 3 and 4, while aliphatic chlorination of Ahda is also possible (Gesner-Apter and Carmeli, 2008; Ishida et al., 2000, 1998; Kodani et al., 1999; Lifshits et al., 2011) (Fig. 1).

Depending on the producer species the analogues are named as microginins, nostoginins, oscillaginins and cyanostatins. Approximately 40 congeners are known, though the number of identified MGs is still increasing (Lodin-Friedman and Carmeli, 2018; Riba et al., 2019; Stewart et al., 2018; Strangman and Wright, 2016). In water blooms, strains of *Microcystis* spp., *Anabaena fertilissima*, and *Woronichinia naegeliiana* (Unger) Elenkin have been isolated as microginin or cyanostatin producers (Bagchi et al., 2016; Bober et al., 2011; Sano et al., 2005; Welker et al., 2006), and *Planktothrix agardhii* as oscillagin producer (Sano and Kaya, 1997), all containing the Ahda moiety. Whereas *Nostoc* species produce nostoginins, with the 3-amino-2-hydroxy-octanoic acid (Ahoa) moiety (Ploutno and Carmeli, 2002). MG congeners produced by *Microcystis aeruginosa* were also reported containing chlorinated or methylated Ahoa moiety (Reshef and Carmeli, 2001; Strangman and Wright, 2016). The production of MGs has been observed worldwide from isolated strains; however, their environmental concentrations up to $2.21 \mu\text{g L}^{-1}$ were reported in eutrophic lakes (Beversdorf et al., 2017; Janssen, 2019).

The biological activity of MGs is not well understood. They are zinc metalloprotease inhibitors and were reported to inhibit angiotensin-converting enzyme (ACE), aminopeptidase M (APM) and leucin aminopeptidase (LAP) (Ishida et al., 2000; Kodani et al., 1999; Lifshits et al., 2011; Lodin-Friedman and Carmeli, 2018; Neumann et al., 1997; Okino et al., 1993; Sano et al., 2005). The mechanism of action on the enzyme inhibition is due to Ahda

moiety, although N-Me-Tyr-Tyr at C-terminus contribute to the binding to the active site of ACE, LAP and APM (Ishida et al., 2000; Kraft et al., 2006). It was observed that tripeptide or tetrapeptide MGs are more potent ACE inhibitors than the pentapeptide congeners (Ishida et al., 2000; Stewart et al., 2018). MGs inhibit the aforementioned enzymes at nanomolar or micromolar concentrations, while no inhibitory effect has been shown on trypsin, chymotrypsin, elastase, thrombin, papain, protein phosphatase 1A (Bober and Bialczyk, 2017). The data on toxic activity of microginins are scarce. To the best of our knowledge, there are only three studies addressing acute toxicity of microginins on animal model organisms with *Thamnocephalus platyurus* larvae (Bober and Bialczyk, 2017) and *Caenorhabditis elegans* (Lenz et al., 2019). The acute toxicity caused by *Microcystis* sp. total extract containing various MGs was demonstrated in freshwater tropical fish *Astyanax altiparanae* larvae where numerous abnormalities including abdominal and pericardial oedema, and spinal curvature were reported (Fernandes et al., 2019).

For a better understanding of the toxicological properties of this group of cyanobacterial secondary metabolites, in the present study we determined *in vitro* cytotoxicity and genotoxicity of a

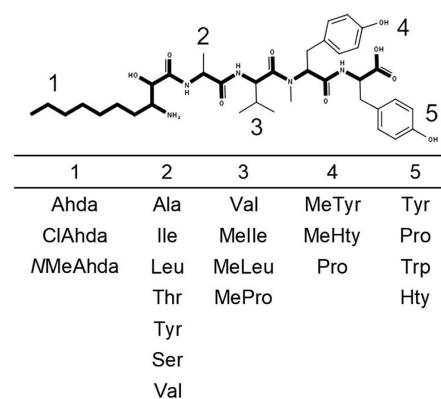


Fig. 1. Schematic general structure of microginins. Bold lines represent the conserved part, which can be found in all of the congeners. Thin lines represent the variable positions with the alternative amino acids, which can be found in the respective positions with the possible modifications (Cl-chlorination; Me-methylation). Amino acids are given in three-letter codes including the homovariant, and 3-amino-2-hydroxy-decanoic acid as Ahda. The stereochemistry is not considered in this figure. Representation is adopted from Janssen (2019).

complex cyanobacterial extract with well identified microginin pattern and selected microginin congeners in the experimental model with human hepatocellular carcinoma cell line HepG2. The cytotoxicity was studied with the MTT assay. The influence on DNA strand break formation and the genomic instability was evaluated with the comet and γ H2AX assays, and cytokinesis block micro-nucleus assay (CBMN), respectively.

2. Materials and methods

2.1. Chemicals

Methanol, citric acid monohydrate, sodium nitrate, magnesium sulfate heptahydrate, sodium hydroxide, hydrochloric acid, sodium chloride, tris(hydroxymethyl)aminomethane, ethanol were obtained from VWR Chemicals BDH Prolabo® Leuven, Belgium. Dipotassium hydrogenphosphate, sodium carbonate, iron (II) sulfate heptahydrate, Ethylenediaminetetraacetic acid (EDTA), boric acid, manganese (II) chloride tetrahydrate, zinc sulfate heptahydrate, sodium molybdate dihydrate, copper (II) sulfate pentahydrate, cobalt (II) chloride hexahydrate were from Reanal, Budapest, Hungary. Calcium chloride dihydrate was from Acidum-2 Ltd., Debrecen, Hungary. Sodium metasilicate pentahydrate, sodium chloride, acetonitrile was from Sigma, St. Louis, US. Trifluoroacetic acid from Merck. Williams' medium E, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), benzo[a]pyrene (BaP), cytochalasin B, and Ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma, St. Louis, USA. Penicillin/streptomycin, foetal bovine serum (FBS), L-glutamine, and phosphate buffered saline (PBS) were from PAA Laboratories, Dartmouth, USA. Triton X-100 was from Fisher Sciences, New Jersey, USA. GelRed™ was from Biotium (Fremont, CA, USA) and trypsin from BD-Difco, Le Pont-De-Claix Cedex, France. Normal melting point agarose (NMP), and low melting point agarose (LMP) were from Invitrogen, Paisley, Scotland, UK. Etoposide (ET) was from Santa Cruz Biotechnology (St. Cruz, USA). Anti-H2AX pS139 antibody as well as running and washing buffers for flow cytometry were from Miltenyi Biotec GmbH, Germany. Cyindrospermopsin (CYN) and microcystin-LR (MCLR) were obtained from Enzo Life Sciences GmbH (Lausen, Switzerland). A 0.5 mg mL⁻¹ and 1 mg mL⁻¹ stock solutions of CYN and MCLR were prepared in 50% methanol and 99.8% ethanol, respectively. All other chemical reagents were of the purest grade available.

2.2. Cyanobacterial cell culture

Cyanobacterial water bloom sample was originated from shallow Lake Balaton, Hungary, according to the parameters described by Riba et al. (2019). *Microcystis* sp. B-14-01 strain was isolated and further cultured in nitrate-containing Allen medium (Allen, 1968) in 1.5 L flasks, at 26 ± 1 °C, under continuous illumination (100 lu m⁻² s⁻¹) and bubbling with sterile air containing 5% CO₂. Cells were harvested after 7 days of cultivation (4420 × g, 5 min, Beckman Avanti J-25) and lyophilized (Christ-Alpha 1-2 LD Plus).

2.3. Sample preparation

Lyophilized cyanobacterial cells (250 mg) for analytical measurements and toxicity bioassays were extracted three times with 99.8% methanol (4 mL-2 mL-2 mL, respectively) while stirring and sonicating. The combined extract was centrifuged at 11400 × g for 15 min (Micro Star 12 VWR Microcentrifuge), the supernatant was evaporated. For further experiments, extract was dissolved in 50% ethanol for stock solution and stored at -20 °C until usage.

For the purification of MGs, lyophilized cyanobacterial cells (10 g) were extracted overnight with 99.8% methanol (200 mL) at room temperature with continuous stirring. After centrifugation (20400 × g, 20 min; Beckman Avanti-J25), another 200 mL 99.8% methanol was added to the pellet and this process was repeated once again. The combined supernatant was concentrated in a rotary evaporator (Sciogex RE100-Pro) at 40 °C. The residue was dissolved in 10 mM Tris-HCl (pH 7.5), centrifuged at 20400 × g for 20 min. The supernatant solution was filtered (GF/C, Whatman) and loaded onto a DEAE-cellulose ion exchange column (Whatman) equilibrated with 10 mM Tris-HCl (pH 7.5). The chromatography was carried out by Teledyne ISCO CombiFlash® Rf + System connected to PeakTrak software. During separation, the column was eluted with 10 mM Tris-HCl containing 0–100% 0.2 M NaCl linear gradient (5 mL min⁻¹ flow rate), the absorbance was measured at 220 nm. Peak fractions were concentrated by evaporation, dissolved in 50% ethanol and loaded onto Toyopearl HW40 (SEC). Mobile phase was 50% ethanol with a flow rate at 3 mL min⁻¹ (Pharmacia FRAC-300 fraction collector). Absorbance of collected fractions was measured at 220 nm (BOECO S-22 UV/VIS) and peaks were identified. Peak fractions were concentrated by evaporation, dissolved in 5% acetonitrile and applied to a C-18 flash column (RediSep C18AQ 50 g Gold). Mobile phase was acetonitrile (B) and water (A), both containing 0.1% (v/v) trifluoroacetic acid, changed from 0 to 5% B in 28.3 min, 5–90% B in 113.2 min, 90–100% B in 28.3 min, 100% for 35.4 min and 100–5% B in 7.6 min, the flow rate was 3 mL min⁻¹ (Teledyne ISCO CombiFlash® Rf + System, PeakTrak software). Collected peak fractions were evaporated and dissolved in 10% acetonitrile for semi-preparative HPLC. The final purification step was performed by using Shimadzu High Performance Liquid Chromatography system equipped with a Shimadzu SPD-M 10AVP diode array detector, GT-154 degasser, CTO-10ASVP oven, SPD-M10AVP PDA detector, SCL-10AVP system control unit, LC-10ADVP pump. Compounds were separated on a Supelco Supelco-sil LC-18 column (250 mm × 10 mm × 5 µm). The gradient mobile phase was consisted of acetonitrile (B) and water (A), both with 0.1% (v/v) trifluoroacetic acid, changed from 10 to 45% B in 50 min, 45–90% B in 10 min, 90–100% B in 5 min and 100–10% B in 2.5 min. The flow rate was 3 mL min⁻¹ and the column temperature was maintained at 40 °C. Chromatograms were monitored at 220 nm. Data acquisition and analysis were performed using Class-VP software. For further experiments, purified compounds were dissolved in 50% ethanol for stock solution and stored at -20 °C until usage.

2.4. Identification of oligopeptides by LC-ESI-MS/MS

The peak fractions collected in each chromatography step as well as cyanobacterial extract composition were analysed with a liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS/MS) method according to Riba et al. (2019). Briefly, LC-MS measurements were carried out on a Thermo Accela HPLC attached to a Thermo LTQ XL Linear Ion Trap MS system, using Kinetex XB-C18 column (100 mm × 2.10 mm × 2.6 µm). ESI source was employed in positive mode, and set as follows: heater temperature, 250 °C; sheath gas, N₂; flow rate, 10 arbitrary units (arb); aux gas flow rate, 5 arb; spray voltage, 5 kV; capillary temperature, 375 °C; capillary voltage, 35.00 V. The gradient mobile phase A was ultrapure water and mobile phase B was acetonitrile, both containing 0.1% (v/v) formic acid. Linear gradient was 10–70% B 0–10 min, 70–100% B 10–11 min, 100% B 11–16 min, 100–10% B 16–18 min, 10% B 18–20 min. Flow rate was 0.25 mL min⁻¹. The injected sample amount was 1 µL. Data acquisition and analysis were performed with Thermo Excalibur version 2.2 SP1.48 and MZmine 2.11 (Pluskal et al., 2010). Molecular ions were scanned in

the range of 300–1500 Da. Due to the lack of microginin standards, the quantification of the MG congeners were not possible. The peak areas of the detected MG congeners within the cyanobacterial extract were calculated and presented as percentages of the detected compounds.

2.5. Cell culture

HepG2 cells were gifted by Dr. Firouz Darroudi (Leiden University Medical Centre, Department of Toxicogenetics, Leiden, The Netherlands). The cells were grown at 37 °C and 5% CO₂ in Williams' medium E (Sigma, St. Louis, USA) supplemented with 15% foetal bovine serum, 2 mM L-glutamine and 100 U mL⁻¹ penicillin/streptomycin. The cells were used at passages between 3 and 11. As positive controls in genotoxicity assays, BaP and etoposide were used as well-established pro-carcinogen and DNA synthesis inhibitor, respectively. In addition, microcystin-LR (MCLR) and cylindrospermopsin (CYN) were included in the study for the comparison of the effects of the cyanotoxins.

2.6. Cell viability and proliferation: MTT assay

The cytotoxicity of cyanobacterial extract and purified compounds was determined by MTT assay according to Mosmann (1983) with minor modifications (Zegura et al., 2008). HepG2 cells were seeded onto 96-well microplates (Nunc, Naperville IL, USA) at a density of 8×10^3 cells and 3×10^3 cell per well for 24 h and 72 h treatment, respectively. Then the cells were incubated for 24 h at 37 °C, in 5% CO₂ humidified atmosphere to attach. Subsequently the growth medium was replaced with fresh medium containing 0, 0.1, 1, 6.25, 10 µg mL⁻¹ of MGs and 0, 1, 6.25, 10, 100, 200 µg mL⁻¹ dry weight of cyanobacterial extract for 24 h exposure and 0, 0.01, 0.1, 1, 6.25, 12.5, 25, 50, 100 µg mL⁻¹ of MGs, and 0, 0.01, 0.1, 1, 6.25, 12.5, 25, 50, 100, 200 µg mL⁻¹ dry weight of cyanobacterial extract for 72 h. The optical density was measured at 570 and 690 nm using spectrofluorimeter (SynergyMx, BioTek, Winooski, USA). The viability of treated cells was determined by comparison to vehicle control (medium containing 0.1% ethanol). Microcystin-LR (1 µg mL⁻¹), cylindrospermopsin (0.5 µg mL⁻¹) and etoposide (50 and 5 µg mL⁻¹, 24 h and 72 h exposure, respectively) were used as positive controls. All experiments were performed twice, independently with 5 replicates each time. Statistical significance between treated and control groups was determined by one-way analysis of variance (1-way ANOVA) and $P < 0.05$ was considered as statistically significant. IC₅₀ values were calculated with non-linear regression (GraphPad Prism 6 software, San Diego, California, USA).

2.7. Alkaline comet assay

HepG2 cells were seeded at a density of 8×10^4 cells/well into 12-well microtiter plates (Corning Costar Corporation, Corning, NY, USA) and were incubated at 37 °C in 5% CO₂ in humidified atmosphere for 24 h. The growth medium was replaced with fresh medium containing cyanobacterial extracts (0, 0.01, 0.1, 1 µg dry weight mL⁻¹) and MGs (0, 0.01, 0.1, 1 µg mL⁻¹) and incubated for 4 h and 24 h. In each experiment, positive controls (0.5 µg mL⁻¹ CYN and 30 µM BaP) were included. At the end of the exposure, the cells were harvested and the DNA damage was determined as described by Singh et al. (1988) with minor modifications (Zegura et al., 2004). Briefly, 30 µL of cell suspension was mixed with 70 µL 1% LMP agarose and added to fully frosted slides coated with 80 µL of 1% NMP agarose. Subsequently, the cells were lysed (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1 h at 4 °C, then placed into an alkaline solution (300 mM NaOH, 1 mM

EDTANa2, pH 13) for 20 min at 4 °C to allow DNA unwinding and electrophoresed for 20 min at 25 (at 1 V/cm). Finally, the slides were neutralized in 0.4 M Tris buffer (pH 7.5) for 15 min, stained with GelRed Nucleic Acid Stain (1:3333) and analysed using a fluorescence microscope (Nikon, Eclipse 800). Fifty randomly selected nuclei per experimental point were analysed with Comet Assay IV (Perceptive Instruments, UK) software. Three independent experiments were performed for each of the treatment conditions. One-way analysis of variance (1-way ANOVA) was used to analyse the differences in the percent of tail DNA between treatments within each experiment. Dunnett's Multiple Comparison test was used for comparing the exposed groups to the control group; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****) was considered as statistically significant.

2.8. Detection of double-strand breaks (DSBs) - γH2AX assay

HepG2 cells were seeded onto T-25 flasks (Corning Costar Corporation, Corning, NY, USA) at a density of 4×10^5 and 7.5×10^5 cells per plate for 24 h and 72 h treatments, respectively. Cells were incubated for 24 h at 37 °C, in 5% CO₂ humidified atmosphere to attach and were subsequently exposed to cyanobacterial extract (1 µg dry weight mL⁻¹) and MGs (1 µg mL⁻¹). In each experiment, etoposide (1 µg mL⁻¹) was used as a positive control and culture medium as a control. At the end of the treatment, floating and adherent cells were collected by trypsinisation, washed twice with ice-cold PBS (phosphate buffered saline), resuspended in 0.5 mL ice-cold PBS and 1.5 mL cold ethanol was added dropwise while vortexing. Cells were left at 4 °C for at least 2 h and stored at -20 °C overnight until analysis. Before analysis, fixed cells were washed with ice-cold PBS and resuspended in PBS containing 9% anti-γH2AX pS139 antibody solution (Hercog et al., 2019). Labelled cells were washed once with cold PBS and resuspended in 0.5 mL PBS for analysis. Analysis was performed on flow cytometer MACSQuant® Analyzer 10 and MACSQuantify™ software (Miltenyi Biotec, Germany). FITC intensity corresponding to DSBs was detected in B1-A channel. In each sample, 10^4 events were recorded. Experiments were repeated independently three times for each exposure time. Statistical significance between control and treatment groups were determined with a linear mixed-effects model using R (R Core Team, 2018), with its packages reshape (Wickham, 2007) and nlme (Pinheiro et al., 2018).

2.9. Cytokinesis block micronucleus (CBMN) assay

CBMN assay was performed according to Fenech (2000) with minor modifications. HepG2 cells were seeded onto T-25 flasks (Corning Costar Corporation, Corning, NY, USA) at a density of 6.5×10^5 cells per plate and incubated for 24 h at 37 °C and 5% CO₂ to attach. They were then exposed to cyanobacterial extract (1 µg dry weight mL⁻¹) and MGs (1 µg mL⁻¹) for 24 h. CYN (0.5 µg mL⁻¹) and BaP (2.5 µM) were used as positive controls and culture medium as a control. After the treatment, fresh medium containing cytochalasin B (2 µg mL⁻¹) was added for 26 h. At the end of the exposure, floating and adherent cells were collected by trypsinisation and were processed according to Straser et al. (2011). For the analysis fixed cells were stained with acridine orange (10 µg mL⁻¹), and evaluated using the fluorescence microscope (Eclipse 800, Nikon, Japan). Micronucleated cells (MNed cells), micronuclei (MNI), nuclear buds (NBUDs), and nuclear bridges (NPBs) were counted in 1000 binucleated cells (BNC) per experimental point at 400× magnification and were scored according to criteria published by Fenech (2000). The nuclear division index (NDI) was estimated by scoring 500 cells with one to four nuclei. The NDI was calculated using the formula $[M1 + 2M2 + 3M3 + 4M4]/500$, where

M1–M4 represent the number of cells with one to four nuclei, respectively. Experiments were repeated three times, independently. In order to compare the number of MNI, MNed cells, NBUDs and NPBs between the control and treated groups, Chi-square test was used; $P < 0.05$ (*). $A \geq 2$ -fold increase compared to control was considered a positive response. For the comparison of the differences in NDI between control and treated groups one-way ANOVA was used.

3. Results

3.1. Identification of cyanobacterial oligopeptides

LC-ESI-MS/MS analysis of the cyanobacterial extract showed that the *Microcystis* sp. B-14-01 strain predominantly produced several MG congeners (Table 1). The compound with m/z 728 $[M + H]^+$ was identified as MG FR3 according to MS² data explained by Welker et al., (2006). MG 565 (m/z 565 $[M + H]^+$) showed a set of identical fragments (m/z 420, m/z 384, m/z 257, m/z 182, m/z 128). Based on the fragmentation pattern, this molecule appeared to be a linear tetrapeptide with a sequence of Ahda-Thr-Pro-Tyr (Riba et al., 2019). MGL 402 microginin-like derivative of the MG 565 congener with m/z 402 $[M + H]^+$ was combined of three amino acids, containing Ahda-Thr-Pro. The fragment peaks at m/z 384, m/z 287, m/z 257, m/z 239, m/z 217 and m/z 128 are corresponding to Ahda-Thr-Pro + H^+ –H₂O, Ahda-Thr + H^+ , Ahda'-Thr-Pro + H^+ , Ahda'-Thr-Pro + H^+ –H₂O, Thr-Pro + H^+ and Ahda, respectively. The microginin congener with m/z 754 $[M + H]^+$ was earlier described as cyanostatin B (Sano et al., 2005; Welker et al., 2006). Deduced from the MS² fragmentation, one intense fragment peak was observed at m/z 476, which was corresponded to Ahda-Tyr-Melle + H^+ . Other fragment peaks at m/z 573, m/z 349, m/z 186 and m/z 59, were corresponded to Ahda-Tyr-Melle-Pro + H^+ , Ahda-Tyr + H^+ , Ahda and Ahda', respectively. The fragmentation pattern of the MG with m/z 788 $[M + H]^+$ was suggested to be a chlorinated congener derivative of cyanostatin B, and was reported as MG GH787 (Lifshits et al., 2011). The most intense fragment peak appeared at m/z 510 as ClAhda-Tyr-Melle + H^+ , while fragment peaks at m/z 607, m/z 383, m/z 220 were corresponded to ClAhda-Tyr-Melle-Pro + H^+ , ClAhda-Tyr-Melle + H^+ and ClAhda, respectively. Other fragment ions, in both congeners, appeared at m/z 569 (Tyr-Melle-Pro-Tyr + H^+), m/z 406 (Melle-Pro-Tyr + H^+), m/z 279 (Pro-Tyr + H^+), and m/z 182 (Tyr + H^+).

Analysis of the cyanobacterial extract showed that the above described MG congeners were produced by *Microcystis* sp. B-14-01 strain in different concentrations. The ratio of detected congeners was determined by the peak area intensities and given in percentages (Table 1). Cyanostatin B was the most dominant among all the detected congeners (51.55%) in the cyanobacterial extract. MG FR3 (21.08%), MG GH787 (17.35%) and MG 565 (8.98%) were less abundant, while MGL 402 (1.02%) was present in a very low quantity. For further toxicological analyses, the complex cyanobacterial extract and the congeners MG FR3, MGL 402, MG GH787

and cyanostatin B were used.

3.2. The influence of cyanobacterial extracts and microginins on the viability of HepG2 cells

The viability and the proliferation of HepG2 cells were determined after 24 h and 72 h of exposure, respectively (Fig. 2). The B-14-01 cyanobacterial extract significantly reduced the cell viability by approximately 14% and 33% at 6.25 and 10 μ g dry weight of cyanobacterial extract mL^{-1} , respectively, with the IC₅₀ 214.8 $\pm$ 1.19 μ g dry weight mL^{-1} , while microginins did not affect cell viability at tested concentrations compared to vehicle control, therefore these concentrations were used in further experiments for genotoxicity assessment.

After 72 h exposure the B-14-01 cyanobacterial extract concentration dependently reduced cell proliferation with the IC₅₀ 211.5 $\pm$ 1.05 μ g dry weight mL^{-1} . Also MGs concentration dependently decreased cell proliferation. The most potent congener was MGL 402 with approximately 22 and 42% decrease of cell viability at 50 and 100 μ g mL^{-1} , respectively. MG FR3 showed lower cytotoxic activity with approximate reduction for 23 and 20% at 50 and 100 μ g mL^{-1} , respectively. MG GH787 and cyanostatin B showed similar cytotoxic activity with significant decrease of cell viability already at 25 μ g mL^{-1} for approximately 25%; however, at the highest tested concentration (100 μ g mL^{-1}) these two congeners showed lower activity (32% and 30%, respectively) compared to MGL 402.

MCLR (1 μ g mL^{-1}) and CYN (0.5 μ g mL^{-1}) were used for the comparison of the effects. After 24 h exposure, no significant effect on cell viability was observed. After 72 h, CYN decreased cell proliferation for approximately 55% compared to control, while MCLR had no significant effect.

3.3. Induction of DNA strand breaks – comet assay

The formation of DNA strand breaks was evaluated after 4 h and 24 h exposure with the comet assay (Fig. 3). HepG2 cells were exposed to the B-14-01 cyanobacterial extract (0, 0.01, 0.1 and 1 μ g dry weight mL^{-1}) and MGs (0, 0.01, 0.1 and 1 μ g mL^{-1}). The cyanobacterial extract induced concentration and time dependent increase of DNA damage that was statistically significant at concentrations ≥ 0.01 μ g dry weight mL^{-1} at both exposure times. Cyanostatin B, MG GH787 and MG FR3 induced statistically significant increase of DNA strand breaks after 4 h exposure at concentrations ≥ 0.1 μ g mL^{-1} , while MGL 402 increased DNA damage at concentrations ≥ 1 μ g mL^{-1} . After 24 h, MGL 402 was the most potent variant inducing DNA damage at concentrations ≥ 0.01 μ g mL^{-1} , while MG FR3 was the least potent variant inducing only slight though statistically significantly increased DNA damage at 1 μ g mL^{-1} compared to the control. Cyanostatin B and MG GH787 significantly increased DNA damage at concentrations ≥ 0.1 μ g mL^{-1} and CYN (0.5 μ g mL^{-1}) at both exposure times.

Table 1

Microginin congeners with the proposed amino acid sequences in the cyanobacterial strain B-14-01 determined by LC-ESI-MS. Leucine and isoleucine cannot be distinguished from LC-MS/MS data, these amino acids were deduced from the nearest literary results. The ratio of the detected microginin congeners within the cyanobacterial extract were calculated as percentages of the total peak area.

Microginin congener	m/z $[M + H]^+$	Proposed amino acid sequences	Percentages of peak area	References
MGL 402	402.2	Ahda-Thr-Pro	1.02%	This study
MG 565	565.5	Ahda-Thr-Pro-Tyr	8.98%	Riba et al. (2019)
MG FR3	728.6	Ahda-Thr-Pro-Tyr-Tyr	21.08%	Welker et al. (2006)
Cyanostatin B	754.4	Ahda-Tyr-Melle-Pro-Tyr	51.51%	Sano et al., (2005); Welker et al., (2006)
MG GH787	788.4	ClAhda-Tyr-Melle-Pro-Tyr	17.35%	Lifshits et al. (2011)

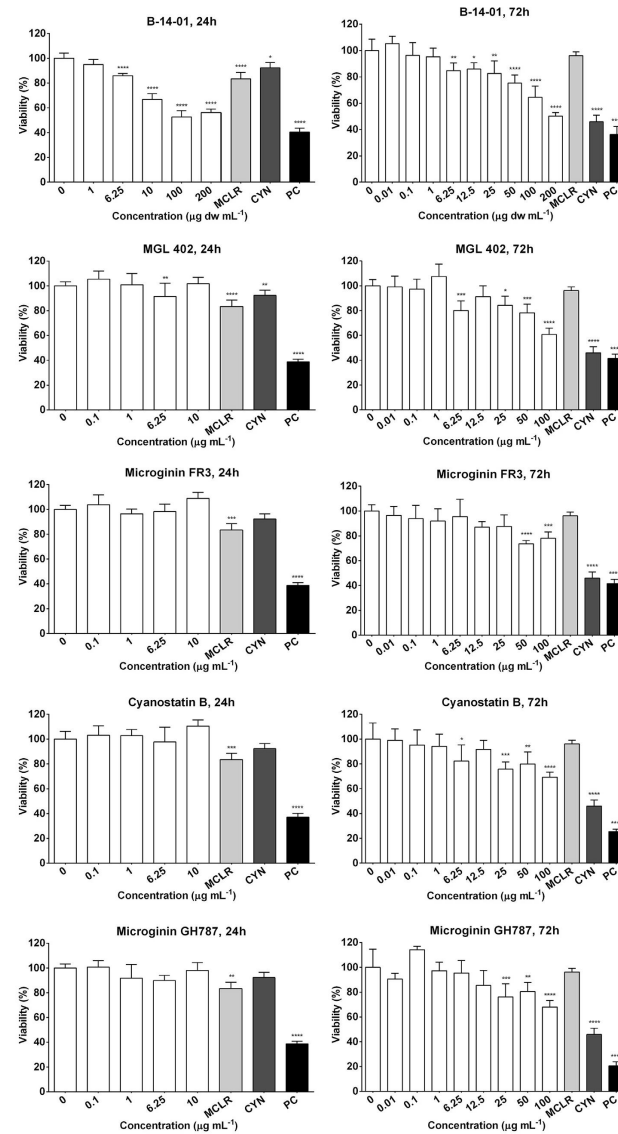


Fig. 2. The effects of the cyanobacterial extract B-14-01 (1–200 µg dry weight mL⁻¹ and 0.01–200 µg dry weight mL⁻¹, 24 and 72 h of exposure, respectively) and microginin congeners MGL 402, MG FR3, cyanostatin B and MG GH787 (0.1–10 µg mL⁻¹ and 0.01–100 µg mL⁻¹, 24 and 72 h of exposure, respectively) on the viability of HepG2 cells. Etoposide (PC) [black] was used as positive control (50 and 5 µg mL⁻¹, 24 and 72 h of exposure, respectively). MCLR (1 µg mL⁻¹) [light grey] and CYN (0.5 µg mL⁻¹) [dark grey] were used as cyanotoxin controls. Vehicle control (0; culture medium containing 0.1% ethanol) was considered as 100%. All experiments were performed twice, independently with 5 replicates each time. Statistically significant difference (1-way ANOVA) between the control and treated groups is indicated by * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001.

3.4. Induction of double-strand breaks (DSBs) - γ H2AX assay

After 24 and 72 h exposure to cyanobacterial extract ($1 \mu\text{g dry weight mL}^{-1}$) and MGs ($1 \mu\text{g mL}^{-1}$) the induction of DSBs was analysed by flow cytometry (Fig. 4). None of the studied MGs statistically significantly increased the formation of γ H2AX foci.

3.5. Induction of genomic instability – CBMN assay

The influence of cyanobacterial extract ($1 \mu\text{g dry weight mL}^{-1}$) and MGs ($1 \mu\text{g mL}^{-1}$) on genomic instability of HepG2 cells was further evaluated with the CBMN assay. The results are summarized in Table 2. After 24 h exposure, B-14-01 extract did not affect the frequencies of MNed cells, MNi and NBUDs, while the number of NPBs was increased for more than 2-fold compared to control group. Only MG GH787 statistically significantly increased the frequencies of the MNed cells, MNi and NPBs that were more than 2-fold higher compared to control group. Cyanostatin B, MGL 402, MG FR3 statistically significantly increased the frequencies of MNed cells and MNi; however, the increase was less than 2-fold compared to control. Nevertheless, we can notice that cyanostatin B, the structural formula of which is very similar to the one of MG GH787 (chlorinated cyanostatin B), significantly increased the frequency of MNi for 1.7-fold compared to control indicating some influence on genomic instability of HepG2 cells. The NDI was not reduced by the cyanobacterial extract B-14-01 and MGs compared to control group (Table 2). CYN and BaP significantly increased the frequencies of the MNed cells, MNi and NBUDs for ≥ 2 -fold compared to control and statistically significantly reduced the NDI.

4. Discussion

MGs, produced by cyanobacteria are zinc metalloprotease inhibitors, and their activities on angiotensin-converting enzyme (ACE), aminopeptidase M (APM) and leucine aminopeptidase (LAP) have been reported (Ishida et al., 2000; Kodani et al., 1999; Lifshits et al., 2011; Lodin-Friedman and Carmeli, 2018; Neumann et al., 1997; Okino et al., 1993; Sano et al., 2005; Stewart et al., 2018). In the environment they are commonly found in co-occurrence with microcystins (MCs) and it is generally believed that toxicity of MGs is much lower than that of MCs (Zanchett and Oliveira-Filho, 2013). To the best of our knowledge, this is the first study to investigate the cytotoxic and genotoxic activities of pure MGs and the cyanobacterial extract containing MG congeners on the human model cell line HepG2.

The LC-ESI-MS/MS chemical analyses of the cyanobacterial strain B-14-01 used in the present study, demonstrated that it produced four MG congeners (cyanostatin B, MG 565, MG FR3, MG GH787) and a MG-like (MGL 402) compound. The oligopeptides produced by *Microcystis* species show high diversity. However, the data regarding different chemotypes are not consistent (Agha et al., 2014) and the co-productions of oligopeptides show variable patterns in *Microcystis* colonies (Carneiro et al., 2012; Fastner et al., 2001; Martins et al., 2009; Riba et al., 2019; Silva-Stenico et al., 2011; Welker et al., 2004). On the other hand, the production of only MG congeners by *Microcystis* species has also been observed (Stewart et al., 2018; Strangman and Wright, 2016). Our results demonstrated that the oligopeptide pattern of the B-14-01 strain was limited to only MG congeners, and co-production of peptides from other classes was not observed. The detected MGs were present in different ratios, cyanostatin B, MG FR3 and MG GH787 being the dominant congeners, while MGL 402 and MG 565 were present in lower quantities. The concentrations that were used for studying the genotoxic effects of the congeners, differ between the congeners when considering the molarity and for $1 \mu\text{g mL}^{-1}$ represent

2.49 μM for MGL 402, 1.37 μM for MG FR3, 1.32 μM for cyanostatin B and 1.27 μM for MG GH787.

In this study, we described the tripeptide congener MGL 402 for the first time, while the structures of other congeners MG FR3, MG GH787, MG 565 and cyanostatin B have already been reported previously (Lifshits et al., 2011; Riba et al., 2019; Sano et al., 2005; Welker et al., 2006). The microginin congeners used in the present study differ by the amino acid sequences. Based on the fragmentation patterns, it was demonstrated that MG FR3, cyanostatin B and MG GH787 shared common characteristics such as the amino acid tyrosine at the C-terminal end. While MG FR3 contained Tyr-Tyr unit, MG 565, cyanostatin B and MG GH787 had Pro-Tyr units. The NMe-Tyr-Tyr at C-terminus is considered to be important for the inhibitions of ACE, LAP and APM (Ishida et al., 2000). On the other hand, cyanostatin B and MG GH787 contained a methylated isoleucine (Ile), compared to the other tested congeners, and the latter had chloro-Ahda as well. It was previously demonstrated that MG FR3, cyanostatin B and MG GH787 exhibit strong aminopeptidase inhibitory activity with an IC_{50} of 6.2 μM , 15.9 nM, 7.7 μM , respectively (Lifshits et al., 2011; Lodin-Friedman and Carmeli, 2018; Sano et al., 2005). Furthermore, the NMeAhda containing MGs inhibited APM more effectively than those counterparts, which were monochlorinated (ClAhda) or were not methylated (Ahda) (Lodin-Friedman and Carmeli, 2018). Additionally, the aminopeptidase inhibitors bestatin and amastatin, which are linear peptides, containing Ahda related α -hydroxy- β -amino acid residues, have been shown to enhance antitumor and antihypertensive activities (Rich et al., 1984), they bind to zinc ion(s) in the active centre of the aminopeptidase. It was concluded that the resemblance in their binding structures suggests similar inhibitory mechanism for microginins (Kraft et al., 2006).

The influence of the cyanobacterial extract and MGs on HepG2 cell viability and proliferation was evaluated with the MTT assay. Only the B-14-01 extract containing all studied MG congeners affected cell viability (Fig. 2) in a concentration and time dependent manner. Pure MGs inhibited cell proliferation after 72 h of exposure at higher concentrations from those previously detected in the environment (Beversdorf et al., 2017). Previously it has been reported that after 48 h exposure an unspecified MG congener did not exert cytotoxic activity at concentrations up to $\geq 30 \mu\text{g mL}^{-1}$ in HepG2 and human colon adenocarcinoma (Caco-2) cell line (Chorus et al., 2006), which is in line with low cytotoxic activity observed in our study.

Further the influence of cyanobacterial extract B-14-01 and MG congeners on the induction of DNA strand breaks was studied after 4 and 24 h of exposure with the comet assay and 24 and 72 h of exposure with the γ H2AX assay. The comet assay detects mostly DNA single strand breaks (SSBs) and/or alkali labile sites and to lesser extend also DNA double strand breaks (DSBs) (Gajski et al., 2019), while γ H2AX assay specifically detects DNA DSBs (Garcia-Canton et al., 2012). The B-14-01 extract induced DNA SSBs already at the lowest concentration tested ($\geq 0.01 \mu\text{g dry weight mL}^{-1}$) in a concentration and time dependent manner. After 4 h of exposure, all studied congeners significantly increased the amount of DNA SSBs at $\geq 0.1 \mu\text{g mL}^{-1}$ with the exception of MGL 402 that was genotoxic at $\geq 1 \mu\text{g mL}^{-1}$. On the contrary, after 24 h of exposure MGL 402 was the most genotoxic congener inducing significant increase of DNA damage at $\geq 0.01 \mu\text{g mL}^{-1}$, while MG FR3 showed the lowest genotoxic potential increasing DNA SSBs at 100-times higher concentrations. Cyanostatin B and MG GH787 induced significant DNA damage at $\geq 0.1 \mu\text{g mL}^{-1}$. From all studied congeners only MG FR3 showed higher genotoxic activity after short-time exposure, which might be related to the transient occurrence of DNA strand breaks due to the repair of DNA damage. Similarly, it has been reported for microcystin-LR (MCLR), which

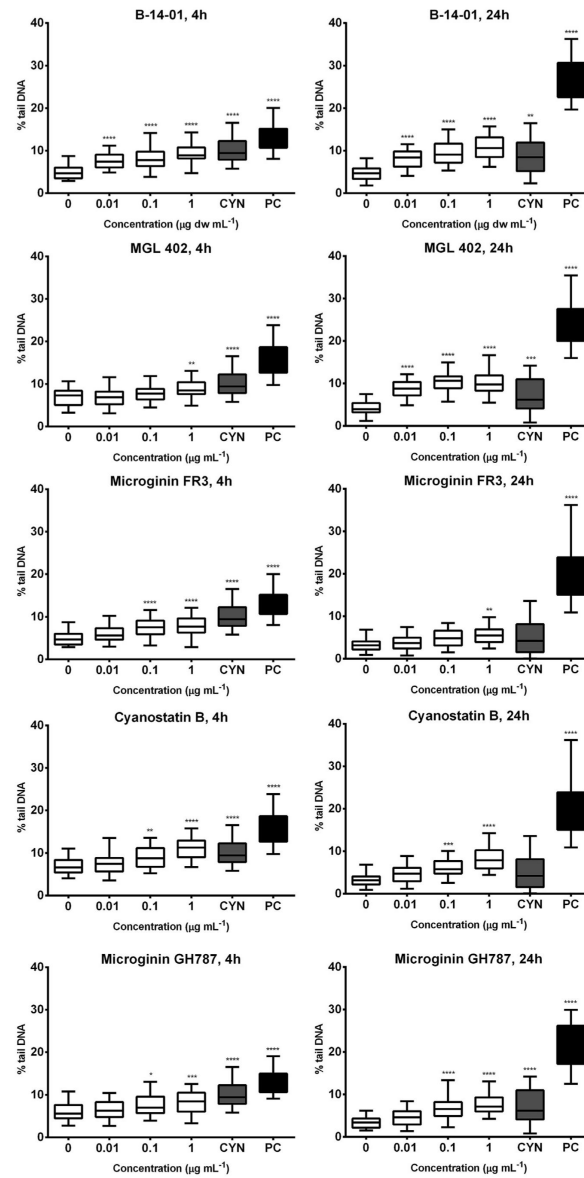


Fig. 3. DNA damage induced by the cyanobacterial extract B-14-01 (0, 0.01, 0.1 and 1 µg dry weight mL⁻¹) and microginin congeners MGL 402, MG FR3, cyanostatin B and MG GH787 (0, 0.01, 0.1 and 1 µg mL⁻¹) in HepG2 cells after 4 and 24 h of exposure. DNA damage is expressed as percentages of comet tail DNA. BaP (30 µM) was used as positive control (PC) [black] and CYN (0.5 µg mL⁻¹) [dark grey] as cyanotoxin control. For each of the treatment conditions three experiments were performed independently. Statistically significant difference (1-way ANOVA; Dunnett's Multiple Comparison test) between the control and treated groups is indicated by *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

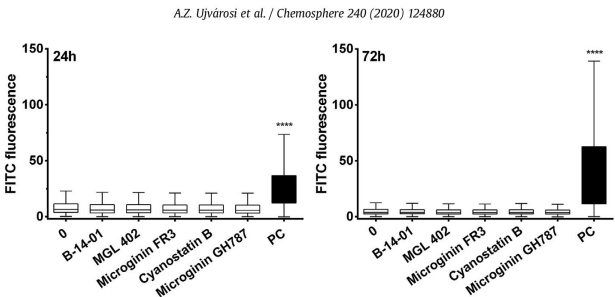


Fig. 4. Induction of DNA double strand breaks (DSBs) by the cyanobacterial extract B-14-01 (1 µg dry weight mL⁻¹) and microglinin congeners MGL 402, MG FR3, cyanostatin B and MG GH787 (1 µg mL⁻¹). HepG2 cells were exposed for 24 and 72 h and the presence of DSBs was determined as FITC fluorescence intensities obtained as FL1-H value by flow cytometry, indirectly through the detection of γH2AX positive sites. In each experiment a control (0; culture medium) and a positive control (PC; 1 µg mL⁻¹ etoposide) [black] were included. In each sample, 10³ events were recorded and for each of the treatment conditions three experiments were performed independently. Statistically significant differences between the control and treated groups were determined with a linear mixed-effects model and indicated by * p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

Table 2
The frequencies of micronucleated cells (MnEd cells), micronuclei (MNI), nuclear bridges (NPBs), nuclear buds (NBUD), and nuclear division (NDI) in HepG2 cells after 24 h of exposure to cyanobacterial extract B-14-01 (1 µg dw mL⁻¹) and MGs (1 µg mL⁻¹) determined with the cytokinesis-block micronucleus (CBMN) assay. The numbers of MnEd cells, MNI, NPBs and NBUDs were determined in 1000 binucleated cells per experimental point in each of the three independent experiments. The significant difference in the number of MNI, NBUDs and NPBs between treated and control cells was determined with Chi-square test (*P < 0.05). The nuclear division index (NDI) was determined by scoring 500 cells with one to four nuclei. The significant difference between treated and control cells was determined by 1-way ANOVA (*p < 0.05). Positive controls used were BaP (2.5 µM) and CYN (0.5 µg mL⁻¹). A ≥ 2-fold increase compared to the control was considered a positive response and is marked with bold.

Sample	MnEd cells/10 ³ BNC	MNI/10 ³ BNC	NPB/10 ³ BNC	NBUD/10 ³ BNC	NDI
Control	26.33 ± 2.08	30.33 ± 3.06	1.67 ± 1.53	15.33 ± 7.64	1.88 ± 0.05
B-14-01	35.33 ± 6.66	40.67 ± 8.14	5.67 ± 2.89*	19.33 ± 13.20	1.89 ± 0.08
MGL 402	34.67 ± 4.93*	41.33 ± 8.14*	3.33 ± 2.08	16.00 ± 9.17	1.83 ± 0.03
Cyanostatin B	44.67 ± 11.59*	51.67 ± 16.74*	3.33 ± 0.58	23.00 ± 9.85	1.80 ± 0.09
Microglinin GH787	53.33 ± 2.52*	65.33 ± 7.23*	6.67 ± 2.08*	25.67 ± 8.62	1.82 ± 0.06
Microglinin FR3	37.33 ± 3.79*	45.00 ± 2.00*	4.33 ± 1.53	18.00 ± 8.19	1.85 ± 0.10
BaP	60.33 ± 39.63*	63.33 ± 36.95*	3.33 ± 0.58	12.33 ± 3.21	1.41 ± 0.31*
CYN	67.67 ± 10.02*	80.67 ± 14.57*	3.00 ± 2.65	38.00 ± 2.65*	1.54 ± 0.31*

induces DNA strand breaks after short-time exposure; however, after prolonged exposure to MCLR DNA strand breaks are reduced. It has been demonstrated that MCLR-induced DNA damage is related to the formation of DNA strand breaks as intermediates of the cellular repair (Zegura et al., 2003) and that DNA damage is of oxidative nature (Zegura, 2016; Zegura et al., 2004). On the other hand, the mechanism of genotoxic activity of another in the environment ubiquitously present cyanotoxin, cylindrospermopsin (CYN), differs from that of MCLR. CYN is known as pro-genotoxic and requires metabolic activation to electrophilic intermediates, which then induce DNA damage (Bazin et al., 2010; Humpage et al., 2005; Štraser et al., 2011) in the form of DNA SSBs and DSBs. Detection of H2AX phosphorylation, which reflects an early reaction to a genotoxic insult resulting in the formation of DNA DSB revealed that non-cytotoxic concentrations of the B-14-01 cyanobacterial extract (1 µg dry weight mL⁻¹) and MGs (1 µg mL⁻¹) did not induce the formation of DNA DSBs, neither after 24 nor 72 h exposure. DNA DSBs generally occur when the DNA repair is impaired. This is the most detrimental form of DNA damage as it can have severe consequences. It can lead to cell death or induction of genomic instability, which can in turn trigger carcinogenesis (Gerić et al., 2014). Previously it has been shown that, similarly to MGs, MCLR at non-cytotoxic concentrations does not induce DSBs (unpublished results) in HepG2 cells, while CYN does (Štraser et al., 2013).

Potential clastogenic activity of cyanobacterial extract B-14-01 and MGs in HepG2 cells was further evaluated using the CBMN assay. Following 24 h exposure, only MG GH787 induced ≥ 2-fold

increase in the frequencies of micronuclei (MNI) and nuclear bridges (NPBs) in binucleated cells compared to control group. Cyanostatin B, which is structurally very similar to MG GH787 (difference is the chlorinated Ahda moiety in MG GH787), also significantly increased the frequency of MNI (1.7-fold compared to control), indicating weaker influence on genomic instability of HepG2 cells. The B-14-01 cyanobacterial extract increased (≥2-fold) only the number of NPBs, but the frequencies of MNI, MnEd cells and NBUDs were not altered. MNI are biomarkers of chromosome breakage or whole chromosome loss, while NPBs are biomarkers of chromosome rearrangement, DNA misrepair and telomere-end fusion and occur when centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase (Fenech, 2000). These results provide new information, as to our knowledge the effects of MGs on the induction of MNI, NBUDs and NPBs formation have never been studied before.

In summary, the present study demonstrated that in human hepatocellular carcinoma HepG2 cells, cyanobacterial extract B-14-01 and all MGs congeners studied, induced DNA damage. The induction of MNI and NPB formation was observed in cells exposed to MG GH787, cyanostatin B induced MNI formation, while the extract B-14-01 induced only increase in NPBs formation.

5. Conclusion

The results from the present study are the first that demonstrated genotoxic potential of MGs indicating that MGs may represent hazard for aquatic environment and humans that has not

been recognized before. In humans, exposure to genotoxic compounds is associated with development of several chronic diseases including cancer, whereas in aquatic environment reproduction of aquatic organisms. This is particularly important as MGs are produced by a common *Microcystis* species that are ubiquitously present in the aquatic environment. Therefore, further studies are needed to explore the mechanisms of the genotoxic activity of MGs as well as studies of their occurrence in the aquatic environment.

Conflicts of interest

The authors declare that they have no conflict of interest.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2019.124880>.

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2.3 Genotoxic Potential of the Binary Mixture of Cyanotoxins Microcystin-LR and Cylindrospermopsin

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Genotoxic potential of the binary mixture of cyanotoxins microcystin-LR and cylindrospermopsin



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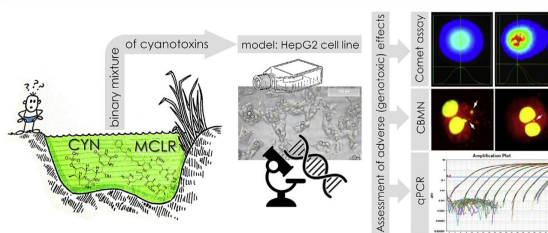
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HIGHLIGHTS

- In HepG2 cells MCLR/CYN mixture induced less DNA strand breaks than MCLR or CYN alone.
- The induction of micronuclei by MCLR/CYN was comparable to that induced by CYN alone.
- Deregulation of genes is similar in MCLR/CYN mixture and CYN exposed HepG2 cells.
- CYN exerts higher genotoxic potential than MCLR.
- Genotoxic potential of the MCLR/CYN mixture is comparable to that of CYN alone.

GRAPHICAL ABSTRACT



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ABSTRACT

Increased eutrophication of water bodies promotes cyanobacterial blooming that is hazardous due to the production of various bioactive compounds. Microcystin-LR (MCLR) is among the most widespread cyanotoxins classified as possible human carcinogen, while cylindrospermopsin (CYN) has only recently been recognized as health concern. Both cyanotoxins are genotoxic; however, the mechanisms of their action differ. They are ubiquitously present in water environment and are often detected together. Therefore, we studied genotoxic potential of the binary mixture of these cyanotoxins. Human hepatoma cells (HepG2) were exposed to a single dose of MCLR (1 µg/mL), graded doses of CYN (0.01–0.5 µg/mL), and their combinations. Comet and Cytokinesis block micronucleus assays were used to detect induction of DNA strand breaks (sb) and genomic instability, respectively, along with the transcriptional analyses of the expression of selected genes involved in xenobiotic metabolism, immediate/early cell response and DNA-damage response. MCLR induced DNA sb that were only transiently present after 4 h exposure,

Abbreviations: BaP, Benzo[a]pyrene; BNC, Binucleated cells; CYN, Cylindrospermopsin; CYP450, Cytochrome P450; CBMN, Cytokinesis block micronucleus assay; DMSO, dimethylsulphoxide; ET, etoposide; FBS, foetal bovine serum; LMP, Low melting point agarose; MCLR, microcystin-LR; MNI, Micronuclei; MTS, thiazolyl blue tetrazolium bromide; NMP, normal melting point agarose; NDI, Nuclear division index; NBUD, Nuclear bud; NPB, Nucleoplasmic bridge; PBS, phosphate buffered saline; qPCR, quantitative real-time polymerase chain reaction.

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Binary mixture
Genotoxicity
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whereas CYN, after 24 h exposure, induced DNA sb and genomic instability. The MCLR/CYN mixture induced DNA sb after 24 h exposure, but to lesser extent as CYN alone. On the other hand, induction of genomic instability by the MCLR/CYN mixture was comparable to that induced by CYN alone. In addition, patterns of changes in the expression of selected genes induced by the MCLR/CYN mixture were not significantly different from those induced by CYN alone. Our results indicate that CYN exerts higher genotoxic potential than MCLR and that genotoxic potential of the MCLR/CYN mixture is comparable to that of CYN alone.

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1. Introduction

Cyanobacteria are producing a broad spectrum of secondary metabolites including toxins with adverse effects in human and wildlife upon exposure. Cyanobacterial toxins have been recognized as a serious and progressive environmental as well as human health concern all over the world since their occurrence is increasing due to water eutrophication and climate change (Paerl et al., 2011). There is a wide spectrum of known cyanotoxins produced by more than 40 genera of cyanobacteria (Buratti et al., 2017; Carmichael, 2001). Cyanobacteria can produce various toxins at the same time and individual cyanotoxins can be produced by different genera of cyanobacteria (Buratti et al., 2017; Rzymski and Poniedziałek, 2014; Żegura et al., 2011c). As in cyanobacterial blooms various species of cyanobacteria can be found (Bláha et al., 2009; Jančula et al., 2014), in naturally occurring blooms mixtures of cyanotoxins are present, which leads to a simultaneous exposure of organisms to several cyanotoxins. The chemical structure of cyanotoxins and their mechanisms of action differ; therefore, the question of synergistic or potentiating toxic effects is relevant for the investigation. In terms of this question the present study was focused on exploring combined genotoxic effects of two cyanotoxins, microcystin-LR (MCLR) and cylindrospermopsin (CYN) that are both hepatotoxins produced by freshwater cyanobacteria and have been reported to be simultaneously present in blooms (Dantas et al., 2011; Fastner et al., 2007; Jančula et al., 2014).

Microcystins (MCs) are hepatotoxic cyclic heptapeptides (Carmichael et al., 1988). There have been over 246 different MCs identified (Meriluoto et al., 2017) with microcystin-LR (MCLR) as the most studied congener. MCLR acts via the inhibition of specific protein phosphatases affecting cytoskeletal proteins. It is known to be tumour-promoter (Humpage et al., 2000a, b; Nishiwaki-Matsushima et al., 1992) and has been classified as possible human carcinogen (IARC, 2010). Its hydrophilic molecule passes cell membranes actively by organic anion transporting polypeptides (OATP) (Fischer et al., 2005); therefore, liver is the main target organ of MCLR toxicity. Non-cytotoxic concentrations of MCLR cause DNA strand breaks *in vitro* and *in vivo* (for review see (Żegura, 2016)), but the mechanisms involved in its genotoxicity are not yet fully understood. There is a strong evidence that MCLR induces DNA damage indirectly through enhancing the intracellular production of reactive oxygen species resulting in oxidation of DNA bases (Bouaicha et al., 2005; Żegura et al., 2006, 2004) and, in addition, it compromises DNA repair mechanisms (Lankoff et al., 2006a), which altogether contribute to genomic instability.

Cylindrospermopsin (CYN), another emerging cyanotoxin, is structurally a polyketide-derived alkaloid with a tricyclic carbon skeleton containing a guanidine functional moiety combined with hydroxymethyluracil attached to it (Ohtani et al., 1992). It is a highly water soluble zwitterionic molecule. Recently it has been described that paracellular transport is involved in CYN intestinal uptake (Pichardo et al., 2017), while the transmembrane transport is

limited (Froschio et al., 2009); however, the mechanism has not been fully elucidated yet. Mainly CYN is affecting the liver; however, other organs can be affected as well (U.S. EPA, 2015). The principle mechanism of its toxicity is irreversible inhibition of protein synthesis (Froschio et al., 2001; Runnegar et al., 2002; Terao et al., 1994). One of the most plausible mechanisms of its toxicity is proposed to be mediated by the uracil moiety (Banker et al., 2001). Numerous studies reported that cytotoxic and genotoxic effects of CYN depend on its metabolic activation by cytochrome P450 enzymes (Bazin et al., 2010; Humpage et al., 2005; Straser et al., 2011); therefore, it is considered as pro-genotoxic. CYN has been shown to be a clastogen inducing DNA fragmentation and loss of whole chromosomes (Bazin et al., 2010; Humpage et al., 2000a, b; Straser et al., 2011; Żegura et al., 2011a) and causing DNA strand breaks (Humpage et al., 2005; Straser et al., 2011; Żegura et al., 2011b) *in vitro* in metabolically active cell models. There are some indications that it can act also as a tumour-initiator (Falconer and Humpage, 2000), but further studies are needed to confirm this assumption.

Data on combined effects of different environmental pollutants or/and cyanotoxins are crucial for relevant risk assessment since in real life organisms are exposed to mixtures of several compounds rather than to a single compound (Dietrich et al., 2008). This lack of knowledge led us to study the genotoxic potential of the binary mixture of two cyanotoxins, MCLR and CYN in an experimental model with metabolically active human hepatoma HepG2 cells. We evaluated the influence of the binary mixture on the formation of DNA strand breaks and genomic stability in comparison to single compounds with the comet assay and cytokinesis block micronucleus (CBMN) assay, respectively. To get insight into the molecular mechanisms of the combined genotoxic effects we applied quantitative real time PCR to explore the influence of the binary mixture on the expression of selected genes involved in xenobiotic metabolism that are relevant for activation and detoxification of CYN and genes involved in immediate-early response/signalling and DNA damage response that are known to be deregulated in response to the exposure to genotoxic chemicals.

2. Materials and methods

2.1. Chemicals

Cylindrospermopsin (CYN) and microcystin-LR (MCLR) were obtained from Enzo Life Sciences GmbH (Lausen, Switzerland). A 0.5 mg/mL and 1 mg/mL stock solutions of CYN and MCLR were prepared in 50% methanol and pure ethanol, respectively. Benzo(a)pyrene (BaP) from Sigma (St. Louis, MO, USA) and etoposide (ET) from Santa Cruz Biotechnology (St. Cruz, USA) were dissolved in dimethylsulphoxide (DMSO) at 9.9 mM and 25 mg/mL stock solutions, respectively. Cytochalasin B, DMSO, ethanol, methanol, phenazine methosulfate (PMS) and William's medium E were obtained from Sigma; thiazolyl blue tetrazolium bromide (MTS) was

from Promega, foetal bovine serum (FBS), L-glutamine, penicillin/streptomycin and phosphate buffered saline (PBS) were from PAA Laboratories (Dartmouth, NH, USA). Trypsin was from BD-Difco (Le Pont-De-Claix Cedex, France). Low melting point agarose (LMP), normal melting point agarose (NMP) and TRIzol[®] reagent were purchased from Invitrogen (Paisley, Scotland, UK). Triton X-100 was obtained from Fisher Sciences (New Jersey, USA) and ethidium-bromide solution from Promega (Madison, USA). cDNA High Capacity Archive Kit, TaqMan Universal PCR Master Mix, TaqMan Gene Expression Assays and TaqMan PreAmp Master Mix Kit were derived from Applied Biosystems (New Jersey, USA).

2.2. Cell culture and exposure conditions

HepG2 cells (human hepatoma cell line) were kindly provided by Dr. Firouz Darroudi (Leiden University Medical Centre, Department of Toxicogenetics, Leiden, The Netherlands). The cells were cultured in William's medium E supplemented with 15% FBS, 100 U/mL penicillin/streptomycin and 2 mM L-glutamine at 37 °C in humidified atmosphere with 5% CO₂.

The cells were seeded at densities of 6000 cells/well, 50000 cells/well and 750000 cells/flask onto 96-well plates for the MTS assay, 12-well tissue culture treated plates for the comet assay and 25 cm² culture flasks for CBMN assay and QRT-PCR gene expression analysis (all Corning Costar Corporation, New York, USA), respectively, and incubated for 24 h to attach. Subsequently the cells were exposed to non-cytotoxic (Straser et al., 2011) graded concentrations of CYN (0.01, 0.05, 0.1 and 0.5 µg/mL), single concentration of MCLR (1.0 µg/mL) and their combinations for 4 and 24 h. For gene expression analyses only the highest two concentrations of CYN (0.01 and 0.5 µg/mL) and their combinations with MCLR (1 µg/mL) were comprised. In all experiments negative control (growth medium), solvent control (medium containing 0.05% methanol and 0.1% ethanol) and positive control were included. The final concentrations of the solvents in the medium were adjusted to the same concentration in all experiments at all tested concentrations.

2.3. Cell viability test

The cytotoxicity of single cyanotoxins and their combinations was tested with the MTS assay. MTS and PMS solutions were mixed in 20:1 ratio. The final solution was added 1:5 to treated cells medium after 21 h and incubated for additional 3 h. Subsequently the biological reduction of the tetrazolium to the formazan by viable cells was analysed by measuring the absorbance of the samples and controls at 490 nm using spectrofluorimeter (SinegyMx, BioTek Instruments). Three independent experiments were performed in five replicates for each experimental point. Statistical significance between treated groups and the solvent control was determined by One-way ANOVA, and $p < 0.05$ was considered as significant.

2.4. Alkaline comet assay

The induction of DNA strand breaks after cyanotoxins exposure and their combinations was analysed with comet assay according to Singh et al. (1988) with minor modifications (Zegura and Filipic, 2004). The nuclei were stained with ethidium bromide (5 µg/mL), the images were captured and analysed using a fluorescence microscope (Eclipse 800, Nikon, Japan) and image analysis software Comet IV (Perceptive Instruments, UK). Three independent experiments were performed wherein fifty randomly selected nuclei were analysed per experimental point. The results are expressed as % of tail DNA.

One-way ANOVA was used to analyse the differences in the percentage of tail DNA between treatments. For comparing the exposed groups to the control group Dunnett's Multiple Comparison test was used. $p < 0.01$ was considered as statistically significant.

2.5. Cytokinesis block micronucleus (CBMN) assay

CBMN assay was performed according to Fenech (2000) with minor modifications. After 24 h exposure to cyanotoxins, the cells were incubated for additional 26 h in the presence of cytochalasin B (2 µg/mL). Subsequently the cells were harvested and processed as described in Straser et al. (2011). For the analysis the slides were stained with acridine orange (10 µg/mL), and examined using the fluorescence microscope (Eclipse 800, Nikon, Japan) at 400× magnification according to the criteria published by Fenech (2000) and OECD guidelines (OECD, 2014). Micronuclei (MNI), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were counted in 1000 binucleated cells (BNC) per experimental point. The nuclear division index (NDI) was estimated by scoring 500 cells with one to four nuclei (M₁–M₄) and using calculation $[M_1 + 2M_2 + 3(M_3 + M_4)]/500$. Three independent experiments were performed. The significant difference in the number of MNI, NBUDs and NPBs between treated and control cells was determined with χ^2 test, and the significant difference in the NDI between the treated and control cells was determined by one-way ANOVA.

2.6. The gene expression analysis

The expression of selected genes was analysed by quantitative real time PCR (qPCR). HepG2 cells were exposed to CYN at 0.5 and 0.1 µg/mL and MCLR at 1 µg/mL alone and in combinations for 4 h and 24 h. In each experiment, a solvent control and a positive control (30 µM BaP) were included.

After the exposure the cells were washed with PBS and total mRNA was isolated using TRIzol reagent according to the manufacturer's instructions with minor modifications (Straser et al., 2011). The concentration and purity of isolated mRNA was determined using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, U.S.A.) according to producer's manual.

One µg of total mRNA per sample was reverse transcribed using cDNA High Capacity Archive Kit, following the manufacturer's protocol. Relative quantification of selected genes was performed using qPCR method where TaqMan Universal PCR Master Mix and the following Taqman Gene Expression Assays were used: *CYP1A1* (Hs01054797_g1), *CYP1A2* (Hs00167927_m1), *CYP1B1* (Hs02382916_s1), *CYP3A4* (Hs00604506_m1), *GSTA1* (Hs00275575_m1), *NAT2* (Hs01854954_s1) and *UGT1A1* (Hs02511055_s1) for metabolism assessment; *FOS* (Hs04194186_s1), *JUNB* (Hs00357891_s1), *MYC* (Hs00153408_m1) and *TGFB2* (Hs00234244_m1) for immediate/early response markers and *CDKN1A* (Hs00355782_m1), *CHEK1* (Hs00967506_m1), *ERCC4* (Hs00193342_m1), *GADD45A* (Hs00169255_m1), *MDM2* (Hs00234753_m1) and *TP53* (Hs01034249_m1) for assessment of DNA-damage response. *GADPH* was used as reference gene in all experiments (Human Endogenous Controls, Cat. No. 4310884E, Applied Biosystems, USA). To evaluate the performance of a primer set and to eliminate the effect of inhibition a serial of 10-fold dilutions of each target gene was analysed in control sample. Due to low gene expression and low quantity ($C_t > 38$) determined in preliminary experiments pre-amplification of the following genes, *CYP1A2*, *CYP3A4*, *CYP1B1*, *NAT2*, was performed using TaqMan PreAmp Master Mix Kit according to producer's protocol. The qPCR experiments were performed on Applied Biosystems 7500 Fast Real-Time PCR System machine. Data were analysed using the $\Delta\Delta C_t$ algorithm giving

relative expression (RE) according to solvent control. Difference greater than 1.5-fold was considered as up/down-regulation (relative expression >1.5 or <0.66 , respectively). To obtain fold change from relative expression for down-regulated genes ($RE < 1$) we calculated the inverse value of relative expression ($1/RE$).

3. Results

3.1. Cell viability

The effects of MCLR and CYN as single compounds and their combinations on the viability of HepG2 cells were evaluated after 24 h exposure. MCLR and CYN and the MCLR/CYN mixtures that contained lower concentrations of CYN did not affect the viability of HepG2 cells (Fig. 1). In cells exposed to MCLR (1 $\mu\text{g/mL}$) in combination with CYN (0.5 $\mu\text{g/mL}$) the viability was decreased to 80% compared to solvent control ($p < 0.026$). The viability of cells in the solvent control did not differ significantly from that of non-treated control cells (data not shown). Based on the viability results these non-cytotoxic concentrations of MCLR and CYN as single compounds and in the MCLR/CYN mixtures were used in further experiments for the determination of DNA damage, genomic instability and gene expression analyses.

3.2. Induction of DNA strand breaks

The formation of DNA strand breaks in HepG2 cells exposed to MCLR, CYN or the MCLR/CYN mixtures was analysed after 4 and

24 h exposure with the comet assay (Fig. 2). After 4 h exposure only MCLR (1 $\mu\text{g/mL}$) induced statistically significant increase of DNA strand breaks, whereas CYN as well as the MCLR/CYN mixtures were not effective. After 24 h of exposure the level of MCLR induced DNA strand breaks was not significantly different from the background level, while CYN induced statistically significant increase in the amount of DNA strand breaks at concentrations ≥ 0.05 $\mu\text{g/mL}$. The levels of DNA strand breaks induced by the MCLR/CYN mixtures were lower from those induced by CYN alone and were not statistically significantly different from the background level.

3.3. Induction of genomic stability

The induction of genomic instability by MCLR, CYN and their binary mixtures was analysed after 24 h exposure with the CBMN cytome assay. The results are presented in Fig. 3. MCLR (1 $\mu\text{g/mL}$) alone did not induce any increase in any of the genomic instability parameters and did not reduce the NDI. CYN alone at the highest tested concentration (0.5 $\mu\text{g/mL}$) induced significant, more than 2-fold increase in the frequencies of MNi, NBUDs and NPBs and significantly reduced the NDI by 20% compared to control cells. The MCLR/CYN mixtures induced similar response as CYN alone. The frequencies of MNi, NBUDs and NPBs induced by the binary mixtures as well as NDI were not significantly different from those induced by CYN alone.

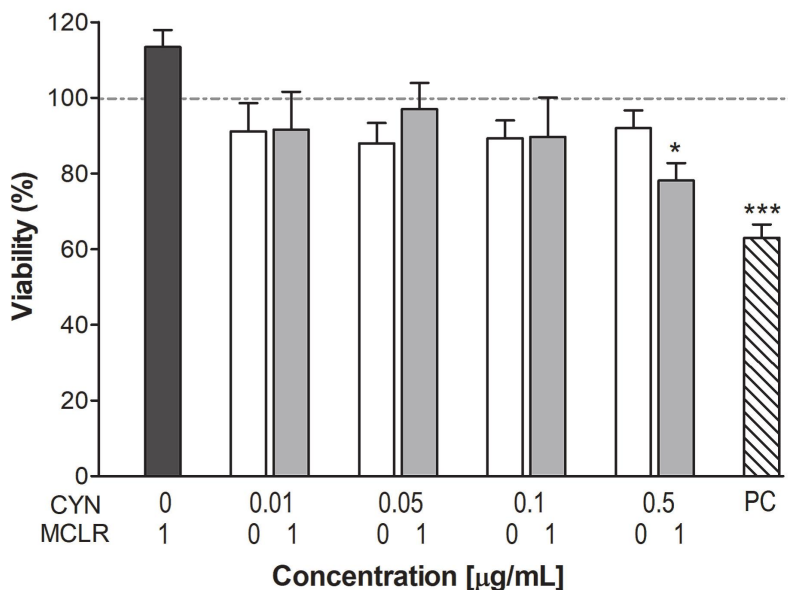


Fig. 1. The effect of MCLR (single concentration, 1 $\mu\text{g/mL}$, [dark grey]), graded concentrations of CYN (0.01–0.5 $\mu\text{g/mL}$, [white]) and MCLR/CYN mixtures [light grey] on the viability of HepG2 cells. Viability was determined with the MTS assay after 24 h exposure. The asterisk denotes a significant difference between solvent control and treated cells (* $p < 0.05$; *** $p < 0.001$). Positive control (PC): 30 $\mu\text{g/mL}$ ET.

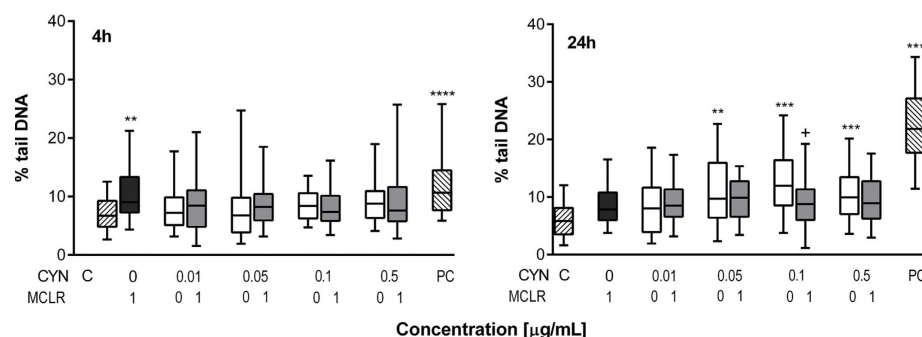


Fig. 2. The effect of MCLR (single concentration; 1 µg/mL, [dark grey]), graded concentrations of CYN (0.01–0.5 µg/mL, [white]) and MCLR/CYN mixtures [light grey] on the formation of DNA strand breaks in HepG2 cells. Percentage of DNA in comet tail was assessed after 4 h and 24 h of exposure. The asterisk (*) denotes statistically significant difference between solvent control and MCLR/CYN exposed groups (* $p < 0.01$, ** $p < 0.001$; *** $p < 0.0001$); (+) denotes significant difference between CYN groups treated in the presence or absence of MCLR (+ $p < 0.01$). Solvent control (C); Positive control (PC): 30 µM BaP.

3.4. Effect of MCLR, CYN and the MCLR/CYN mixtures on gene transcription

The mRNA expression of selected genes was analysed after 4 and 24 h of exposure to 1 µg/mL MCLR, 0.1 and 0.5 µg/mL CYN and their combinations by qPCR. The values presented in Table 1 are relative quantities of mRNA of selected genes in exposed groups compared to solvent control group.

3.4.1. Transcription of genes involved in the metabolism

After 4 h exposure of HepG2 cells to the two cyanotoxins and their mixtures the expression of most of the selected genes involved in the metabolism was not significantly different from that of the control group. The exception was 1.8-fold down-regulation (relative expression 0.56) of *CYP3A4* in cells exposed to the MCLR/CYN mixture with 0.1 µg/mL CYN and 1 µg/mL MCLR. After 24 h of exposure, the expression of *CYP1A1* and *CYP1A2* was 2.15 and 1.8-fold up-regulated, respectively, in cells incubated with 0.5 µg/mL CYN as well as its combination with 1 µg/mL MCLR compared to control. A 1.6-fold down-regulated expression of *CYP1B1* compared to control was observed in cells exposed to the MCLR/CYN mixture with 0.5 µg/mL CYN and 1 µg/mL MCLR, while the expression of *CYP3A4* was not deregulated. From the group of phase II enzymes, the expression of *NAT2* was 2.3-fold down-regulated (relative expression < 0.43) upon exposure to higher concentration of CYN alone and in combination with MCLR. In addition, 1.5-fold down-regulation of *NAT2* was determined in cells exposed to 1 µg/mL MCLR. However, the difference between the effect of 0.5 µg/mL CYN alone and a mixture with 1 µg/mL MCLR was not significant. The expressions of *GSTA1* and *UGT1A1* mRNA were not affected by the exposure to the two cyanotoxins or their mixtures.

3.4.2. Transcription of genes involved in immediate-early response/ signalling

After 4 h treatment, the expression of *FOS* was up-regulated in cells exposed to CYN (0.1 and 0.5 µg/mL) alone (1.6-fold and 1.9-fold, respectively) and the MCLR/CYN mixtures (1.5-fold and 2.1-fold, respectively). After 24 h treatment at the higher concentration CYN alone and the MCLR/CYN mixture induced up-regulation of *FOS* (2.3-fold and 2.7-fold, respectively). The expression of *JUNB* was not deregulated in the short time exposure experiments;

whereas, after 24 h it's expression was up-regulated by 3.3- and 2.5-fold in cells exposed to 0.5 µg/mL CYN alone and to the MCLR/CYN mixture, respectively. The expression of *TGFB2* was, upon short time exposure, down-regulated by MCLR alone (1.6-fold) and in combination with 0.1 µg/mL CYN (1.9-fold). After 24 h-exposure the expression of *TGFB2* remained 1.7-fold down-regulated in cells exposed to MCLR, whereas in cells exposed to 0.5 µg/mL CYN it was 2.0-fold up-regulated. The MCLR/CYN mixture up-regulated the expression of *TGFB2* (1.7-fold), but to a lesser extent than CYN alone (2.0-fold). The expression of *MYC* was not deregulated after the exposure to cyanotoxins.

3.4.3. Transcription of genes involved in DNA damage response

After 4 h exposure to the two cyanotoxins and their mixtures none of the genes involved in DNA damage response was affected. After 24 h exposure to 0.5 µg/mL CYN alone up-regulated the expressions of the *CDKN1A*, *GADD45A* and *ERCC4* (2.0-, 2.8- and 2.5-fold, respectively) and slightly less in combination with 1 µg/mL MCLR (1.9-, 2.2- and 2.4-fold, respectively). *MDM2* gene was up-regulated (1.5-fold) only by 0.5 µg/mL CYN. The gene expression of *CHEK1* and *TP53* was after the exposure to cyanotoxins not affected.

4. Discussion

Cyanobacterial blooms have been recognized as a serious threat to human health and the environment since their occurrence is related to the presence of their toxic secondary metabolites - cyanotoxins. MCLR and recently CYN are among the most studied cyanotoxins as they frequently occur in cyanobacterial blooms worldwide. There are more and more reports of their simultaneous occurrence (Brient et al., 2009; Jancula et al., 2014; Oehrlé et al., 2010) as well as co-occurrence of their producers (Bláhová et al., 2009; Kokociński et al., 2009; Miller and McMahon, 2011), which is consequently associated with increased risk of co-exposure to both cyanotoxins. In the literature, the evidence of MCLR and CYN genotoxicity has been well documented (Buratti et al., 2017; Zegura et al., 2011c), while in the present study for the first time we report their combined genotoxic effects in target hepatic cells.

It is known that MCLR and CYN induce DNA strand breaks *in vitro* and *in vivo* (Zegura et al., 2011c); however, the mechanisms

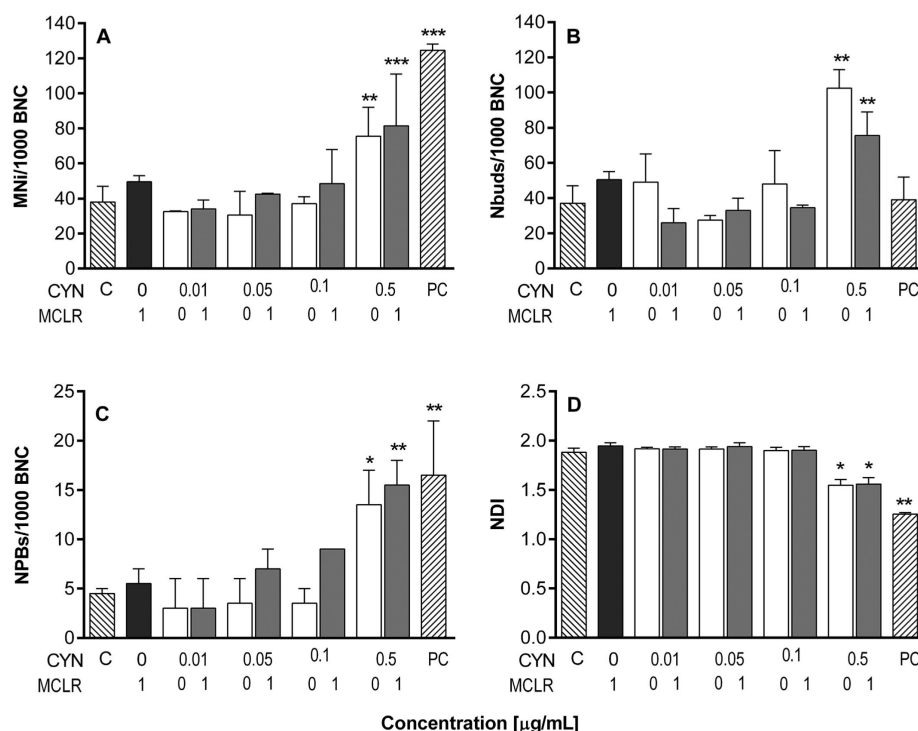


Fig. 3. Induction of micronuclei (MNI; A), nuclear buds (NBUDs; B), nuclear bridges (NPBs; C) and reduction of nuclear division (NDI; D) in HepG2 cells after 24 h exposure to MCLR (1 µg/mL), CYN (0.01–0.5 µg/mL) and MCLR/CYN mixtures determined with the cytokinesis-block micronucleus (CBMN) assay. The numbers of MNI, NBUDs and NPBs were determined in 1000 binucleated cells per experimental point in each of the three independent experiments. The significant difference in the number of MNI, NBUDs and NPBs between treated and control cells was determined with χ^2 test (* P < 0.01, ** P < 0.001, *** P < 0.0001). The nuclear division index (NDI) was determined by scoring 500 cells with one to four nuclei. The significant difference between treated and control cells was determined by One-way ANOVA (* p < 0.01 and ** p < 0.001). Positive control (PC): 2.5 µM BaP.

of their genotoxic activity differ. MCLR induces DNA damage indirectly through intracellular production of reactive oxygen species resulting in oxidation of DNA bases (Zegura et al., 2004; Zegura, 2016), while CYN is pro-genotoxic and requires metabolic activation to electrophilic intermediates that induce DNA damage (Bazin et al., 2010; Humpage et al., 2005; Straser et al., 2011). The results of the present study corroborate with previously published data, showing that MCLR induces transiently present DNA strand breaks after short term exposure probably due to oxidative stress (Palus et al., 2007; Zegura et al., 2008, 2003), while CYN induces DNA strand breaks after longer exposure in metabolically active cells (Humpage et al., 2005; Straser et al., 2011). The results of the comet assay with the MCLR/CYN mixture demonstrated lower levels of DNA strand breaks from the levels induced by MCLR after 4 h exposure and CYN after 24 h exposure, respectively. The observed lower DNA damage in combined exposure could be due to the attenuated DNA repair by MCLR as from the literature it is known that MCLR can compromise DNA repair mechanisms (Lankoff et al., 2006a,b).

In the next step, we studied single and combined effects of MCLR and CYN on genomic stability of HepG2 cells. In HepG2 cells MCLR alone, at the tested concentration, did not induce genomic instability, while CYN induced significant increase in genomic instability at 0.5 µg/mL. The result is in line with previous studies. It has been reported that at low concentrations equivalent to those naturally occurring in cyanobacterial blooms MCLR does not induce MNI formation (Abramsson-Zetterberg et al., 2010; Lankoff et al., 2006b), while at higher concentrations (up to 80 µg/mL) induction of MNI was reported in human TK6 cells (Zhan et al., 2004) and Vero cells (Dias et al., 2014). For CYN it has been reported that it induces MNI formation *in vitro* in metabolically active cells (Bazin et al., 2010; Humpage et al., 2000a, b; Straser et al., 2011), while it does not affect chromosomal stability in CHO-K1 cells (Lankoff et al., 2007). The MCLR/CYN mixtures induced genomic instability did not differ significantly from that induced by CYN alone indicating that MCLR has no effect on CYN induced genomic instability.

To our knowledge, the present study is the first where the influence of the combination of MCLR and CYN on the mRNA

Table 1

The mRNA expression of selected genes involved in the metabolism, immediate-early response/signalling and DNA damage response in HepG2 cells after 4 and 24 h exposure to single concentration of MCLR (1 µg/mL), two concentrations of CYN (0.1 and 0.5 µg/mL) and MCLR/CYN mixtures.

Mechanism involved	Gene symbol	Treatment (µg/mL)	4 h Mean ± SD	24 h Mean ± SD	Entrez gen description
Metabolism (activation, detoxification)	<i>CYP1A1</i>	1 MCLR	1.03 ± 0.04	1.00 ± 0.09	cytochrome P450 family 1 subfamily A member 1
		0.1 CYN	1.15 ± 0.03	1.13 ± 0.18	
		0.5 CYN	1.13 ± 0.04	2.15 ± 0.46	
		1 MCLR/0.1CYN	1.08 ± 0.14	1.13 ± 0.17	
		1 MCLR/0.5CYN	1.15 ± 0.14	2.15 ± 0.39	
		B(a)P 30 µM	2.96 ± 1.14	390.50 ± 53.86*	
	<i>CYP1A2</i>	1 MCLR	0.89 ± 0.07	1.16 ± 0.82	cytochrome P450 family 1 subfamily A member 2
		0.1 CYN	0.87 ± 0.01	1.03 ± 0.42	
		0.5 CYN	0.83 ± 0.07	1.86 ± 0.72	
		1 MCLR/0.1CYN	0.89 ± 0.20	0.76 ± 0.31	
		1 MCLR/0.5CYN	0.94 ± 0.05	1.82 ± 0.57	
		B(a)P 30 µM	1.36 ± 0.36	22.20 ± 1.50**	
	<i>CYP3A4</i>	1 MCLR	0.98 ± 0.13	0.73 ± 0.37	cytochrome P450 family 3 subfamily A member 4
		0.1 CYN	1.04 ± 0.26	0.75 ± 0.45	
		0.5 CYN	0.73 ± 0.06	0.71 ± 0.31	
		1 MCLR/0.1CYN	0.56 ± 0.21	1.06 ± 0.11	
		1 MCLR/0.5CYN	0.72 ± 0.37	0.72 ± 0.14	
		B(a)P 30 µM	0.74 ± 0.19	2.59 ± 1.09	
	<i>CYP1B1</i>	1 MCLR	0.92 ± 0.25	0.70 ± 0.12	cytochrome P450 family 1 subfamily B member 1
		0.1 CYN	0.77 ± 0.05	0.78 ± 0.14	
		0.5 CYN	0.75 ± 0.05	0.82 ± 0.12	
		1 MCLR/0.1CYN	0.66 ± 0.29	1.02 ± 0.66	
		1 MCLR/0.5CYN	0.79 ± 0.35	0.61 ± 0.22	
		B(a)P 30 µM	3.28 ± 1.23	38.22 ± 2.73**	
	<i>NAT2</i>	1 MCLR	0.92 ± 0.46	0.61 ± 0.29	N-acetyltransferase 2
		0.1 CYN	0.91 ± 0.11	0.70 ± 0.25	
		0.5 CYN	0.94 ± 0.24	0.42 ± 0.26	
		1 MCLR/0.1CYN	0.90 ± 0.43	1.20 ± 0.05	
		1 MCLR/0.5CYN	0.90 ± 0.12	0.43 ± 0.03**	
		B(a)P 30 µM	0.94 ± 0.17	0.76 ± 0.08	
	<i>GSTA1</i>	1 MCLR	1.09 ± 0.12	1.08 ± 0.27	glutathione S-transferase alpha 1
		0.1 CYN	1.06 ± 0.04	0.91 ± 0.09	
		0.5 CYN	1.01 ± 0.01	0.82 ± 0.35	
		1 MCLR/0.1CYN	1.00 ± 0.06	0.90 ± 0.12	
		1 MCLR/0.5CYN	1.01 ± 0.07	0.73 ± 0.35	
		B(a)P 30 µM	0.92 ± 0.07	0.44 ± 0.55	
	<i>UGT1A1</i>	1 MCLR	0.90 ± 0.20	0.84 ± 0.02	UDP glucuronosyltransferase 1 family, polypeptide A1
		0.1 CYN	0.95 ± 0.13	0.92 ± 0.07	
		0.5 CYN	1.03 ± 0.08	1.19 ± 0.04	
		1 MCLR/0.1CYN	0.93 ± 0.14	0.90 ± 0.01	
		1 MCLR/0.5CYN	1.02 ± 0.03	1.11 ± 0.00	
		B(a)P 30 µM	1.22 ± 0.06	4.22 ± 0.95*	
Immediate-early response/signaling	<i>FOS</i>	1 MCLR	0.95 ± 0.06	0.93 ± 0.27	FBJ murine osteosarcoma viral oncogene homolog
		0.1 CYN	1.60 ± 0.62	1.41 ± 0.51	
		0.5 CYN	1.92 ± 0.72	2.27 ± 1.41	
		1 MCLR/0.1CYN	1.52 ± 0.72	1.11 ± 0.54	
		1 MCLR/0.5CYN	2.08 ± 0.52	2.71 ± 1.66	
		B(a)P 30 µM	2.78 ± 0.08***	109.52 ± 34.15*	
	<i>JUNB</i>	1 MCLR	1.20 ± 0.14	0.95 ± 0.05	jun B proto-oncogene
		0.1 CYN	0.93 ± 0.13	0.90 ± 0.18	
		0.5 CYN	1.11 ± 0.21	3.27 ± 0.08**	
		1 MCLR/0.1CYN	1.03 ± 0.01	0.91 ± 0.07	
		1 MCLR/0.5CYN	1.40 ± 0.23	2.52 ± 0.11**	
		B(a)P 30 µM	1.46 ± 0.12	6.64 ± 1.14*	
	<i>MYC</i>	1 MCLR	0.98 ± 0.20	0.95 ± 0.01	v-myc avian myelocytomatosis viral oncogene homolog
		0.1 CYN	0.97 ± 0.17	0.89 ± 0.04	
		0.5 CYN	0.96 ± 0.17	0.75 ± 0.08	
		1 MCLR/0.1CYN	0.94 ± 0.14	0.89 ± 0.00	
		1 MCLR/0.5CYN	1.02 ± 0.10	0.72 ± 0.14	
		B(a)P 30 µM	0.80 ± 0.00	0.21 ± 0.06***	
	<i>TGFB2</i>	1 MCLR	0.61 ± 0.16	0.58 ± 0.23	transforming growth factor beta 2
		0.1 CYN	1.12 ± 0.20	0.89 ± 0.53	
		0.5 CYN	0.96 ± 0.06	2.02 ± 1.38	
		1 MCLR/0.1CYN	0.52 ± 0.11*	0.85 ± 0.66	
		1 MCLR/0.5CYN	0.79 ± 0.31	1.74 ± 0.64	
		B(a)P 30 µM	1.06 ± 0.05	0.81 ± 0.03	
DNA damage responsive	<i>TP53</i>	1 MCLR	1.07 ± 0.10	1.21 ± 0.24	tumour protein p53
		0.1 CYN	1.14 ± 0.03	1.02 ± 0.06	
		0.5 CYN	1.02 ± 0.10	1.01 ± 0.13	
		1 MCLR/0.1CYN	1.02 ± 0.06	1.01 ± 0.08	
		1 MCLR/0.5CYN	1.04 ± 0.01	0.95 ± 0.07	

(continued on next page)

Table 1 (continued)

Mechanism involved	Gene symbol	Treatment ($\mu\text{g/mL}$)	4 h Mean $\pm$ SD	24 h Mean $\pm$ SD	Entrez gen description
	<i>MDM2</i>	B(a)P 30 μM	1.02 $\pm$ 0.05	1.14 $\pm$ 0.21	MDM2 proto-oncogene. E3 ubiquitin protein ligase
		1 MCLR	1.08 $\pm$ 0.00	1.02 $\pm$ 0.07	
		0.1 CYN	1.30 $\pm$ 0.27	1.16 $\pm$ 0.38	
		0.5 CYN	1.17 $\pm$ 0.17	1.53 $\pm$ 0.32	
		1 MCLR/0.1 CYN	1.13 $\pm$ 0.10	1.19 $\pm$ 0.09	
	<i>CDKN1A</i>	1 MCLR/0.5 CYN	1.23 $\pm$ 0.28	1.28 $\pm$ 0.12	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
		B(a)P 30 μM	0.85 $\pm$ 0.05	0.60 $\pm$ 0.02**	
		1 MCLR	1.13 $\pm$ 0.15	1.00 $\pm$ 0.04	
		0.1 CYN	1.20 $\pm$ 0.09	0.98 $\pm$ 0.04	
		0.5 CYN	1.08 $\pm$ 0.07	2.04 $\pm$ 0.25*	
	<i>GADD45A</i>	1 MCLR/0.1 CYN	1.11 $\pm$ 0.09	1.05 $\pm$ 0.09	growth arrest and DNA damage inducible alpha
		1 MCLR/0.5 CYN	1.08 $\pm$ 0.11	1.87 $\pm$ 0.32	
		B(a)P 30 μM	1.74 $\pm$ 0.31	19.38 $\pm$ 2.33*	
		1 MCLR	1.37 $\pm$ 0.41	1.00 $\pm$ 0.13	
		0.1 CYN	1.38 $\pm$ 0.62	1.03 $\pm$ 0.20	
	<i>CHEK1</i>	0.5 CYN	1.34 $\pm$ 0.58	2.79 $\pm$ 0.50*	checkpoint kinase 1
		1 MCLR/0.1 CYN	1.21 $\pm$ 0.27	0.90 $\pm$ 0.02	
		1 MCLR/0.5 CYN	1.18 $\pm$ 0.27	2.20 $\pm$ 0.71	
		B(a)P 30 μM	1.49 $\pm$ 0.29	5.84 $\pm$ 0.27**	
		1 MCLR	0.93 $\pm$ 0.12	0.99 $\pm$ 0.04	
	<i>ERCC4</i>	0.1 CYN	0.90 $\pm$ 0.06	0.93 $\pm$ 0.11	excision repair cross-complementation group 4
		0.5 CYN	0.94 $\pm$ 0.09	0.88 $\pm$ 0.01	
		1 MCLR/0.1 CYN	0.92 $\pm$ 0.11	0.94 $\pm$ 0.06	
		1 MCLR/0.5 CYN	1.00 $\pm$ 0.04	0.85 $\pm$ 0.12	
		B(a)P 30 μM	0.94 $\pm$ 0.09	0.46 $\pm$ 0.03**	
		1 MCLR	0.93 $\pm$ 0.09	1.07 $\pm$ 0.05	
		0.1 CYN	0.96 $\pm$ 0.01	1.48 $\pm$ 0.31	
		0.5 CYN	1.16 $\pm$ 0.00	2.51 $\pm$ 0.48*	
		1 MCLR/0.1 CYN	0.94 $\pm$ 0.07	1.31 $\pm$ 0.20	
		1 MCLR/0.5 CYN	1.43 $\pm$ 0.34	2.40 $\pm$ 0.16**	
		B(a)P 30 μM	0.92 $\pm$ 0.05	2.20 $\pm$ 0.10**	

The results are shown as relative mRNA expression normalized to the solvent control group. Data are means $\pm$ SD of two independent experiments each performed in two replicates. Bold values indicate up- or down-regulation of genes with the threshold set on 1.5-fold, which is more than 1.5 or less than 0.66 relative expression, respectively. Statistically significant differences between cells treated with MCLR, CYN or MCLR/CYN mixtures and the solvent control group is indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's t-test).

expression of genes involved in the metabolism of xenobiotics, immediate/early response, and DNA-damage response has been evaluated. There is evidence that human cytochrome P450 (CYP P450s) enzymes, a superfamily of hemoproteins that catalyze the metabolism of xenobiotics, are involved in the metabolism of CYN (Humpage et al., 2005), while MCLR, according to the literature data is not metabolically transformed to an active form (Zegura et al., 2011c). The most important P450 enzymes that in humans participate in the metabolism of the majority of xenobiotics and are involved in the bio-activation of several pro-carcinogens to reactive metabolites are CYP1A1, CYP1A2 and CYP1B1 and CYP3A4 (Gajjar et al., 2012). The results of the present study suggest the involvement of CYP1A1 and CYP1A2 in CYN metabolism, which is in accordance with our earlier findings (Straser et al., 2013b, 2011; Zegura et al., 2011a). As expected MCLR alone did not affect the expression of genes coding for CYP 450 enzymes. In the literature only CYP2E1 was reported to be involved in MCLR metabolism (Zhang et al., 2015); this enzyme has a role in reactive oxygen species formation resulting in cell damage including DNA damage and carcinogenicity (Koop, 1992). The MCLR/CYN binary mixture induced changes in the expression of CYPs was comparable to those induced by CYN alone confirming that MCLR does not affect the expression of these enzymes.

The phase II enzymes are generally responsible for detoxification of xenobiotics; however, depending on the properties of the substrate their activity can also results in bio-activation of xenobiotics (Grillo, 2012). Here, we analysed three genes belonging to phase II enzymes (*GSTA1*, *NAT2*, and *UGT1A1*) wherein the only

observed deregulation was down-regulation of *NAT2* by CYN and MCLR alone as well as by their combination after 24 h of exposure. CYN induced deregulation of *NAT2* was also reported from Stel-IARay experiment, where in contrary to the results obtained in the present study it was upregulated for 1.94-fold, while similarly as shown in the present study, CYN had no significant effect on *UGT1A1* gene (Straser et al., 2013b).

One of the main pathways activated in the immediate-early response to stress signals, infections, as well as oncogenic stimuli is gene regulation through activator protein 1 (AP-1); the transcription factor, which is composed of JUN and FOS protein dimers, recognized as homologues of retroviral onco-proteins (Hess et al., 2004). *MYC* is another oncogene recognized as key regulator of cell proliferation, and its deregulation is related to the genesis of most human tumours (Adhikary and Eilers, 2005). Straser et al. (2013b) reported that in HepG2 cells CYN up-regulated the expression of *FOS* and *JUN* family genes as well as *TGFB2* after 12 h and 24 h incubation. Likewise, with the same CYN concentration (0.5 $\mu\text{g/mL}$) and time-point (24 h) we detected similar up-regulation of these genes. Moreover, we showed that in co-exposure with MCLR gene expression of *FOS*, *JUN* and *MYC* did not significantly differ from the gene expression caused by CYN alone at corresponding concentration. Additionally, we observed up-regulation of *FOS* in all samples containing CYN already after 4 h exposure, while MCLR alone did not deregulate *FOS*, *JUN* or *MYC* transcription under the experimental conditions applied in the study. On the contrary to the results of the present study it has been reported that MCLR induced up-regulation of proto-oncogenes

from *FOS* and *JUN* family as well as *MYC* at transcriptional and protein level in primary rat hepatocytes (Sueoka et al., 1997) and in *in vivo* studies on rats (Clark et al., 2007; Li et al., 2009). *C-FOS* and *JUN-B* are implicated in many important cell processes, including proliferation, differentiation and tumorigenesis (Hess et al., 2004); however, to our knowledge there is no existing literature data about the influence of MCLR on the regulation of these genes in human cells. The transcription profile of *FOS* has also been shown to differentiate between DNA-reactive and DNA-nonreactive genotoxic mechanisms (Dickinson et al., 2004). Therefore, we can confirm previous findings on genotoxic properties of CYN, but based on present results we observed no additive, synergistic or antagonistic effects of its co-exposure with MCLR at applied conditions. On the other hand, at both studied time-points MCLR down-regulated transcription of *TGFβ2*, encoding transforming growth factor with cytostatic effects that are important in tissue regeneration and homeostasis (Massagué, 2012). CYN alone caused up-regulation of *TGFβ2* after longer exposure similarly as previously published by Straser et al. (2013b). Therefore, in the case of *TGFβ2* we cannot exclude the influence of co-exposure on gene regulation, since the quantity of transcript was lower in samples treated with mixtures of both toxins compared to CYN alone although the difference was not statistically significant. In the context of tumour progression, the function of *TGFβ* depends on developmental state of tumour – it can act as a powerful tumour suppressor or an enhancer of invasion and metastasis (Massagué, 2012). According to complex mechanisms of *TGFβ2* regulation, the adverse de-regulative effect obtained with MCLR and CYN is not surprising and would be interesting for further research.

DNA damage triggers the p53 network activation leading to transcription of p53 downstream regulated genes, which, directly or indirectly, leads to cell death or the inhibition of cell proliferation (Vogelstein et al., 2000). The main mechanism of raising the level of p53 in stressed cell is the inhibition of its degradation rather than initiation of synthesis. Therefore it is not unusual that, upon exposure to non-cytotoxic concentrations of CYN or MCLR, no change in *TP53* mRNA expression were observed in the present as well as previous studies (Straser et al., 2013b, 2011; Zegura et al., 2008). MDM2, one of the enzymes involved in degradation of tumour suppressor p53 that stimulates cell cycle progression and enhances tumorigenic potential (Wade et al., 2013) was up-regulated by the highest concentration of CYN, similarly to results of Straser et al. (2011), but not in the combination with MCLR. Checkpoint kinase 1 (*CHEK1*) plays role in normal cell cycle regulation as well as a mediator of DNA damage response resulting in activation of DNA damage check points, cell cycle arrest, DNA repair and/or apoptosis (Patil et al., 2013). Deregulation of *CHEK1* expression was not detected in the present study at any experimental condition, while up-regulation by 0.5 µg/mL CYN was reported by Straser et al. (2013b).

Three genes involved in DNA damage response (*CDKN1A*, *GADD45A* and *ERCC4*), were up-regulated upon 24 h exposure to 0.5 µg/mL CYN and its mixture with MCLR in a very similar manner, while their expression was not affected by MCLR alone. Cyclin-dependent kinase inhibitor p21 encoded by *CDKN1A* plays an important role in p53 mediated DNA damage response by preventing G1-S transition, and inhibiting DNA replication. Moreover, it is involved in DNA repair mechanisms (Cazzalini et al., 2010). *GADD45A* gene is inducible by DNA-reactive genotoxins (Dickinson et al., 2004). Besides its product is importantly involved in the cell cycle control at G2-M checkpoint, induction of apoptosis and DNA repair (Fornace et al., 1992). *ERCC4* acts as a crucial part of nucleotide excision repair (NER) of DNA, induced by DNA damaging agents that cause covalent helix-distorting adducts (Manandhar et al., 2015). Previously it was reported that in HepG2 cells CYN

caused up-regulation of the expression of these genes as well as cell cycle arrest (Straser et al., 2013a, 2011). On the contrary to the present study MCLR has been reported to up-regulate expression of *CDKN1A* and *GADD45A* in HepG2 cells, however not for more than 1.5-fold compared to control group (Zegura et al., 2008). The results obtained with the MCLR/CYN mixtures show no significant effect of MCLR on deregulation of gene expression caused by CYN alone.

Taken together, exposure of HepG2 cells to CYN alone resulted in the changes in the expression of genes involved in xenobiotic metabolism (*CYP1A1*, *CYP1A2*, *NAT2*), genes involved in immediate-early response/signalling (*FOS*, *JUN* and *TGFβ2*) and DNA damage response (*MDM2*, *CDKN1A*, *GADD45A* and *ERCC4*), while exposure to MCLR alone down-regulated the expression of *NAT2* and *TGFβ2*. The pattern of the changes in gene expression induced by the exposure to the binary MCLR/CYN mixture did not differ significantly from that induced by CYN alone. This indicates that MCLR has no effect on CYN induced deregulation of the selected genes that reflect the mechanisms of its pro-genotoxic activity and also explains no effect of MCLR on CYN induced genomic instability.

5. Conclusions

MCLR and CYN are two cyanobacterial toxins that in the environment often occur simultaneously. Both toxins are genotoxic; however, MCLR predominantly induces oxidative DNA damage, while CYN requires metabolic activation to electrophilic intermediates that cause DNA damage. In HepG2 cells exposed to low, non-cytotoxic concentrations MCLR induced DNA strand breaks that were only transiently present after 4 h exposure, whereas CYN, after 24 h exposure, induced DNA strand breaks and genomic instability. The binary MCLR/CYN mixture induced DNA strand breaks after 4 and 24 h exposure; however, their levels were lower from those induced by MCLR or CYN alone, indicating an antagonistic interaction. On the other hand, induction of genomic instability by MCLR/CYN mixture was comparable to that induced by CYN alone. In addition, the changes in the expression of selected genes involved in xenobiotic metabolism, genes involved in immediate-early response/signalling and DNA damage responsive genes induced by the MCLR/CYN mixture were not significantly different from those induced by CYN alone. Based on these results we conclude that at concentrations that are relevant for environmental contamination CYN represent higher genotoxic hazard than MCLR and that the genotoxic hazard of the MCLR/CYN mixture is comparable to that of CYN alone. However, MCLR is a well-known tumour promoter classified as possible human carcinogen, while evidence indicates that CYN may act as tumour initiator; therefore, it cannot be excluded that in combination they may represent higher carcinogenic hazard, which remains to be explored.

Conflicts of interest

None.

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2.4 Genotoxic Activity of Bisphenol A and its Analogues Bisphenol S, Bisphenol F and Bisphenol AF and their Mixtures in Human Hepatocellular Carcinoma (HepG2) Cells

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Genotoxic activity of bisphenol A and its analogues bisphenol S, bisphenol F and bisphenol AF and their mixtures in human hepatocellular carcinoma (HepG2) cells

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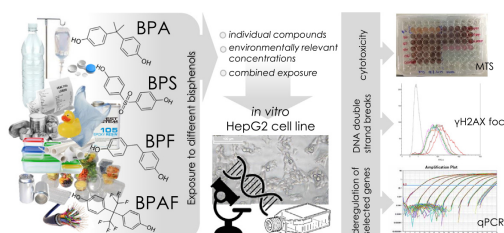
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HIGHLIGHTS

- BPF and BPAF showed higher cytotoxic/genotoxic potential than BPA in HepG2 cells.
- BPS showed the lowest cytotoxic/genotoxic potential compared to BPA.
- At low environmentally relevant concentrations, BPs did not induce DNA damage.
- At combined exposure, BPs induced additive effects in the expression of specific genes.
- Not all BPA analogues present safer replacement for BPA.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of bisphenol A (BPA) in manufacturing of plastics is being gradually replaced by presumably safer analogues such as bisphenol S (BPS), bisphenol F (BPF) and bisphenol AF (BPAF). Despite their widespread occurrence in the environment, there is a knowledge gap in their toxicological profiles. We investigated cytotoxic/genotoxic effects as well as changes in the expression of selected genes involved in the xenobiotic metabolism, response to oxidative stress and DNA damage upon exposure to BPs and their mixtures in human hepatocellular carcinoma HepG2 cells.

BPS and BPF slightly decreased the viability of HepG2 cells, while BPAF was the most cytotoxic compound tested. BPA, BPF and BPAF induced the formation of DNA double strand breaks determined with γH2AX assay, while BPS was inactive (5–20 μg/mL). All four BPs up-regulated the expression of *CYP1A1* and *UGT1A1*, while BPS up-regulated and BPAF down-regulated also the expression of *GST1A*. Only BPA up-regulated oxidative stress responsive gene *GCLC*, while BPAF up-regulated the expression of *CDKN1A* and *GADD45a*.

At concentrations relevant for human exposure (ng/mL range) BPA and its analogues as individual compounds and in mixtures did not exert genotoxic activity, whereas BPA and BPAF as well as the mixtures up-regulated the expressions of *CYP1A1* and *UGT1A1*.

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1. Introduction

Bisphenol A (BPA) is one of the most widely used materials in the production of polycarbonate plastics, epoxy resins and phenolic resin in the manufacture of a variety of consumer products such as plastic food packaging products, can linings, baby feeding bottles, medical tubings, toys, water pipes, dental sealants, eyeglass lenses, several paper products etc. (Park et al., 2018; Vandenberg et al., 2007). The global consumption of BPA is estimated to be around 8 million metric tons in 2016, and the global BPA demand is projected to increase to 10.6 million tons by 2022 (Lehmler et al., 2018). Consequently, it is ubiquitously present in the environment, which causes huge concern as many studies reported that BPA induces adverse environmental and human health effects. It is considered that food is the major contributor to the total exposure to BPA for most population groups (EFSA, 2015), however, recently thermal paper has been identified as important source of exposure through dermal transfer (Björnsdóttir et al., 2017; Eckardt and Simat, 2017). In 2017 The European Chemical Agency (ECHA) added BPA to the Candidate List of substances of very high concern. Due to its known endocrine disrupting potential the use of BPA has been banned for the manufacturing of child care products including baby bottles in European Union, the United States and Canada (Lehmler et al., 2018). In the EU, the use of BPA in thermal paper has been limited since 2016 and the ban will come into force in 2020. Since March 2018 products containing BPA must be classified and label as toxic to reproduction. The industry responded to these restrictions by development and gradual replacement of BPA by its chemical analogues such as bisphenol S (BPS), bisphenol F (BPF) and bisphenol AF (BPAF), presumably as safer alternatives to BPA.

Bisphenols (BPs) are a class of chemicals known as diphenylmethanes, which contain two benzene rings separated by one central carbon atom, usually with a 4-OH substituent on both benzene rings. BPS, BPF and BPAF have similar chemical structure as BPA (Table 1). BPS is the most commonly used replacement monomer in BPA-free products (Chen et al., 2016; Qiu et al., 2019). BPS became ubiquitous in various consumer products and environmental compartments although generally its concentrations are lower than concentrations of BPA (Wu et al., 2018; Zhang et al., 2019). BPF has a broad range of industrial applications such as lacquers, varnishes, liners, adhesives plastics, water pipe and is also used in dental sealants, oral prosthetic devices, tissue substitutes and coatings for food packaging (Liao and Kannan, 2013). BPAF is used as a cross-linker in fluoroelectronics, electronics and optical fibers, and as a high-performance monomer specialty polymers including membranes and plastic optical fibers (for review see (Chen et al., 2016)).

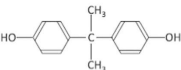
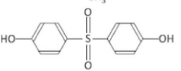
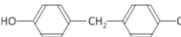
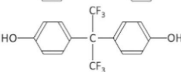
Massive production of BPs and broad spectrum of their use results in their presence in the environment. The average concentrations of BPA detected in surface waters are mostly around 100 ng/L but individual

measurements up to 44,000 ng/L were recorded (Corrales et al., 2015). There is less data about the concentrations of BPA analogues in the environment but they are in the range between 1 and 100 ng/L; however, even 100-fold higher concentrations were detected downflow from industrial effluents (Yamazaki et al., 2015). In humans BPA was detected in >90% of tested urine samples with average concentration 2600 ng/L (Calafat et al., 2008), while its uptake is estimated to 50 ng/kg bw/day (Huang et al., 2017). BPA analogues are detected in urine less frequently and in lower concentrations; however, they occur simultaneously in the same samples (Ye et al., 2015).

The information about toxicological properties and adverse health impacts of BPA is quite comprehensive, while such information for BPA analogues is limited. Due to structural similarities and physico-chemical properties, it can be hypothesized that analogues might exhibit similar toxicological profile and similar or even stronger toxic potential as BPA. Related to the endocrine disrupting potential the studies demonstrated that BPS and BPF exhibited estrogenic, anti-estrogenic, androgenic, and anti-androgenic activities comparable to those of BPA (Björnsdóttir et al., 2017; Cabaton et al., 2009; Chen et al., 2016; Perera et al., 2017), while for BPAF it was reported that it has even stronger estrogenic and anti-androgenic potential than BPA (Chen et al., 2016; Fic et al., 2014; Usman and Ahmad, 2016). Another concern is potential genotoxicity of BPs (for review see Usman and Ahmad, 2016). The mechanisms of BPA genotoxicity have not been fully elucidated, while for its analogues the data on their genotoxic activities are scarce. BPA does not induce gene mutations in bacteria (Fic et al., 2013; Schweikl et al., 1998; Tiwari et al., 2012; Xin et al., 2015) and in mammalian cells *in vitro* (Schweikl et al., 1998; Tsutsui et al., 1998), while evidence is accumulating that it induces DNA and chromosomal damage. BPA and its metabolites have been shown to form DNA adducts *in vitro* (Kolšek et al., 2012; Tsutsui et al., 1998; Wu et al., 2017; Xin et al., 2015) and *in vivo* (Izzotti et al., 2009), to induce DNA strand breaks *in vitro* (Fic et al., 2013; García-Córcoles et al., 2018; Li et al., 2017; Xin et al., 2015) and *in vivo* (Tiwari et al., 2012) as well as structural and numerical chromosomal aberrations *in vitro* (Santovito et al., 2018; Xin et al., 2015) and *in vivo* (Aghajani-pour-Mir et al., 2016; Santovito et al., 2018; Tiwari et al., 2012; Xin et al., 2015).

It is considered that BPA analogues are safer than BPA; however, this may also be due to the lack of sufficient toxicological data to support the risk assessment. Therefore, the aim of our study was to evaluate cytotoxic and genotoxic potential of commonly used analogues BPS, BPF and BPAF in comparison to BPA in the experimental model with metabolically competent human hepatocellular carcinoma (HepG2) cells. Genotoxicity was determined by detection of H2AX phosphorylation, which reflects an early reaction to a genotoxic insult resulting in the formation of DNA double strand breaks. Formation of γ -H2AX foci correlates with the formation of micronuclei (Watters et al., 2009), and the

Table 1
BPA and its structural analogues.

	Abbreviation	Synonym	CAS N°	Chemical structure	Formula	MW (g/mol)
Bisphenol A	BPA	2,2-bis(4-hydroxyphenyl)propane	080-05-7		C15H16O2	228.29
Bisphenol S	BPS	4,4'-Sulfonyldiphenol	080-09-1		C12H10O4S	250.27
Bisphenol F	BPF	bis(4-hydroxyphenyl)methane	620-92-8		C13H12O2	200.23
Bisphenol AF	BPAF	4,4'-(hexafluoroisopropylidene)diphenol	1478-61-1		C15H10F6O2	336.23

γ -H2AX assay is more precise and sensitive than the comet assay (Kuo and Yang, 2008). The underlying mechanisms of potential toxicity of BPs analogues have been studied by analysing changes in mRNA expression of selected genes involved in the xenobiotic metabolism (*CYP1A1*, *UGT1A1* and *GSTA1*), response to oxidative stress (*GCLC*, *GPX1*, *GSR*, *SOD1A* and *CAT*) and DNA damage response (*TP53*, *MDM2*, *CDKN1A*, *GADD45A*, *CHEK1* and *ERCC4*).

The number of chemicals and combinations thereof to which humans and the environment are continuously exposed is potentially enormous. Concerns about the current limitations of assessing compounds individually without proper understanding of risks associated with chemical mixtures are arising. *In vitro* methods are one of potential tools enabling better understanding of the underlying mechanisms of mixture effect (Kienzler et al., 2016). Given that in the environmental compartments, foodstuff and consumer products BPA analogues co-exist with BPA, we also investigated the genotoxicity and mechanisms of action of the mixtures of BPA with its analogues at low concentrations that may be relevant for human exposure.

2. Materials and methods

2.1. Chemicals

Bisphenol A (BPA, 99% analytical purity), bisphenol S (BPS, 99.7% analytical purity), bisphenol F (BPF, 99.7% analytical purity), bisphenol AF (BPAF, 99.7% analytical purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA) (Table 1).

A 25 mg/mL stock solutions of BPA, BPS, BPF and BPAF were prepared in dimethylsulphoxide (DMSO): BPA (109.5 mM), BPS (99.89 mM), BPF (124.86 mM) and BPAF (74.35 mM); aliquoted and stored at -20°C . Minimal Essential Medium Eagle, non-essential aminoacids, methanol, DMSO, phenazine methosulfate (PMS), NaHCO_3 , sodium pyruvate and Benzo(a)pyrene (BaP) were obtained from Sigma, USA. Penicillin/streptomycin, foetal bovine serum, L-glutamine and phosphate buffered saline (PBS) were from PAA Laboratories, USA. CellTiter 96® AQueous cell proliferation assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTS) was from Promega, USA. Etoposide (ET) was from Santa Cruz Biotechnology, USA. Human recombinant Anti-H2AX pS139, FITC conjugate antibodies were from Miltenyi Biotec GmbH, Germany. Trypsin was from Invitrogen™, Life Technologies, USA. TRIzol® reagent was purchased from Thermo Fisher Scientific, USA. cDNA High Capacity Archive Kit, TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays were derived from Applied Biosystems, USA. All chemical reagents were of the purest grade available and all solutions were made using Milli-Q water.

2.2. Cell culture

The HepG2 cell line was obtained from American Type Culture Collection, USA at passage 108. The cells were grown at 37°C and 5% CO_2 in MEME medium supplemented with 10% FBS, 2 mM L-glutamine, 1% NEAA, 2.2 g/L NaHCO_3 , 1 mM sodium pyruvate and 100 IU/mL penicillin/streptomycin. The experiments were performed on cells between passage 120 and 130. The cells were seeded on culture plates and left overnight to attach. Subsequently the growth medium was replaced with fresh medium containing appropriate concentrations of bisphenols and incubated for 24 or 72 h, respectively.

2.3. Concentrations of the tested bisphenols for determination of cytotoxicity, genotoxicity and mRNA expression

The cell viability was studied after 24 and 72 h exposure of HepG2 cells to single compounds BPA, BPF, BPS and BPAF at concentrations 5, 10, 15 and 20 $\mu\text{g/mL}$. Genotoxicity of BPs was determined by the γ -H2AX foci assay after 24 and 72 h exposure to non-cytotoxic

concentrations (BPA, BPF and BPS at 2.5, 5, 10 and 20 $\mu\text{g/mL}$; BPAF at 1.25, 2.5, 5 and 10 $\mu\text{g/mL}$). The mRNA expression of selected genes was evaluated after 24 h exposure of cells to 10 $\mu\text{g/mL}$ BPA, BPS, BPF or BPAF.

Genotoxicity and mRNA expressions in HepG2 cells upon combined exposure to BPs were determined at low concentrations, in the range of ng/mL that may be relevant for human exposure. HepG2 cells were exposed to single BPs, binary mixtures of BPA/BPS, BPA/BPF and BPA/BPAF, and quaternary mixture consisting of BPA/BPS/BPF/BPAF. In all experiments the concentration of BPA was 10 ng/mL and the concentrations of other BPs were 1 ng/mL. Tenfold higher concentration of BPA was selected to better represent the expected exposure as BPA is the most often detected bisphenol in different environmental and food matrices and therefore expected levels of BPA in human biomonitoring are higher compared to other BP analogues (Chen et al., 2016; Lehmler et al., 2018).

In each experiment, a solvent control (0.08% DMSO) was included.

2.4. Cell viability - MTS assay

Cell viability was determined with MTS tetrazolium reduction assay. The cells were seeded onto 96-well plates at a density of 8000 and 4000 cells/well for 24 and 72 h exposure, respectively. After incubation, 40 μL of freshly prepared MTS: PMS solution (20:1) was added to the 200 μL of medium and incubated for 3 h. Finally, the absorbance was measured at 490 nm using a Microplate Reader (Synergy MX, BioTek, USA). Cell viability is expressed as a percentage of the solvent control at each exposure time point. Three independent experiments were performed, with five replicates per treatment point. ET (30 $\mu\text{g/mL}$) was used as a positive control. Statistical significance between treated groups and the control was determined by One-way analysis of variance and Dunnett's Multiple Comparison Test, using GraphPad Prism v6.00 (GraphPad Software, USA).

2.5. Analyses of the induction of DNA DSB by γ -H2AX assay

The cells were seeded on 6-well plates (Corning Inc., USA) at a density of 400,000 or 250,000 cells/well for 24h and 72h exposure, respectively. At the end of the exposure the cells were collected by trypsinization, washed twice with cold PBS, resuspended in 0.5 mL cold PBS and ethanol (1.5 mL) was added drop wise into the cell pellet. The cells were stored at -20°C until analysis. Fixed cells were washed with ice cold PBS and labelled with Anti-H2AX pS139 antibodies according to the manufacturer's protocol. Flow cytometric analysis was carried out on a MACSQuant Analyzer 10 (Miltenyi Biotec, Germany). FITC intensity was recorded for 10^4 single cells in each sample. Independent experiments were repeated three times. ET (1 $\mu\text{g/mL}$) was used as a positive control. For further analysis the raw data (FITC intensities of each cell obtained as FL1-H values) was exported from MACSQuantify software to GraphPad Prism 5 to generate the box and whiskers plots. For the γ -H2AX foci analysis the statistical significance between treated groups and the vehicle control was determined with a linear mixed-effects model. Calculations were done with the statistical program R (R Core Team, 2018) and its packages reshape (Wickham, 2007), and nlme (Pinheiro et al., 2018).

2.6. Real-time quantitative PCR (qPCR) analysis

For mRNA analyses 600,000 cells per T25 plate were seeded exposed for 24 h. After the exposure of cells to BPs, total RNA was isolated using TRIzol reagent according to the manufacturer's protocol with minor modifications described in Maisanaba et al. (2016). The RNA was transcribed to cDNA using 1 μg of total RNA and cDNA High Capacity Archive Kit, according to the manufacturer's protocol. Gene expression was quantified by RT-qPCR using TaqMan Universal PCR Master Mix and the following Taqman Gene Expression Assays: *CYP1A1* (cytochrome P450, family 1, subfamily A, polypeptide 1), Hs01054797 g1; *GSTA1* (glutathione S-transferase alpha 1), Hs00275575 m1; *UGT1A1* (UDP

glucuronosyltransferase 1 family, polypeptide A1), Hs02511055 s1; *CDKN1A* (cyclin-dependent kinase inhibitor 1A) Hs00355782 m1; *CHEK1* (checkpoint kinase 1) Hs00967506 m1; *CAT* (catalase), Hs00156308 m1; *GCLC* (glutamate-cysteine ligase, catalytic subunit), Hs00155249 m1; *GPX1* (glutathione peroxidase 1), Hs00829989 gH; *GSR* (glutathione reductase), Hs00167317 m1; *SOD1A* (superoxide dismutase 1), Hs00533490 m1; *ERCC4* (ERCC excision repair 4, endonuclease catalytic subunit) Hs00193342 m1; *GADD45A* (growth arrest and DNA damage inducible gene alpha), Hs00169255 m1; *MDM2* (MDM2 proto-oncogene), Hs01066930 m1; *TP53* (tumor protein P53), Hs01034249 m1; all using FAM reporter and non-fluorescent quencher. Amplification of *GAPDH* probe (VIC-TAMRA, Cat. No.: 4310884E, Applied Biosystems, USA) was performed as an internal control. All experiments were performed at 384-well plates with a single probe per well using the Viia™ 7 Real-Time PCR System (Applied Biosystems™). The conditions for the PCR were 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The expression levels of target mRNAs were normalized to *GAPDH*. The relative quantification of gene expression was done by comparison of the Ct values of treated and control groups considering the actual efficiency of each assay using the QuantGenius protocol (Baebler et al., 2017). BaP (30 µM) was used as a positive control. Three independent experiments were performed each time in duplicates. Statistical difference between treated groups and controls was determined by two tailed Student's *t*-test. A ≥ 1.5 -fold change in gene expression compared to control was considered as up-regulation and down-regulation, respectively.

3. Results

3.1. The effect of BPA, BPS, BPF, BPAF on cell viability

The effects of BPA and its analogues BPS, BPF and BPAF on the viability of HepG2 cells were evaluated with the MTS assay. After 24 h

exposure, BPAF caused decrease in cell viability of HepG2 cells at the highest tested concentrations, 15 and 20 µg/mL by 10.4% and 13%, respectively. None of the other studied BPs affected the viability after short time exposure. After 72 h exposure, BPF and BPS at the highest tested concentration 20 µg/mL decreased cell viability by approximately 15%, whereas BPAF decreased cell viability by 22% and 47% at 15 and 20 µg/mL, respectively (Fig. 1). To avoid false-positive results due to cell death, in further experiments DNA damage and gene expression analyses were tested at concentrations that did not reduce cell viability by >20%.

3.2. The induction of DSBs by BPA, BPS, BPF and BPAF and their mixtures

The induction of DNA double strand breaks (DSBs) by BPA, BPS, BPF and BPAF was determined indirectly through the detection of γ H2AX formation, by measuring the fluorescent signals of individual cells using flow cytometry. Twenty-four hour exposure of cells to BPF and BPAF increased the γ H2AX formation at 10 and 20 µg/mL dose dependently (Fig. 2). After 72 h exposure, the increase of γ H2AX expression was detected in cells exposed to 20 µg/mL BPA and in cells exposed to 20 µg/mL BPF. Due to its cytotoxicity after 72 h exposure, BPAF was tested at lower concentrations (up to 10 µg/mL) at which it did not increase the formation of DNA DSBs compared to control group (Fig. 2). At low for human exposure relevant concentrations, BPA (10 ng/mL), BPS, BPF and BPAF (1 ng/mL), binary mixtures of BPA with analogues and quaternary mixture of all four studied BPs did not induce increased formation of DNA DSBs (Fig. 3).

3.3. The influence of BPA, BPS, BPF and BPAF and their mixtures on mRNA expression of selected genes

The mRNA expression of selected genes involved in the xenobiotic metabolism (*CYP1A1*, *UGT1A1*, *GSTA1*), oxidative stress (*GCLC*, *GPX1*,

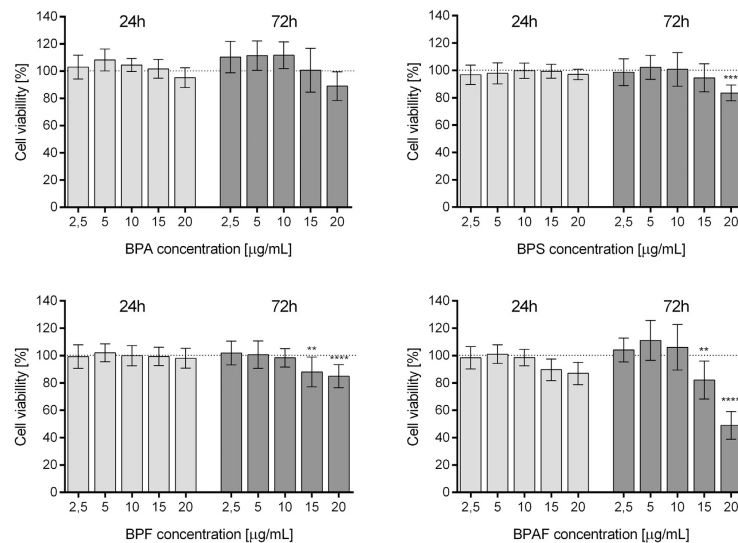


Fig. 1. The effect of BPA, BPS, BPF and BPAF on the viability of HepG2 cells after 24 h (light grey) and 72 h (dark grey) exposure, expressed in percentage of solvent control (0.08% DMSO = dotted line). The asterisk denotes a significant difference between solvent control and treated cells (***p* < 0.01; ****p* < 0.001; *****p* < 0.0001).

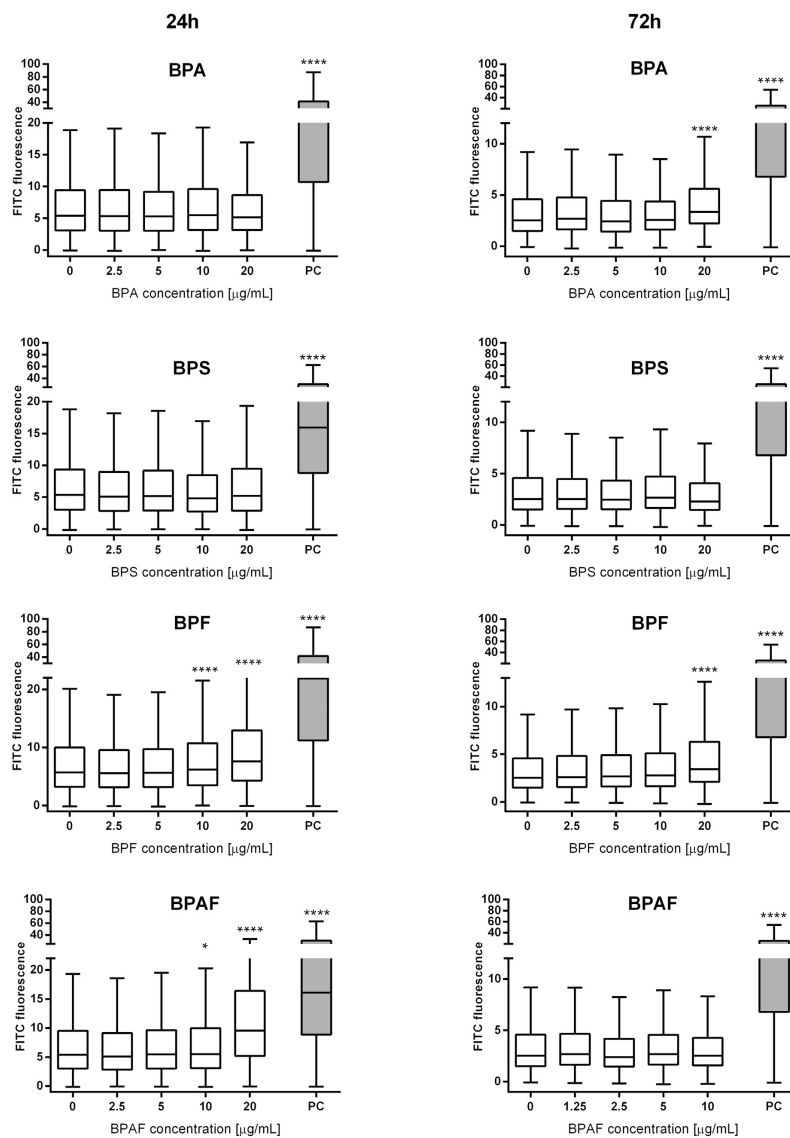


Fig. 2. The induction of DNA DSB by BPA, BPS, BPF and BPAF in HepG2 cells after 24 h (left column) and 72 h (right column) exposure. The presence of DSB was analysed by flow cytometry of anti-H2AX pS139 stained cells. 0 = 0.08% DMSO, PC = 1 $\mu\text{g/mL}$ ET. The asterisk denotes a significant difference between solvent control and treated cells (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$).

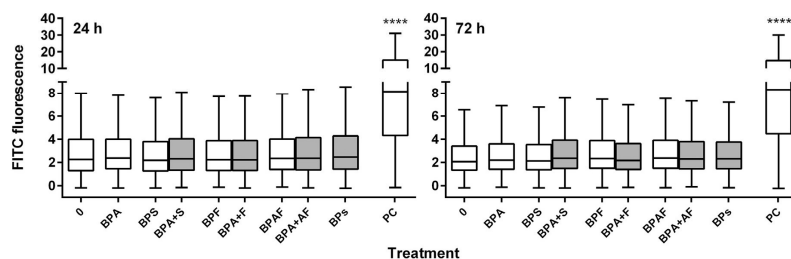


Fig. 3. The induction of DNA DSB by BPA, BPS, BPF and BPAF (white box) and their mixtures (grey box) at low for human exposure relevant concentrations after 24 h and 72 h exposure. In all experiments the concentration of BPA was 10 ng/mL and the concentrations of other BPs were 1 ng/mL. FITC fluorescence of anti-H2AX pS139 stained cells was measured by flow cytometry. 0 = 0.08% DMSO. PC = 1 μ g/mL ET. The asterisk denotes a significant difference between solvent control and treated cells (**** $p < 0.0001$).

GSR, *SOD1A*, *CAT*) and DNA damage response (*TP53*, *MDM2*, *CDKN1A*, *GADD45A*, *CHEK1*, *ERCC4*) was investigated after 24 h exposure to BPA, BPS, BPF and BPAF and to binary and quaternary mixtures by qPCR method (Table 2).

All four studied BPs (10 μ g/mL) up-regulated the expression of *CYP1A1* and *UGT1A1*. The highest up-regulation of the expression of *CYP1A1* was induced by BPAF (11.6-fold), followed by BPA (8.2-fold), BPF (3.1-fold) and BPS (3-fold). Similarly, also the expression of *UGT1A1* was more up-regulated by BPAF (2.7-fold) and BPA (2.5-fold), while lower effect was observed by BPF (1.5-fold) and BPS (1.6-fold). Gene expression of *GST1A1* was up-regulated in cells exposed to BPS (1.6-fold), while BPAF down-regulated the expression of *GST1A1*. BPA and BPF did not affect the mRNA level of *GST1A1*. From the selected genes involved in the response to oxidative stress the expressions of *GPX1*, *GSR*, *SOD1A* and *CAT* were not deregulated by the tested BPs, whereas *GCLC* was up-regulated only by BPA (1.5-fold). From the group of selected genes that are involved in DNA damage response, BPAF up-regulated the expression of *CDKN1A* (1.8-fold) and *GADD45A* (1.9-fold), while other BPs did not affect the expression of these two genes. The mRNA levels of *TP53*, *MDM2*, *CHEK1* and *ERCC4* were not affected by the 24-h exposure to the studied BPs.

At low for human exposure relevant concentrations BPs and their binary as well as quaternary mixtures up-regulated the expression of genes involved in xenobiotic metabolism, while the expressions of genes involved in oxidative stress and DNA damage response were not affected. The expression of *CYP1A1* was up-regulated by the exposure to 10 ng/mL BPA (5.3-fold) and 1 ng/mL BPAF (1.8-fold). Also BPS and BPF at this low concentration up-regulated the mRNA expression of *CYP1A1*, however by <1.5-fold. In combined exposure to binary mixtures slightly higher induction of *CYP1A1* gene expression (5.6-fold) was determined compared to BPA alone, while the highest increase was detected in cells exposed to the quaternary mixture (6.5-fold). The expression of *UGT1A1* was up-regulated only by 10 ng/mL BPA (~2-fold), while other studied BPs at 1 ng/mL did not affect the expression of this gene. The up-regulation of *UGT1A1* expression by exposure to binary and quaternary mixtures was slightly higher than after the exposure to BPA alone.

4. Discussion

In the present study, genotoxic potential of BPA and its analogues BPS, BPF and BPAF as well as their mixtures was explored and their effects on the expression of selected genes involved in xenobiotic metabolism, response to oxidative stress and DNA damage were studied in metabolically active HepG2 cells.

The cytotoxicity testing of the four BPs demonstrated that at the applied exposure conditions, BPA, BPS or BPF only marginally reduced the viability of HepG2 cells, whereas BPAF was significantly more toxic. This

is in line with the results of a recent study by Russo et al. (Russo et al., 2018) who compared cytotoxicity of BPA and seven analogues in four cell lines and found the BPAF as the most toxic. Higher cytotoxicity of BPAF compared to BPA and BPF has been observed also in SHE cells (Tsutsui et al., 2000).

Genotoxic potential of the four BPs was evaluated as their ability to induce DNA DSBs determined by γ -H2AX assay. Among the four compounds examined BPF and BPAF exerted stronger genotoxic potential than BPA, while BPS did not induce DNA DSBs. Published data on genotoxicity of BPs reported that in HepG2 cells, human renal cell adenocarcinoma cells (ACHN) and human epithelial colorectal adenocarcinoma cells (LS174T), BPA at concentrations up to 100 μ M (22.8 μ g/mL) did not induce increase in γ -H2AX foci, while BPF induced DNA DSBs in HepG2 cells but not in ACHN and LS174T cells (Audebert et al., 2011). In another study with HepG2 cells BPF induced dose dependent increase in DNA single strand breaks (SSB) at concentrations higher than 78.8 μ M (15.8 μ g/mL), while it failed to induce micronuclei (Cabaton et al., 2009). Mokra et al. (Mokra et al., 2017) compared genotoxic potential of BPA, BPS, BPF and BPAF in human peripheral blood mononuclear cells (PBMCs) using the alkaline and neutral versions of the comet assay. They showed that the four BPs induced predominantly DNA SSBs. Like in our study, BPAF was the most and BPS the least potent. The same authors reported that the studied BPs induced oxidative DNA damage (Mokra et al., 2018). Again, the most potent was BPAF and the least potent BPS. Higher DNA damaging potential of BPAF compared to BPA has been reported also in MCF-7 cells (Lei et al., 2018). Furthermore, in SHE cells BPA and BPAF, but not BPF exhibited transforming activity and induction of aneuploidy with BPAF being more potent than BPA (Tsutsui et al., 2000). Similar differences in potencies were reported by Pfeiffer et al. (Pfeiffer et al., 1997), who studied BPA, BPAF and BPF induced disruption of mitotic spindle, mitotic arrest and aneuploidy in Chinese hamster V79 lung fibroblasts. So far we can conclude, that BPAF poses higher genotoxic threat than BPA and other tested analogues.

To get an insight into the mechanisms underlying the observed genotoxic effects of BPA and its analogues we explored their influence on the expression of selected genes involved in xenobiotic metabolism, oxidative stress and DNA damage response.

BPs are metabolized predominantly by the glucuronidation reaction catalysed by UDP glucuronosyltransferases (UGTs) (for review see (Gramec Skledar and Peterlin Mašič, 2016)). However, BPs also undergo cytochrome P450 (CYP) mediated oxidative transformations. It has been demonstrated that BPA and BPF are oxidized to reactive intermediates (Cabaton et al., 2008; Jaeg et al., 2004), which have been reported to form DNA adducts (Atkinson and Roy, 1995a; Atkinson and Roy, 1995b; Izzotti et al., 2009; Kolšek et al., 2012). The expressions of *CYP1A1* and *UGT1A1* were upregulated by all four BPs tested after 24 h exposure to 10 μ g/mL indicating activation of oxidative metabolism

Table 2
Effect of BPA, BPS, BPF and BPAF at different concentrations and combinations on the expression of mRNAs of selected genes involved in the metabolism, DNA damage and oxidative stress response in HepG2 cells.

Metabolism	DNA damage response									
	Oxidative stress					DNA damage response				
CYP1A1	UGT1A1	GSTA1	GCLC	GPX1	GSR	SOD1A	CAT	TP53	MDM2	CDKN1A
Effects of single compounds at 10 µg/mL										
BPA	8.16 ± 1.09**	2.47 ± 0.45*	1.51 ± 0.51	1.05 ± 0.23	1.23 ± 0.03	0.99 ± 0.17	1.11 ± 0.01	0.97 ± 0.2	1.08 ± 0.19	1.05 ± 0.28
BPS	3.04 ± 0.54*	1.62 ± 0.47	1.26 ± 0.36	1.19 ± 0.25	1.11 ± 0.02	1.05 ± 0.24	1.13 ± 0.05	1.23 ± 0.24	1.11 ± 0.26	1.08 ± 0.32
BPF	3.13 ± 0.82	1.54 ± 0.48	1.37 ± 0.57	1.10 ± 0.05	1.12 ± 0.07	1.05 ± 0.04	1.10 ± 0.07	1.10 ± 0.24	1.13 ± 0.20	1.28 ± 0.37
BPAF	11.64 ± 0.91***	2.66 ± 0.31*	1.33 ± 0.39	0.99 ± 0.10	0.97 ± 0.08	0.91 ± 0.16	0.94 ± 0.03	1.09 ± 0.19	1.25 ± 0.11	1.75 ± 0.56
PC	280.6 ± 95.1	4.49 ± 0.004***	0.31 ± 0.17*	1.50 ± 0.42	1.99 ± 0.07*	1.21 ± 0.15	0.46 ± 0.08	1.40 ± 0.32	0.61 ± 0.10	15.27 ± 2.65*
Effects of BPA (10 µg/mL) and BPS, BPF and BPAF (1 µg/mL) and their combinations										
BPA	5.31 ± 1.89	1.96 ± 0.14*	0.98 ± 0.3	1.20 ± 0.19	0.97 ± 0.14	0.97 ± 0.01	1.08 ± 0.15	1.04 ± 0.10	1.04 ± 0.12	0.94 ± 0.08
BPS	1.49 ± 0.17	1.37 ± 0.17	1.09 ± 0.13	1.10 ± 0.11	0.99 ± 0.09	1.07 ± 0.02	1.05 ± 0.05	1.06 ± 0.04	1.14 ± 0.09	1.06 ± 0.02
BPF	1.37 ± 0.15	0.97 ± 0.02	1.04 ± 0.03	1.02 ± 0.02	0.98 ± 0.03	1.04 ± 0.02	1.00 ± 0.04	1.04 ± 0.10	1.06 ± 0.02	1.09 ± 0.03
BPAF	1.83 ± 0.36	1.10 ± 0.23	0.99 ± 0.04	1.01 ± 0.12	0.99 ± 0.03	1.04 ± 0.09	0.96 ± 0.02	1.08 ± 0.04	1.10 ± 0.11	1.08 ± 0.06
BPA + BPS	5.63 ± 2.02	2.18 ± 0.01**	1.27 ± 0.06*	1.04 ± 0.08	1.09 ± 0.03	1.09 ± 0.02	1.17 ± 0.03	1.08 ± 0.07	1.07 ± 0.11	0.96 ± 0.06
BPA + BPF	5.58 ± 2.05	2.15 ± 0.44	1.29 ± 0.22	0.98 ± 0.29	1.08 ± 0.15	1.07 ± 0.14	1.17 ± 0.15	1.12 ± 0.10	1.07 ± 0.28	1.00 ± 0.21
BPA + BPAF	5.53 ± 2.14	2.39 ± 0.17*	1.31 ± 0.04*	1.00 ± 0.14	1.10 ± 0.05	1.10 ± 0.01	1.01 ± 0.02	1.08 ± 0.05	1.21 ± 0.09	1.01 ± 0.12
4 BPs mix	6.47 ± 3.03	2.32 ± 0.48	0.89 ± 0.25	1.27 ± 0.18	1.15 ± 0.21	1.03 ± 0.27	1.19 ± 0.20	1.09 ± 0.06	1.18 ± 0.31	1.10 ± 0.20
PC	135.8 ± 49.8	4.83 ± 0.55*	1.54 ± 0.06	1.90 ± 0.50	0.67 ± 0.03	1.35 ± 0.14	0.55 ± 0.07	1.23 ± 0.08	0.82 ± 0.03*	11.9 ± 2.8*

The results are expressed as relative mRNA expression normalized to the control group. Data are means ± SD of two independent experiments. Significant differences between bisphenols treated cells and the control group is indicated by *P<0.05, **P<0.01, ***P<0.001 (Two-tailed Student's t-test comparison). Bold values indicate up or down-regulation of genes. PC = BPA/BP at 30 µM, 4 BPs mix = BPA + BPS + BPF + BPAF.

and detoxification pathway. The highest up-regulation of both genes was induced by BPAF, followed by BPA. The induction of CYP1A1 as well as UGT1A1 expression is directed by a ligand activated transcriptional factor aryl hydrocarbon receptor (AhR) (Ramadoss et al., 2005; Williams et al., 2014). The observed up-regulated expression of CYP1A1 and UGT1A1 most probably occurred via AhR, while it was demonstrated that BPA activates AhR-dependent mechanism via formation of AhR-DNA-binding complex (Vrzal et al., 2015). It is notable that this upregulation was observed also after the exposure to the low, for human exposure relevant concentrations of BPA and BPAF as well as to the mixtures of BPA with its analogues.

Glutathione S-transferases (GSTs) play an important role in detoxification and cellular protection against xenobiotics and oxidative stress. Human GSTA1, a cytosolic isoform, is the most abundant GST in the liver and catalyses glutathione conjugation of a wide range of electrophiles (Williams et al., 2014). In the present study, BPS up-regulated (1.6-fold) and BPAF down-regulated (3.1-fold) mRNA expression of GSTA1, while BPA and BPF had no significant effect. Previously, it has been reported that in liver of rats exposed to BPA the gene expression and activity of GST were down-regulated (Williams et al., 2014), whereas in murine embryos exposed to BPA *in utero* the mRNA expression of GST was up-regulated (Nishizawa et al., 2005). Altogether this shows that BPs deregulate the expression and the activities of GSTs.

In our study BPAF was the strongest inducer of CYP1A1 (11.4-fold) among the four tested BPs, the up-regulation of the expression of UGT1A1 by BPAF (2.66-fold) was comparable to that induced by BPA (2.47-fold). At the same time the expression of GSTA1 was even down-regulated by BPAF (3.1-fold). Therefore at the gene expression level, the balance between the expression of activating enzyme and detoxifying enzymes in cells exposed to BPAF was more in favour of activation compared to cells exposed to other three BPs. Thus, the highest genotoxic potential of BPAF may be explained by higher rate of the formation of DNA reactive metabolites by CYP1A and relatively lower rate of detoxification by UGTs and GSTs compared to the other three BPs. Oppositely, BPS, which did not induce DNA DSBs, was the weakest inducer of CYP1A1 and the only inducer of GSTA1 expression, which also supports the same assumption.

The possible mechanism behind the observed DNA damage induced by BPs could be the increase in the cellular oxidative stress, which results in the formation of reactive oxygen species (ROS) that induce damage to DNA. Although the majority of BPA is detoxified via glucuronidation and sulfatation to less toxic intermediates the remaining free BPA induces formation of ROS through various enzymatic and nonenzymatic reactions (Babu et al., 2013; Sakuma et al., 2010; Yoshida et al., 2001). BPA induced ROS formation has been demonstrated *in vivo* in rat liver and testis (Bindhumol et al., 2003; Ullah et al., 2016) and *in vitro* in different cell models (Gassman et al., 2015; Huc et al., 2012; Kim et al., 2018; Li et al., 2017; Xin et al., 2014). Xin et al. (2014) demonstrated that the induction of DNA strand breaks by BPA has been linked to the induction of ROS. More recently, it has been reported that also BPA analogues BPS, BPF and BPAF induce ROS formation in human peripheral blood mononuclear cells (Michalowski et al., 2015; Mokra et al., 2017) and in human erythrocytes (Macczak et al., 2017). Our results demonstrated that gene expressions of major antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX1) that play a critical role in maintaining intracellular redox homeostasis by scavenging and catalytic removal of the generated ROS were not affected by the exposure to the tested BPs. Also the expression of glutathione reductase (GSR), which catalyses the reduction of GSSG to GSH, was not affected, whereas the expression of GCLC was up-regulated by BPA. The latter indicates that exposure to BPA triggered *de novo* synthesis of GSH, which may be due to its depletion. Depletion of intracellular GSH after BPA exposure has been previously observed *in vivo* in rodents (Kabuto et al., 2003; Wu et al., 2013) and different cell lines *in vitro* (Gassman et al., 2015; Ge et al., 2014; Xin et al., 2014). Macczak et al. (2017) measured the activities of

antioxidant enzymes SOD, CAT and GPX in human erythrocytes exposed to bisphenols and observed changes after the exposure to BPA, BPF and BPAF, while BPS had no effect. However, changes were observed at >10-fold higher concentrations than used in our study. The lack of the changes in the expression of antioxidant enzymes at the transcriptional level may be due to low level of oxidative stress induced by the tested BPs at the applied exposure conditions (10 µg/mL for 24 h).

It has been demonstrated that changes in the expression of DNA damage responsive genes can be considered as molecular markers of genotoxic and carcinogenic stress (Ellinger-Ziegelbauer et al., 2005; Hreljac et al., 2008; Petković et al., 2011; Pezdirc et al., 2013; Žegura et al., 2008). Therefore, the effect of BPs on the expression of *TP53*, *MDM2*, *CDKN1A*, *GADD45A*, *CHEK1* and *ERCC4* has been analysed. The tumor-suppressor gene, *P53* plays central role in DNA damage response pathways and cancer development via regulation of cell cycle progression, DNA damage repair, senescence, differentiation and apoptosis (Oren, 2003). The MDM2 protein is a negative regulator of the TP53 involved in its degradation. It is a product of oncogene that is over-expressed in a significant number of human tumours (Michael and Oren, 2003). At the applied exposure conditions, BPs did not affect the transcription of *TP53*. However, in response to DNA damage, TP53 protein is predominantly activated through its phosphorylation by DNA damage responsive kinases and, to lesser extent through upregulation of gene expression (Lakin and Jackson, 1999); therefore, it is not unusual that its expression is not up-regulated at the mRNA level. At the level of protein expression Xin et al. (2014) reported, elevated expression of TP53 protein and checkpoint kinase 2 (Chk2) in response to BPA induced ROS formation and DNA damage in rat insulinoma cells. The main TP53 down-stream activated genes in response to DNA damage are *CDKN1A* and *GADD45a*. *CDKN1A* prevents G1-M cell cycle transition and inhibits DNA replication (Cazzalini et al., 2010). *GADD45α* is implicated in the control of cell cycle G2-M transition, induction of cell death/survival, DNA repair process, chromatin assembly, and genome stability (Zhan, 2005). The expressions of *CDKN1A* and *GADD45a* were up-regulated only by BPAF, which most likely reflects its higher genotoxic activity compared to BPF and BPA. Another central protein involved in the DNA damage response is Checkpoint Kinase 1 (CHK1) encoded by *CHEK1* that regulates cell cycle checkpoints following genotoxic stress to prevent cells with damaged DNA to enter mitosis and coordinates various aspects of DNA repair (McNeely et al., 2014). The ERCC4 protein plays a vital role in the nucleotide excision repair (NER) pathway and is crucial for maintaining DNA integrity (Manandhar et al., 2015). None of the four tested BPs deregulated expression of these two genes. Similarly, no up-regulation of the gene expression of ERCC family proteins, including ERCC4, has been observed in mouse embryonic fibroblasts after 25 h exposure to BPA (34 µg/mL) (Gassman et al., 2016). Taken together the gene expression analyses of DNA damage responsive genes confirmed BPAF as the most potent inducer of DNA damage among the four tested BPs.

In the next step, we studied genotoxicity and modulation of gene expression induced by BPA and its analogues as single compounds and their combinations at low concentrations (10 or 1 ng/mL, respectively) that reflect human exposure conditions. At these concentrations BPs did not induce DNA damage and did not induce changes in the expression of genes involved in oxidative response and response to DNA damage. However, BPA (10 ng/mL) and BPAF (1 ng/mL) as well as the binary and quaternary mixtures of BPA with its analogues induced increased expression of *CYP1A1* and *UGT1A1* mRNA. The up-regulation of the expression of both genes induced by the binary and quaternary mixtures was higher from that induced by BPA alone, indicating an additive effect on the expression of these genes which was observed also with higher concentrations.

The observed up-regulation of the expression of *CYP1A1* and *UGT1A1* by BPA, BPAF and the mixtures in HepG2 cells means, that exposure to these low concentrations that reflect human exposure conditions, triggered cellular defence by activating their metabolic transformation

system. On the other hand, CYP1A enzymes are known to play critical role in activating indirect acting genotoxic carcinogens such as polyaromatic hydrocarbons and heterocyclic aromatic amines to electrophilic reactive intermediates, leading to DNA damage, mutations and cancer (Miller and Miller, 1981). It has been demonstrated that polychlorinated biphenyls, known inducers of CYP1A enzymes, increased genotoxic activity of BaP in the metabolically active HepG2 cells (Wei et al., 2009; Wu et al., 2003; Yuan et al., 2009). As polycyclic aromatic hydrocarbons and heterocyclic aromatic amines are, like BPs, ubiquitous environmental pollutants combined human exposure is inevitable. Therein, it is possible that also BPs can increase the genotoxic potency of indirect acting genotoxic pollutants in combined exposure by inducing the expression of CYP1A enzymes, which could, at least theoretically, increase cancer risk.

5. Conclusions

In conclusion, our results show that in the metabolically active HepG2 cells BPAF exhibited the highest cytotoxic and genotoxic potential among the four tested BPs, while BPS, which is the main substituent of BPA in the manufacture, was not genotoxic. The gene expression analyses of the selected genes involved in the metabolism of xenobiotics showed that all four BPs triggered their up-regulation. The balance between the gene expression of activating (*CYP1A1*) and detoxifying enzymes (*UGT1A1* and *GSTA1*) apparently correlated with the highest genotoxic activity of BPAF and the lack of genotoxic activity of BPS. At concentrations relevant for human exposure BPA and its analogues as individual compounds and in mixtures did not exert genotoxic activity. However, BPA and BPAF as well as the mixtures at these low concentrations triggered increased gene expression of xenobiotic metabolic enzymes *CYP1A1* and *UGT1A1*. Of particular concern is the observed up-regulation of *CYP1A1* that catalyse metabolic activation of procarcinogens to ultimate carcinogens, which may result in increased susceptibility for genotoxic injury by indirect acting genotoxic pollutants to which humans may also be exposed. Taken together, our results indicate, that regarding the genotoxic potential, BPA analogues BPF and BPAF cannot be considered as safer alternatives to BPA. Further studies on their possible adverse effects and mechanisms of action are warranted focusing on not only the individual compounds but also their mixtures as well as combined effects with other genotoxic pollutants.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

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2.5 Plastics in Cyanobacterial Blooms - Genotoxic Effects of Binary Mixtures of Cylindrospermopsin and Bisphenols in HepG2 Cells

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Article

Plastics in Cyanobacterial Blooms—Genotoxic Effects of Binary Mixtures of Cylindrospermopsin and Bisphenols in HepG2 Cells

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Abstract: Ever-expanding environmental pollution is causing a rise in cyanobacterial blooms and the accumulation of plastics in water bodies. Consequently, exposure to mixtures of cyanotoxins and plastic-related contaminants such as bisphenols (BPs) is of increasing concern. The present study describes genotoxic effects induced by co-exposure to one of the emerging cyanotoxins—cylindrospermopsin (CYN)—(0.5 µg/mL) and BPs (bisphenol A (BPA), S (BPS), and F (BPF); (10 µg/mL)) in HepG2 cells after 24 and 72 h of exposure. The cytotoxicity was evaluated with an MTS assay and genotoxicity was assessed through the measurement of the induction of DNA double strand breaks (DSB) with the γH2AX assay. The deregulation of selected genes (xenobiotic metabolic enzyme genes, DNA damage, and oxidative response genes) was assessed using qPCR. The results showed a moderate reduction of cell viability and induction of DSBs after 72 h of exposure to the CYN/BPs mixtures and CYN alone. None of the BPs alone reduced cell viability or induced DSBs. No significant difference was observed between CYN and CYN/BPs exposed cells, except with CYN/BPA, where the antagonistic activity of BPA against CYN was indicated. The deregulation of some of the tested genes (*CYP1A1*, *CDKN1A*, *GADD45A*, and *GCLC*) was more pronounced after exposure to the CYN/BPs mixtures compared to single compounds, suggesting additive or synergistic action. The present study confirms the importance of co-exposure studies, as our results show pollutant mixtures to induce effects different from those confirmed for single compounds.

Keywords: cylindrospermopsin; CYN; bisphenols; BPA; BPS; BPF; BPAF; co-exposure; genotoxicity; cytotoxicity

Key Contribution: Antagonistic activity of bisphenol A (BPA) against DNA double-strand break induction by cylindrospermopsin (CYN) was indicated in HepG2 cells following 72 h co-exposure. Additive or synergistic activity of CYN and BPs (BPA, BPS, BPF, and BPAF) on the expression of genes involved in the metabolism of xenobiotics (*CYP1A1*), DNA damage response (*CDKN1A*, *GADD45A*) and oxidative stress response (*GCLC*) in HepG2 cells was indicated following 24 h co-exposure.

1. Introduction

Environmental pollution and accumulation of plastic in the environment are becoming an increasing concern, given the steady rise in plastic production and the release of diverse anthropogenic contaminants into the environment. Organic waste is causing eutrophication of water bodies, which, together with climate change, creates favorable conditions for extensive cyanobacterial proliferation.

Cyanobacterial blooms in surface waters are, therefore, globally increasing in extension, frequency, and magnitude [1]. Cyanobacteria produce an impressive plethora of bioactive substances, including a variety of toxins. Among them is the cyanotoxin cylindrospermopsin (CYN), which is considered an emerging health threat worldwide. Compared to other cyanotoxins, humans are more likely to be exposed to CYN, as it is highly water-soluble, very stable and persistent in aquatic environments [2,3], and is predominantly extracellular, especially in older blooms [2,4]. It is produced by a wide variety of species from the genera *Cylindrospermopsis*, *Raphidiopsis*, *Aphanizomenon*, *Chrysosporum*, and *Dolichospermum* (*Anabaena*) [1,5]. The distribution of CYN producing cyanobacteria is expanding globally, also into temperate zones [1,6]. The toxin has been detected in surface fresh and brackish waters in America, Asia, Europe, Oceania, and even in Antarctica, at concentrations up to 200 µg/L (for a review see: [5]). It has even been detected in water used for drinking in the United States of America (USA), China, and Taiwan, at concentrations 0.41 to 36 µg/L [7–9]. Furthermore, bioaccumulation of CYN in various aquatic animals and plants has been reported (for a review see: [10]) and the predicted exposure of humans, consuming such organisms, could exceed the provisional tolerable daily intake (TDI) proposed for CYN (0.03 µg/kg body weight) [11].

CYN is an alkaloid with a cyclic guanidine moiety bound to a hydroxymethyluracil group [12]. Its structure alone suggests it could exert a wide range of adverse effects in mammalian cells. In fact, all the main functional groups (uracil, hydroxyl, and guanidine) are crucial for CYN toxicity [13]. CYN is a potent protein synthesis inhibitor [14–16] and has been shown to induce oxidative stress [17–19]. It has traditionally been classified as a hepatotoxin but has subsequently been shown to target various other organs (for a review see: [20]) and was even reported to have endocrine-disrupting potential [21,22]. The toxin is genotoxic and potentially carcinogenic and needs metabolic activation by cytochrome P450 enzymes to exert genotoxic effects (for a review see: [20,23]). CYN has been shown to induce DNA, and chromosome damage in vitro, suggesting CYN has clastogenic activity [17,24–28]. Its genotoxicity has also been demonstrated in vivo [29–31]. Moreover, there are indications that it can also act as a tumor-initiator [29]. However, CYN has not yet been classified for its carcinogenic potential by the International Agency for Research on Cancer (IARC) due to insufficient information on its carcinogenic activity, and the mechanisms potentially involved are still under investigation.

Concomitantly with the rising emergence of toxic cyanobacterial blooms, the accumulation of plastic in the environment is also increasing. It is estimated that 12,000 million metric tons of plastic waste will be released into the environment by 2050 [32]. Consequently, plastic constituents are becoming ubiquitously present in marine and terrestrial water environments [33], adding to the mixture of present pollutants. A study recently suggested that cyanobacterial blooms could act as a sink for such pollutants [34]. The authors detected three of the plastic-related pollutants bisphenol A (BPA), S (BPS), and F (BPF) in bloom samples in a heavily eutrophic lake in China, at relatively high concentrations of 3954 ng/g dry weight (d.w.), 547 ng/g d.w., and 324 ng/g d.w., respectively.

Among the plastic-related contaminants, BPA is the most common, as it is the most widely-used material in the production of polycarbonate plastics, epoxy resins, and phenolic resins [35]. BPA is an organic synthetic compound belonging to the group of diphenylmethane derivatives, with two hydroxyphenyl groups. The global consumption of this compound in 2016 was estimated to be around 8 million tons, and the global BPA demand is projected to increase to 10.6 million tons by 2022 [36]. However, due to its known endocrine-disrupting activity and its other potential hazardous effects, the use of BPA is being restricted [36], resulting in the gradual replacement of BPA by presumably safer alternatives, its chemical analogues (BPS, BPF, and BPAF). BPS is the most commonly used replacement in various consumer BPA-free products, but BPF and BPAF are used as well in a broad range of industrial applications [37].

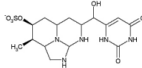
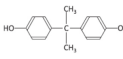
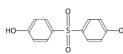
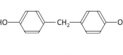
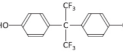
Due to their massive production, BPs can at present be detected in the environment at alarming concentrations [33,38,39]. They leach off during production, treatment, processing, and hydrolysis of the polymers into the ground water, wastewater, air, and food [40]. BPA tends to elute easily from plastic waste and move rapidly into the aqueous environment, due to its relatively low hydrophobicity

(for a review see: [41]). Human exposure is thus unavoidable. The average BPA concentration detected in surface waters is approximately 100 ng/L, but was recorded to be as high as 44,000 ng/L [38]. There are fewer data about the concentrations of BPA analogues, but they are in the range between 1 and 100 ng/L; however, up to 100-fold higher concentrations were detected in the down flow from industrial effluents [33]. Nevertheless, the dietary ingestion of free BPA accounts for its major route of exposure. The daily uptake rate of BPA for humans is estimated to be 50 ng/kg bw/day [42]. It was found to prevail in diverse human tissues and body fluids [43]. Similar data for BPA analogues are scarce. It has to be emphasized that BPA analogue consumption is rising because they are considered safer than BPA. However, the latter assumption is based on insufficient toxicological data to support the risk assessment. Considering the structural similarities and physicochemical properties, BPA analogues are expected to exhibit similar or even stronger endocrine-disrupting and toxic potential as BPA, which is also gradually being confirmed (for a review see: [37]).

Apart from the well-known endocrine disruption effects of BPA, evidence for its potential genotoxicity is accumulating (for a review see: [37,40]). BPA and its metabolites have been reported to induce DNA strand breaks in vitro [44–46] and in vivo [47], chromosomal aberrations in vitro [45,48] and in vivo [45,47,48], and to form DNA adducts in vitro [45,49,50] and in vivo [51]. Similar data for BPA analogues are again scarce. BPF and BPAF were found to induce DNA double-strand breaks in vitro, while BPS was inactive at concentrations up to 20 µg/mL in hepatic cells [46].

Data on the adverse effects of single compound exposure for CYN and BPA are accumulating. However, combined exposure to these pollutants has not been studied thus far. Recently, a review highlighted the importance of BPA co-exposure studies with other chemicals and environmental stressors for the assessment of outcomes that common co-exposures can exert on human health [52]. Considering their simultaneous presence in the environment, exposure to mixtures of different pollutants is the only realistic exposure scenario. Humans can thus be exposed to cyanotoxin/BPs mixtures following recreational activities and/or through the consumption of contaminated water and food. Co-exposure to various pollutants can induce effects that differ from those observed for single compounds due to unknown interactions that can occur between the compounds. The aim of this study was, therefore, to evaluate the cytotoxic and genotoxic potential of mixtures of CYN, BPA, and its commonly used analogues BPS, BPF, and BPAF (Table 1). The co-exposure was studied in the metabolically competent human hepatocellular carcinoma cell line HepG2. This cell line is considered one of the in vitro models of choice when studying the genotoxic effects of progenotoxic agents and is also recommended by Organization for Economic Co-operation and Development (OECD) standards (e.g., 487) [53], as it retained several phase I and II metabolic enzymes, involved in the metabolism of xenobiotics [54]. The genotoxic effects of the mixtures were determined by the detection of H2AX histone phosphorylation, which reflects an early reaction to a genotoxic insult resulting in the formation of DNA double-strand breaks (DNA DSBs). The cellular response to the exposure to these mixtures was further studied by analysis of the transcriptional response—deregulation of selected genes (genes involved in the metabolism of xenobiotics, immediate-early response, and DNA damage response), using qPCR.

Table 1. CYN, BPA, and its structural analogues.

	Abbreviation	CAS N ^o	Chemical Structure	Formula	MW (g/mol)
Cylindrospermopsin	CYN	143545-90-8		C ₁₅ H ₂₁ N ₅ O ₇ S	415.4
Bisphenol A	BPA	080-05-7		C ₁₅ H ₁₆ O ₂	228.29
Bisphenol S	BPS	080-09-1		C ₁₂ H ₁₀ O ₄ S	250.27
Bisphenol F	BPF	620-92-8		C ₁₃ H ₁₂ O ₂	200.23
Bisphenol AF	BPAF	1478-61-1		C ₁₅ H ₁₀ F ₆ O ₂	336.23

2. Results and Discussion

2.1. The Influence of CYN, BPs, and Their Combinations on Cell Viability

The influence of single compounds and CYN/BPs combinations on HepG2 cell viability was evaluated with the tetrazolium-based MTS assay. The concentrations of CYN (0.5 $\mu\text{g/mL}$) and BPs (10 $\mu\text{g/mL}$) were chosen based on previous findings on the genotoxic effects of CYN [25,27,28] and BPs [46] in HepG2 cells, also considering reported environmental concentrations and the significant daily uptake rate through dietary ingestion in the case of BPA. Although these concentrations are still higher than those reported in the environment and are not expected to be directly relevant for human exposure, they serve the aim of this study, which was to identify the possible DNA damaging effects (induction of DNA DSBs) and potential mechanisms of action of non-cytotoxic concentrations of CYN/BPs binary mixtures. It is thought that chemicals, directly inducing DNA damage, have no safe exposure threshold dose, only that the probability of potentially harmful mutations, resulting from the induced DNA damage (e.g., DNA DSBs), decreases with lower concentrations [55].

After 24 h of exposure, neither single compounds nor the combinations induced any measurable effect on HepG2 cell viability (Figure 1A). At the same time, the positive control (etoposide—ET) reduced cell viability for roughly 16% on average. After longer exposure (72 h), CYN (0.5 $\mu\text{g/mL}$) and all of its combinations with BPs (10 $\mu\text{g/mL}$) significantly reduced cell viability by 23% to 38%; however, there was no significant difference between CYN treated cells and cells treated with any of the CYN/BPs combinations (Figure 1B). None of the BPs (10 $\mu\text{g/mL}$) alone induced a measurable reduction in cell viability. BPS and BPAF even had a slight proliferating or more likely cell metabolic activity enhancing effect, which can commonly be observed after exposure to various toxic agents in cell lines as the consequence of increased mitochondrial activity in cell-cycle arrested cells [56]. Our results are in line with previous findings, showing a reduction in cell viability only after exposure to higher concentrations (15 $\mu\text{g/mL}$) of BPs (BPA, BPS, BPF, and BPAF) in HepG2 cells [46]. Low cytotoxicity was, for these BPs, also reported in other in vitro model systems (human breast adenocarcinoma cells (MCF-7), human 189 cervical epithelial cancer cells (HeLa), mouse fibroblasts (3T3-L1), and rat glioma cells (C6)), with the calculated half-maximal inhibitory concentrations (IC_{50}) generally being in the range of 20–75 $\mu\text{g/mL}$ [57]. The exception was BPAF, which was slightly more toxic (IC_{50} : 4–20 $\mu\text{g/mL}$) in some of the cell models. Thus, we can conclude that the decrease in cell viability observed in cells exposed to the CYN/BPs combinations was the consequence of CYN activity. CYN was previously shown to decrease cell viability and cell proliferation in HepG2 cells after prolonged exposure (96 h) at concentrations of up to 0.5 $\mu\text{g/mL}$, which was found to be due to the induction of cell cycle arrest

rather cell death [28]. Therefore, the observed effects of CYN and the CYN/BPs combinations in this study were probably predominantly the consequence of cell cycle arrest induced by CYN. Compared to the control, CYN at 0.5 $\mu\text{g/mL}$ and CYN/BPs combinations did not reduce cell viability for more than 40%, which is considered the limit value for genotoxicity assessment; thus, these concentrations were used for further studies of their genotoxic effects.

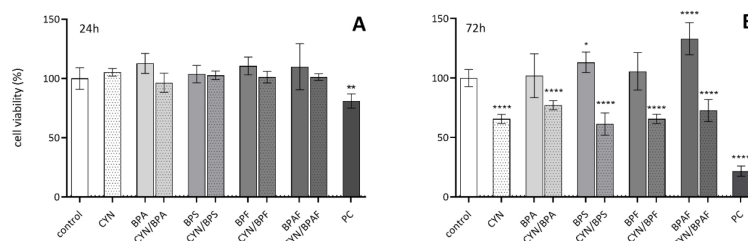


Figure 1. The effects of single compounds and binary mixtures of CYN (0.5 $\mu\text{g/mL}$) and BPs (BPA, BPS, BPF, BPAF; 10 $\mu\text{g/mL}$) on the viability of HepG2 cells after 24 h (A) and 72 h (B) exposure, expressed in percentage of the solvent control (0.05% methanol and 0.04% DMSO = dotted line at value 1.0). PC is the positive control—etoposide ET (30 $\mu\text{g/mL}$). The lower dotted line in figure B denotes the average viability reduction by CYN. The asterisks (*) denote statistically significant difference between solvent control and treated cells (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.0001$).

2.2. Induction of DNA Double-Strand Breaks by CYN, BPs, and Their Combinations

DNA DSB induction in HepG2 cells after exposure to CYN, BPs, and the CYN/BPs combinations was measured indirectly through the measurement of $\gamma\text{-H2AX}$ formation, using flow cytometry. $\gamma\text{-H2AX}$ is the phosphorylated form of the histone H2AX, which becomes phosphorylated on serine residue 139 in response to DNA DSB induction and forms nuclear foci adjacent to the sites of the DSBs [58]. Because the phosphorylation of H2AX is rapid, abundant, and correlates well with the number of DSBs [59], it is a very sensitive marker for DNA DSB induction (for a review see: [60]).

No increase in γH2X formation was detected after 24 h exposure to CYN, BPs, or CYN/BPs combinations (data not shown). After 72 h of exposure, a significant increase in DSB formation was observed in cells exposed to CYN and its combinations with BPS, BPF, and BPAF (Figure 2). CYN-induced DNA DSB formation in HepG2 cells has also been reported in a previous study [28]. No induction of γH2X was detected in cells exposed to the tested BPs alone except for BPAF, which slightly increased DSB formation. Additionally, in our previous study [46], no induction of DSBs in HepG2 cells at comparable exposure conditions by BPs was observed. BPF and BPAF were found to increase the formation of γH2X at higher concentrations ($\geq 10 \mu\text{g/mL}$) or at an earlier time point (24 h). Similarly, Audebert et al. [61] reported that BPA does not induce $\gamma\text{-H2AX}$ in HepG2 cells, human renal cell adenocarcinoma cells (ACHN), and human epithelial colorectal adenocarcinoma cells (LS174T), while BPF (10–50 μM) induced DNA DSBs in HepG2 cells, but not in ACHN and LS174T cells. Comparing the effects of CYN alone and its combination with BPS, BPF, or BPAF, on DSB formation, no significant difference could be detected, indicating that the formed DSBs were the consequence of CYN activity. Interestingly, CYN in combination with BPA induced slightly but statistically significantly less DNA DSB compared to the induction by CYN alone, indicating an antagonistic effect of BPA.

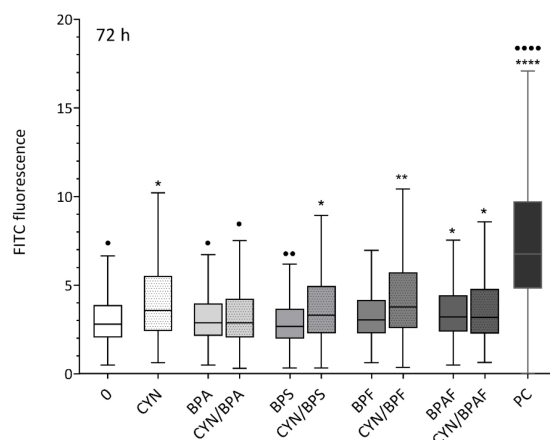


Figure 2. The effect of single compounds and binary mixtures of CYN (0.5 µg/mL) and BPs (BPA, BPS, BPF, BPAF; 10 µg/mL) on the induction of γ-H2AX formation (a sensitive marker for DNA double-strand breaks (DSBs)) in HepG2 cells after 72 h of exposure, using flow cytometry. The data are presented as quantile box plots. The edges represent the 25th and 75th percentiles, the solid line through the box is the median, and the error bars represent 95% confidence intervals. The solvent control (0 = 0.05% methanol and 0.04% DMSO) and a positive control (Etoposide ET, 1 µg/mL) were included in the experiment. The asterisks (*) denote statistically significant difference between solvent control and treated cells (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.0001$). The dots (•) denote a statistically significant difference between CYN alone and CYN/BPs treated cells (• $p \leq 0.05$; •• $p \leq 0.01$; ••• $p \leq 0.0001$).

CYN is considered to be a pro-genotoxin [17,18,24,25,62], predominantly activated by CYP450 enzymes [62,63]. BPA is also known to be metabolized by phase I and II xenobiotic-metabolizing enzymes [64,65]. Furthermore, BPA was reported to suppress or inhibit certain human hepatic cytochrome P450s activities [66–68]. Thus, the observed antagonistic effects of BPA in the CYN/BPA combination may be due to BPA-mediated suppression of the metabolic activation of CYN.

2.3. Gene Deregulation in Response to CYN/BPs Exposure

To get an insight into the response of HepG2 cells to the exposure to CYN in combination with BPs at the molecular level, expression of the metabolic enzyme gene *CYP1A1* (involved in the metabolism of CYN and BPs), and the expression of selected genes involved in DNA damage (*TP53*, *MDM2*, *CDKN1A*, *GADD45A*) and oxidative stress response (*GCLC*, *GPX1*, *GSR*, *SOD1A*, and *CAT*) was analyzed after 24 h of exposure by quantitative real-time PCR.

2.4. Xenobiotic Metabolism—*CYP1A1*

All of the tested compounds either alone or in combination up-regulated *CYP1A1* expression (Figure 3). The *CYP1A1* gene encodes a member of the family of cytochrome P450 enzymes that are involved in phase I of xenobiotic and drug metabolism. CYN alone up-regulated *CYP1A1* expression by 2.7-fold on average, while the BPs induced an even higher up-regulation. The highest up-regulation was observed in BPAF exposed cells (11.6-fold up-regulation). The exposure to the CYN/BPs combinations exerted stronger response than single compounds and induced *CYP1A1* up-regulation in an additive manner. In the CYN/BPA treated cells, even synergistic action was indicated as *CYP1A1* up-regulation

induced by CYN/BPA (12.4-fold) was higher than the sum up-regulation induced by the single compounds (CYN 2.7-fold and BPA 5.3-fold).

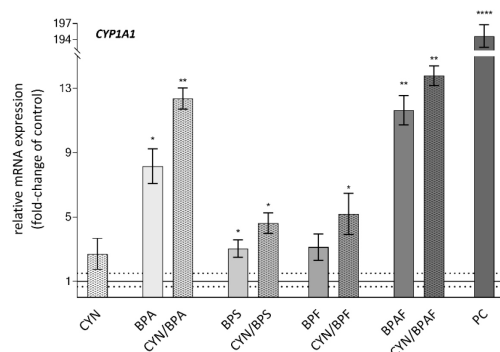


Figure 3. The effect of CYN (0.5 µg/mL), BPs (BPA, BPS, BPF, BPAF; 10 µg/mL) and their binary mixtures on the expression of the *CYP1A1* gene, involved in xenobiotic metabolism, in HepG2 cells after 24 h of exposure. The deregulations are expressed in the fold-change of expression of the gene in the solvent control group (0.05% methanol and 0.04% DMSO = solid line at value 1.0). PC is the positive control—Benzo[a]pyrene BaP (30 µM). The dotted line denotes biologically significant differences in gene expression (1.5-fold change). The asterisks (*) denote a statistically significant difference between solvent control and treated cells (* $p \leq 0.05$; ** $p = 0.01$; **** $p = 0.0001$).

Although the main enzymes involved in BPA detoxification are uridine 5'-diphospho-glucuronosyltransferases (UGT) and sulfotransferases (SULT) (for a review see: [66]), they also undergo CYP450-mediated oxidative transformations, as does CYN. The involvement of CYP450 enzymes in the toxic and genotoxic activation of CYN has been demonstrated using different broad-spectrum CYP450 inhibitors, which showed protective effects against toxicity [14,63,69] and genotoxicity [24,62] of CYN. Besides, CYN genotoxic effects were observed only in metabolically competent test systems (for a review see: [20,23]). Furthermore, BPA and BPF have been reported to be oxidized to reactive intermediates as well [70,71], which have been reported to form DNA adducts [51,72,73]. In line with our results, CYN was previously shown to up-regulate *CYP1A1*, and other CYP isoforms (*CYP1B1* and *CYP1A2*) in HepG2 cells [18,25–27] and human peripheral blood lymphocytes (HPBLs) [26]. Recently, the up-regulation of the expression of *CYP1A1* was reported following the exposure to BPs (BPA, BPS, BPF, and BPAF) in HepG2 cells [46] and the up-regulation of *CYP1A1* on the protein level following exposure to BPA in human placental JEG-3 choriocarcinoma cells was reported [74].

As our results show a more pronounced up-regulation of *CYP1A1* by all tested CYN/BPs combinations compared to the up-regulation of this gene by single compounds, this indicates an even stronger induction of the xenobiotic metabolism. [75–77] Meaning that also in addition, CYN and the BPs could potentially increase each other's genotoxic potency through the increased induction of CYP enzymes. However, as the results from the γ H2AX assay demonstrate otherwise, showing no further increase in DNA DSB formation in the CYN/BPs exposed; on the contrary, it even reduced in the case of CYN/BPA. Thus, BPs may impair CYN activation through the inhibition of CYP activity.

2.5. DNA Damage Response Genes

Deregulations of crucial DNA damage response genes (*TP53*, *MDM2*, *GADD45A*, *CDKN1A*), which were analyzed in the present study (Figure 4) are considered as molecular markers of genotoxic

and carcinogenic stress [78–82]. The protein P53 plays a central role in the major DNA damage response pathways: the regulation of DNA damage repair, cell cycle progression, senescence, differentiation, and apoptosis [83]. In response to DNA damage, P53 protein is predominantly activated through its phosphorylation by DNA damage responsive kinases and, to a lesser extent, through the up-regulation of gene expression [84]. The expression of the tumor-suppressor gene, *TP53*, was not significantly altered by BPs exposure, whereas it was slightly (<1.5-fold) down-regulated by CYN and the CYN/BPs combinations. Previous studies on the expression of the *TP53* gene reported that CYN [18,25,27] and BPs [46] did not influence *TP53* expression in HepG2 cells. Our results indicate that the combined exposure to CYN/BPs does not affect the expression of this gene differently than exposure to CYN as a single compound.

The main P53 down-stream regulated genes are *CDKN1A* and *GADD45A*. The gene *CDKN1A* encodes the cyclin-dependent kinase inhibitor P21^{WAF1/CIP1}, an essential regulator of cell cycle progression, and mediator of the P53-dependant G1 and G2 phase arrests (for a review see: [85]). *CDKN1A* was more than 1.5-fold up-regulated only after exposure to BPAF (1.75-fold) alone and the CYN/BPAF (1.59-fold) and CYN/BPF (1.55-fold) combinations. The up-regulation of *CDKN1A* by CYN alone in the present study was only 1.3-fold, which is lower than found previously [18,25].

The protein encoded by *GADD45A* (Growth arrest and DNA damage-inducible protein alpha) is implicated in the control of cell cycle G2-M transition, induction of cell death/survival, DNA repair process, chromatin assembly, and genome stability [86]. In contrast to *CDKN1A*, the expression of the gene *GADD45A* was up-regulated more than 1.5-fold in all CYN exposed groups (CYN alone 1.66-fold; CYN/BPs combinations 1.78–2.42-fold). The BPs alone did not influence *GADD45A* expression, with the exception of BPAF, which up-regulated its expression by approximately 1.9-fold. Among the tested BPA analogues, BPAF was previously found to be the only one that induced up-regulation of the expression of *CDKN1A1* and *GADD45A* and was also the most potent inducer of DNA DSBs in HepG2 cells [46]. Nevertheless, no additive or synergistic effect of BPAF in combination with CYN on the *CDKN1A1* or *GADD45A* expression was indicated. The highest up-regulation of *GADD45A* was observed in cells exposed to the CYN/BPF mixture (2.42-fold), much higher than BPF (1.37-fold) or CYN alone, which suggests synergistic action of the compounds in the mixture.

The MDM2 and CHEK1 genes were the most un-responsive to the exposure to the tested compounds. The expression of the gene encoding the MDM2 protein, a negative regulator of P53 [87], was stable in all the tested cell populations, regardless of the exposure to CYN, BPs, or their binary mixtures. The same was observed with the CHEK1 gene, the protein product of which is a checkpoint kinase that is activated in response to DNA damage and can thereafter modulate the activity of a number of proteins, including P53, providing a link between DNA damage and P53 checkpoint activity [88]. The results observed in single compound exposure groups are in agreement with previous reports, showing no deregulation of these genes by CYN or BPs in HepG2 cells [25,27,46], and the tested binary mixtures did not induce any different deregulation patterns from single compounds.

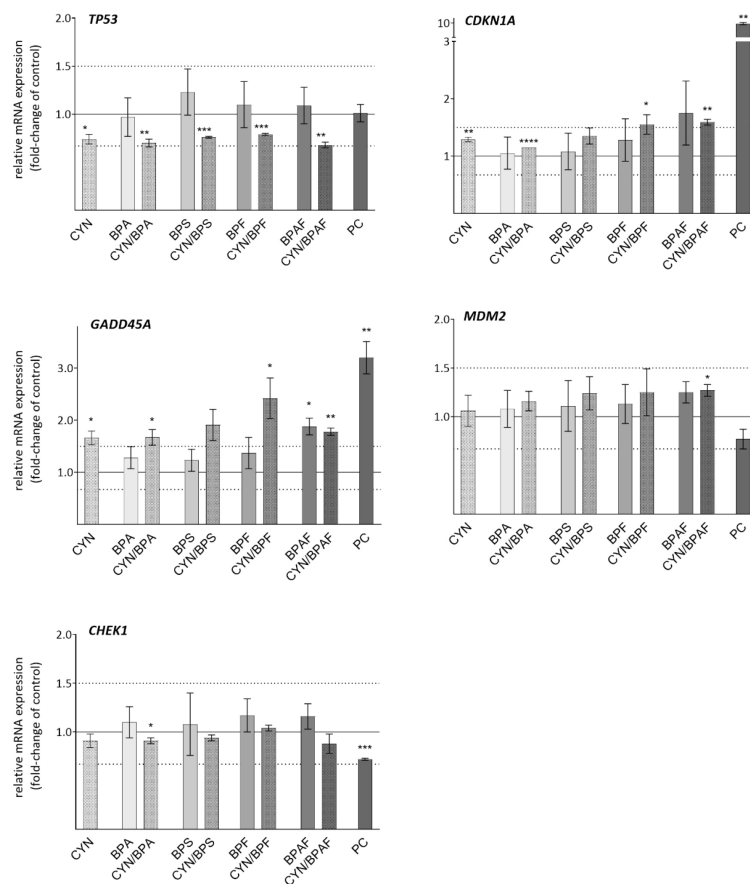


Figure 4. The effect of CYN (0.5 µg/mL), BPs (BPA, BPS, BPE, BPAF; 10 µg/mL), and their binary mixtures on the expression of DNA damage response genes (*TP53*, *MDM2*, *CDKN1A*, *GADD45A*) in HepG2 cells after 24 h of exposure. The deregulations are expressed in fold-change of expression of the gene in the solvent control group (0.05% methanol and 0.04% DMSO = solid line at value 1.0). PC is the positive control—Benzo[a]pyrene BaP (30 µM). The lower and upper dotted lines denote biologically significant differences in gene expression (1.5-fold change). The asterisks (*) denote a statistically significant difference between solvent control and treated cells (* $p = 0.05$; ** $p = 0.01$; *** $p = 0.001$).

2.6. Oxidative Stress Response Genes

The influence of single compounds and CYN/BPs binary mixtures on oxidative stress induction in HepG2 cells was evaluated by analyzing the deregulation of selected oxidative stress response genes. One of the possible mechanisms of CYN genotoxicity is postulated to be the formation of reactive

oxygen species (ROS) that can induce DNA damage. However, the published data are not consistent. While there are studies reporting increased ROS formation by CYN in various test systems, oxidative damage was observed rarely (for a review see: [20,23]).

There is growing evidence for ROS induction by BPA that may contribute to its toxicity and carcinogenic potential (for a review see: [89]). Huc et al. [90] reported mitochondria-dependent ROS generation, cytosolic oxidative stress, and lipid peroxidation in HepG2 cells following exposure to low doses (10^{-6} –100 μ M) of BPA. Moreover, BPA and its analogues (BPS, BPF, and BPAF) have been reported to induce oxidative stress and oxidative damage in human peripheral mononuclear cells and human red blood cells [91,92].

The reported increase in ROS formation following exposure to CYN and BPs could be, at least in part, the consequence of their metabolic activation. It is known that reactions catalyzed by CYP450 enzymes, which are involved in CYN and BPs metabolism, are a significant source of ROS formation (for a review see: [93]). Given that our results show increased up-regulation of *CYP1A1* by CYN/BPs binary mixtures, a higher increase in ROS formation and enhanced oxidative stress response could be expected in cells exposed to the mixtures compared to single compound exposure. The cellular response to an increase in intracellular ROS formation is the induction of enzymatic and non-enzymatic defensive mechanisms. The enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) play critical roles in maintaining intracellular redox homeostasis by scavenging and catalytic removal of the generated ROS [94]. The most abundant intracellular antioxidant, central to the non-enzymatic oxidative stress defense, is glutathione (GSH). During oxidative stress, reduced GSH is oxidized to glutathione disulfide (GSSG); therefore, the ratio of GSH to GSSG reflects cellular oxidative stress [95]. Two of the major enzymes involved in the regulation of the intracellular GSH content are glutathione reductase (GSR), which catalyzes the reduction of GSSG to GSH and γ -glutamylcysteine synthetase (GCL), which is recognized as the rate-limiting enzyme in GSH de-novo biosynthesis [96]. Thus, the deregulation of the major antioxidant enzymes and enzymes involved in the GSH detoxification and antioxidant pathways can be considered as a marker for oxidative stress.

In the present study, among the tested oxidative response genes (Figure 5), the *GCLC* gene, which encodes the catalytic subunit of GCL, was the most affected by the exposure to CYN and all CYN/BPs mixtures. The gene was significantly up-regulated by CYN (1.66-fold) alone but not by the single BPs (<1.39-fold). The co-exposure to CYN/BPA (1.96-fold), CYN/BPS (1.72-fold), CYN/BPF (1.94-fold), and CYN/BPAF (1.73-fold) up-regulated *GCLC* gene expression to a higher extent than exposure to CYN alone, suggesting additive effects of the compounds in the binary mixtures. *GCLC* gene up-regulation indicates a possible cell response to GSH depletion, increasing its biosynthesis. Decreased GSH content in the cells can result from either increased oxidation, increased efflux, the formation of GSH-conjugates, or decreased synthesis (for a review see: [97]). CYN [62,69] and BPA (for a review see: [89]) have both been shown to reduce intracellular GSH. However, in the case of CYN, the GSH depletion might be the consequence of GSH synthesis inhibition, as has been shown in primary rat and mouse hepatocytes [62,69]. Thus, our results indicate that exposure to the CYN/BPs binary mixtures might reduce intracellular GSH levels in an additive manner.

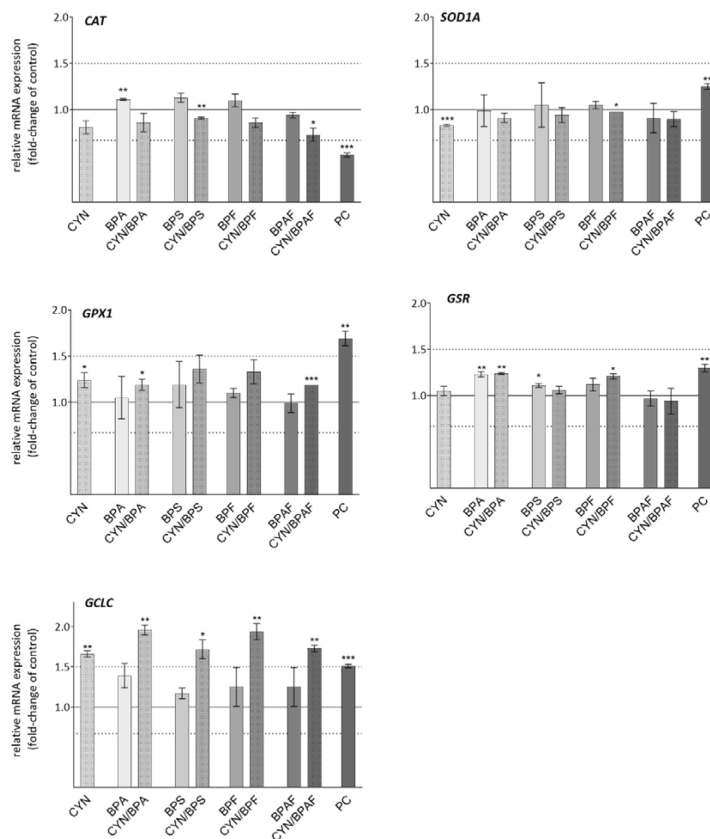


Figure 5. The effect of CYN (0.5 µg/mL), BPs (BPA, BPS, BPF, BPAF; 10 µg/mL), and their binary mixtures on the expression of oxidative stress response genes (*GCLC*, *GPX1*, *GSR*, *SOD1A*, *CAT*) in HepG2 cells after 24 h of exposure. The deregulations are expressed in fold-change of expression of the gene in the solvent control group (0.05% methanol and 0.04% DMSO = solid line at value 1.0). PC is the positive control—Benzo[a]pyrene BaP (30 µM). The lower and upper dotted lines denote biologically significant differences in gene expression (1.5-fold change). The asterisks (*) denote a statistically significant difference between solvent control and treated cells (* $p = 0.05$; ** $p = 0.01$; *** $p = 0.001$).

The other studied oxidative stress-responsive genes were generally not significantly deregulated by the tested compounds and/or the deregulation was less than 1.5-fold (Figure 5). *SOD1* was slightly down-regulated by CYN, while BPs and the combinations did not influence the expression of this gene. There was an indication of slight down-regulation of *CAT* and slight up-regulation of *GPX1* by CYN and all CYN/BPs combinations. Our results suggest that single or combined exposure to CYN and BPs after 24 h in HepG2, at the tested conditions, did not cause major oxidative stress. Except in the case of the gene *GCLC*, there was no indication for additive, synergistic, or antagonistic interactions of the

compounds in the binary mixtures that would affect oxidative response gene expression. In line with previous findings [46] in the HepG2 cell line, BPA and its analogues (BPS, BPF, and BPAF) as single compounds had no significant influence on the expression of the selected oxidative stress response genes. On the other hand, the activities of the enzymes SOD, CAT, and GPX were reported to be increased in human erythrocytes following exposure to BPA, BPF, and BPAF, but not BPS [92]. However, changes were observed at 10-fold higher concentrations than used in the present study. Therefore, the slight deregulations of *CAT* and *GPX1* in the cells exposed to the CYN/BPs combinations seem to be the consequence of CYN activity and reflect previously confirmed findings, showing CYN to induce only minor oxidative stress in HepG2 cells at the tested time point and concentration range used [18,19]. In addition, CYN induced a significant increase in the expression of genes *GCLC*, *GSR*, *GPX1*, and *SOD1* after 24 h of exposure in HPBLs. In contrast, the expression of *CAT* was not changed [26], indicating that lymphocytes might be more sensitive to CYN in terms of oxidative stress than hepatocytes.

3. Conclusions

In the present study, the cyto/genotoxic effects after co-exposure to emerging aquatic contaminants, the cyanobacterial toxin CYN in combination with BPA, and its analogues BPS, BPF, and BPAF were studied in the metabolically competent HepG2 cell line, for the first time. Exposure to CYN and the CYN/BPs binary mixtures significantly reduced the viability of HepG2 cells and increased the formation of DNA DSBs. The BPs alone did not decrease HepG2 cell viability and had no effect on DNA DSB induction. Generally, no significant differences were observed between cells treated with CYN alone and the CYN/BPs combinations, suggesting that the observed effects were the consequence of CYN activity. The only exception was the CYN/BPA combination, where significantly lower DNA DSBs were induced compared to the induction by CYN alone, suggesting antagonistic action of BPA against CYN in the mixture. The gene expression analysis, on the other hand, indicated additive or synergistic interactive effects in the CYN/BPs mixtures in several of the tested genes. The most pronounced effects were detected for the gene *CYP1A1*, where additive effects of CYN and BPs in all of the tested binary mixtures on gene up-regulation were indicated. In the case of the CYN/BPA mixture, the observed combined effect on *CYP1A1* up-regulation might have even been synergistic. The up-regulation of *CYP1A1* is of particular concern as the enzyme product of this gene catalyzes metabolic activation of pro-carcinogens to carcinogens that may result in increased susceptibility for genotoxic injury by indirect-acting genotoxic compounds to which humans can be exposed. The other genes, found differentially deregulated upon exposure to CYN/BPs mixtures compared to single compounds were *CDKN1A* and *GADD45A*, involved in DNA damage response, and *GCLC*, involved in oxidative stress response. The deregulation of these genes was more pronounced after exposure to the CYN/BPs binary mixtures compared to single compound exposure, suggesting an additive action of CYN and BPs. Our results confirm that co-exposure to pollutant mixtures can exert effects different from those caused by single compounds, even at relatively low concentrations, relevant for human exposure. Additionally, the results highlight the importance of co-exposure studies.

4. Materials and Methods

4.1. Chemicals

Cylindrospermopsin (CYN) was from Enzo Life Sciences GmbH (Lausen, Switzerland) (Table 1). A 0.5 mg/mL stock solution of CYN was prepared in 50% methanol and stored at -20°C . Bisphenol A (BPA), bisphenol S (BPS), bisphenol F (BPF), and bisphenol AF (BPAF) were from Sigma-Aldrich (St. Louis, MO, USA) (Table 1). Stock solutions of all the BPs (25 mg/mL) were prepared in dimethylsulphoxide (DMSO): BPA (109.5 mM), BPS (99.89 mM), BPF (124.86 mM), and BPAF (74.35 mM), and stored at -20°C . Minimal Essential Medium Eagle (MEM), non-essential amino acids (NEAA), Benzo(a)pyrene (BaP), methanol, DMSO, NaHCO_3 , phenazine methosulfate (PMS), and sodium pyruvate were from Sigma, USA. Penicillin/streptomycin, fetal bovine serum,

L-glutamine, and phosphate-buffered saline (PBS) were from PAA Laboratories, USA. Etoposide (ET) was from Santa Cruz Biotechnology, USA. The CellTiter 96® AQueous cell proliferation assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTS) was from Promega, Madison, WI, USA. Human recombinant Anti-H2AX pS139, FITC conjugate antibodies were from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany. Trypsin was from Invitrogen™, Life Technologies, Waltham, MA, USA. The TRIzol® reagent was from Thermo Fisher Scientific, Waltham, MA, USA. The cDNA High Capacity Archive Kit, TaqMan Universal PCR Master Mix, and Taq Man Gene Expression Assays (Table 1) were from Applied Biosystems, Waltham, MA, USA. All chemical reagents were of the purest grade available, and all solutions were made using Milli-Q water. The chemical structures shown in Table 1 were prepared with the ChemDraw Prime software from PerkinElmer Informatics, Waltham, MA, USA.

4.2. Cell Culture

The HepG2 cell line was obtained from the American Type Culture Collection (ATCC), USA, at passage 108. The cells were grown in MEM medium supplemented with 10% FBS, 2 mM L-glutamine, 1% NEAA, 2.2 g/L NaHCO₃, 1 mM sodium pyruvate, and 100 IU/mL penicillin/streptomycin, at 37 °C and 5% CO₂. Cell passages between 120 and 130 were used in the experiments. The cells were seeded on culture plates and left overnight to attach. Subsequently, the growth medium was replaced with fresh medium containing appropriate concentrations of single compounds CYN (0.5 µg/mL) and BPA, BPE, BPS, and BPAF (10 µg/mL) and binary mixtures of CNY and the different BPs, and incubated for 24 and 72 h. A negative control (growth medium containing 0.05% methanol and 0.04% DMSO) and assay-specific positive controls were included in all experiments. The final concentration of the solvent in the medium was adjusted in all experimental points.

4.3. Cell Viability—MTS Assay

Cell viability was studied after 24 and 72 h exposure of HepG2 cells to CYN, the BPs, and combinations thereof. ET (30 µg/mL) was used as a positive control. Cell viability was evaluated using the MTS tetrazolium reduction assay, as previously described by Hercog et al. [46]. Three independent experiments were performed, with five replicates per treatment point.

4.4. Analyses of the Induction of DNA DSB by the γ -H2AX Assay

Double-strand break (DSB) induction was evaluated through measuring γ H2X formation, using flow cytometry (MACSQuant Analyzer 10; Miltenyi Biotech, Germany). The experiments were performed as described by Hercog et al. [46]. The cells were seeded on six-well plates (Corning Inc., USA), exposed to the tested compounds and their mixtures, and then collected and fixed using ethanol (70%). Fixed cells were washed and labeled with Anti-H2AX pS139 antibodies (130-118-339) according to the manufacturer's protocol. Etoposide (ET, 1 µg/mL) was used as a positive control. FITC intensity was recorded for 10⁴ single cells in each sample. Independent experiments were repeated three times. GraphPad Prism 8 was used to generate box and whiskers plots. Statistical significance between treated groups and the vehicle control was determined with a linear mixed-effects model with the statistical program R [98] and its packages reshape [99], and nlme [100].

4.5. Real-Time Quantitative PCR (qPCR) Analysis

The mRNA expression of selected genes was analyzed by quantitative real-time PCR (qPCR). HepG2 cells were seeded on T25 plates (600,000 cells/plate) exposed to CYN, BPs alone, and to combinations thereof for 24 h. Total RNA was isolated using the TRIzol reagent according to the manufacturer's protocol with minor modifications described by Maisanaba et al. [101]. The RNA was transcribed to cDNA using 1 µg of total RNA and cDNA High Capacity Archive Kit, according to the manufacturer's protocol. Relative quantification of the selected genes was performed using

qPCR, where the TaqMan Universal PCR Master Mix and Taqman Gene Expression Assays were used (Table 2).

Table 2. List of Taqman Gene Expression Assays used.

Gene Symbol	Assay ID	Entrez Gene Name	Cellular Function
<i>CYP1A1</i>	Hs01054797_g1	Cytochrome P450, family 1, subfamily A, polypeptide 1	Metabolism of xenobiotics, detoxification response
<i>GCLC</i>	Hs00155249_m1	Glutamate-cysteine ligase, catalytic subunit	Oxidative stress response
<i>GPX1</i>	Hs00829989_gH	Glutathione peroxidase 1	Oxidative stress response
<i>GSR</i>	Hs00167317_m1	Glutathione reductase	Oxidative stress response
<i>SOD1A</i>	Hs00533490_m1	Superoxide dis-mutase 1	Oxidative stress response
<i>CAT</i>	Hs00156308_m1	Catalase	Oxidative stress response
<i>CDKN1A</i>	Hs00355782_m1	Cyclin-dependent kinase inhibitor 1A (p21. Cip1)	DNA-damage response genes
<i>GADD45A</i>	Hs00169255_m1	Growth arrest and DNA damage-inducible, alpha	DNA-damage response genes
<i>MDM2</i>	Hs00234753_m1	MDM2 proto-oncogen	DNA-damage response genes
<i>TP53</i>	Hs01034249_m1	Tumor protein P53	DNA-damage response genes
<i>CHEK1</i>	Hs00967506_m1	Checkpoint kinase 1	DNA-damage response genes

GAPDH (VIC-TAMRA, Cat. No.:4310884E, Applied Biosystems, USA) was used as a reference gene in all experiments. All experiments were performed on 384-well plates, with a single probe per well using the ViiA™ 7 Real-Time PCR System (Applied Biosystems™). The conditions for the PCR were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative quantification of gene expression was done by comparison of the Ct values of treated and control groups considering the actual efficiency of each assay using the Quant Genious protocol [102]. BaP (30 µM) was used as a positive control. Three independent experiments were performed each time in duplicates. Statistical difference between treated groups and controls was determined by two-tailed Student's *t*-test. A ≥ 1.5 -fold change in gene expression compared to control was considered as up-regulation and down-regulation, respectively.

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2.6 Application of Advanced HepG2 3D Cell Model for Studying Genotoxic Activity of Cyanobacterial Toxin Cylindrospermopsin

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Application of advanced HepG2 3D cell model for studying genotoxic activity of cyanobacterial toxin cylindrospermopsin[☆]

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ABSTRACT

Cylindrospermopsin (CYN) is an emerging cyanotoxin increasingly being found in freshwater cyanobacterial blooms worldwide. Humans and animals are exposed to CYN through the consumption of contaminated water and food as well as occupational and recreational water activities; therefore, it represents a potential health threat. It exhibits genotoxic effects in metabolically active test systems, thus it is considered as pro-genotoxic. In the present study, the advanced 3D cell model developed from human hepatocellular carcinoma (HepG2) cells was used for the evaluation of CYN cyto-/genotoxic activity. Spheroids were formed by forced floating method and were cultured for three days under static conditions prior to exposure to CYN (0.125, 0.25 and 0.5 µg/mL) for 72 h. CYN influence on spheroid growth was measured daily and cell survival was determined by MTS assay and live/dead staining. The influence on cell proliferation, cell cycle alterations and induction of DNA damage (γH2AX) was determined using flow cytometry. Further, the expression of selected genes (qPCR) involved in the metabolism of xenobiotics, proliferation, DNA damage response, apoptosis and oxidative stress was studied. Results revealed that CYN dose-dependently reduced the size of spheroids and affected cell division by arresting HepG2 cells in G1 phase of the cell cycle. No induction of DNA double strand breaks compared to control was determined at applied conditions. The analysis of gene expression revealed that CYN significantly deregulated genes encoding phase I (CYP1A1, CYP1A2, CYP3A4, ALDH3A) and II (NAT1, NAT2, SULT1B1, SULT1C2, UGT1A1, UGT2B7) enzymes as well as genes involved in cell proliferation (PCNA, TOP2α), apoptosis (BBC3) and DNA damage response (GADD45a, CDKN1A, ERCC4). The advanced 3D HepG2 cell model due to its more complex structure and improved cellular interactions provides more physiologically relevant information and more predictive data for human exposure, and can thus contribute to more reliable genotoxicity assessment of chemicals including cyanotoxins.

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1. Introduction

Cyanobacterial blooms are of increasing concern since their occurrence in the environment is expanding due to water eutrophication and climate change (Mantzouki et al., 2018; O'Neil et al., 2012; Visser et al., 2016). Cyanobacteria produce a wide range of secondary metabolites including highly toxic cyanotoxins that can pose threat to human and animal health. One of the emerging

cyanotoxins is cylindrospermopsin (CYN) that is produced by many cyanobacterial species including *Anabaena* sp., *Aphanizomenon* sp., *Chroosporum ovalisporum*, *Raphidiopsis raciborskii*, *Lyngbya wollei*, *Oscillatoria* sp., *Raphidiopsis* sp., *Umezakia natans* etc (Rzymiski and Poniedzialek, 2014). Its producers are mainly native to subtropical areas (de la Cruz et al., 2013), but CYN has recently been identified also in inland waters of North America and Europe (Suklenik et al., 2012; Svirčev et al., 2019) as well as in reservoirs for urban water supply (Buratti et al., 2017; Miller et al., 2017; Poniedzialek et al., 2012), indicating that species producing the toxin are spreading to temperate regions.

Humans may be exposed to CYN through several routes, where dermal and inhalation exposure may occur with professional (e.g. fishing), recreational (sports) or domestic (e.g. showering) use of contaminated water. The most frequent way of human exposure, is

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the oral route, which occurs through the ingestion of contaminated drinking water or accidental swallowing of water during recreational activities. In the environment, the concentrations of CYN in surface waters can reach up to several tens of $\mu\text{g/L}$ (Mohamed and Al-Shehri, 2013; Moreira et al., 2017), while during the blooming season the concentrations of CYN can reach concentrations such as 589 $\mu\text{g/L}$ (Saker and Eaglesham, 1999) and up to 800 $\mu\text{g/L}$ (Shaw et al., 2000).

CYN is a polyketide-derived alkaloid with a guanidine moiety combined with a hydroxymethyluracil group attached to its tricyclic carbon skeleton (Ohtani et al., 1992). The primary target organs of CYN toxicity are the liver and kidney; however, also other organs (e.g. spleen, thymus, lung, heart, and lymphocytes) can be affected (Falconer and Humpage, 2006). CYN displays a wide range of toxic effects, with protein synthesis inhibition (Frosio et al., 2001) being the main known mechanism of action. An interplay between the hydroxyl and guanidine functional groups appears to be the key for CYNs toxicity (Evans et al., 2019); however, detailed information of its molecular mode of action remains unclear. In addition to being hepatotoxic (Runnegar et al., 2002) and generally cytotoxic, it is developmentally toxic (Berry et al., 2009), immunotoxic (Moosova et al., 2019; Poniedzialek et al., 2014), dermatotoxic, and there are indications of its endocrine disrupting (Liu et al., 2018; Young et al., 2008) and neurotoxic activity (for review see: Hinojosa et al., 2019).

At the molecular level, CYN is inhibiting glutathione synthesis (Runnegar et al., 1994, 1995) resulting in generation of reactive oxygen species and lipid peroxidation (Evans et al., 2019; Liebel et al., 2011; Poniedzialek et al., 2015). Its genotoxic activity is manifested in the form of DNA strand breaks (Humpage et al., 2005; Sieroslawska and Rymuska, 2015; Straser et al., 2013b, 2013a; Żegura et al., 2011a, 2011b) and chromosomal damage (Bazin et al., 2010; Herczeg et al., 2017; Humpage et al., 2000; Sieroslawska and Rymuska, 2015; Straser et al., 2011; Żegura et al., 2011a, 2011b). CYN is considered to be pro-genotoxic, meaning that metabolic activation is required for its cytotoxic and/or genotoxic effects; however, the exact metabolic pathway remains unclear. Numerous indirect evidences are showing that various isoforms of cytochrome P450 (CYP450) family are involved in its metabolic activation (Bazin et al., 2010; Frosio et al., 2003; Herczeg et al., 2017; Humpage et al., 2005; Kittler et al., 2016; Straser et al., 2013c, 2013a, 2011; Żegura et al., 2011a, 2011b), while not much literature data exists on the involvement of phase II enzymes in CYN detoxification.

Recently US Environmental Protection Agency (EPA, 2015) and European Food Safety Agency (Testai et al., 2017) published documents prioritizing the identification of CYN adverse health effects, thus there is an urgent need to elucidate its molecular mechanisms of action in detail.

In the present study, cytotoxic and genotoxic activity of CYN was studied in three dimensional (3D) *in vitro* cell model (spheroids) developed from human hepatocellular carcinoma cell line (HepG2). Compared to the traditional two dimensional (2D) cell cultures, cell line derived hepatospheres possess improved cell-cell and cell-matrix interactions, resulting in higher cell differentiation, cell morphology and cytoskeleton architecture that more closely resemble *in vivo* cellular functions (Fey and Wrzesinski, 2012; Godoy et al., 2013; Zhang et al., 2016). Primary liver cells are considered the best approximation to *in vivo* situation; however, spheroids formed from hepatocyte cell lines appear equally useful for determination of cytotoxic effects *in vitro*, while the classical 2D cultured cell models show significantly lower correlation (Fey and Wrzesinski, 2012). Another advantage of 3D cell models is that they allow prolonged exposures due to their higher stability as they retain cell viability, morphology, and functionality over a period of several weeks (Bell et al., 2016).

The spheroids obtained from HepG2 cells show increased level

of liver-specific functions and expression of markers of differentiated and polarized hepatocytes (Kelm et al., 2003; Luckert et al., 2017; Mueller et al., 2011; Ramaiahgari et al., 2014). In HepG2 spheroids bile canaliculi-like structures are formed (Kelm et al., 2003; Ramaiahgari et al., 2014) and thus spheroids possess functional bile transport (Luckert et al., 2017; Mueller et al., 2011). Moreover, gene expression patterns including liver-specific genes and genes involved in xenobiotic metabolism are markedly different compared to monolayer cultures (Chang and Hughes-Fulford, 2009; Hurrell et al., 2019; Luckert et al., 2017; Stampar et al., 2019).

In our study, the HepG2 spheroids were exposed to non-cytotoxic concentrations of CYN and the influence on spheroid growth, cell division, and DNA damage was studied with microscopy and flow cytometry. To explore molecular mechanisms underlying CYN induced cellular effects, deregulation of the expression of selected genes involved in metabolism of xenobiotics (phase I and II), DNA damage response, cell proliferation, apoptosis and oxidative stress upon exposure of 3D spheroids to CYN was analysed using the qPCR.

2. Materials and methods

2.1. Chemicals

Cylindrospermopsin (CYN) was obtained from Enzo Life Sciences GmbH (Lausen, Switzerland). A 0.5 mg/mL stock solution of CYN was prepared in 50% methanol and stored at -20°C . Minimum essential medium eagle (MEME) and its supplements, benzo(a) pyrene (BaP), fluorescein diacetate (FDA) and propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA), while the etoposide (ET) was from Santa Cruz Biotechnology (St. Cruz, CA, USA). Hoechst 33258 dye was purchased from Invitrogen (Waltham, MA, USA). Methanol and phosphate buffered saline (PBS) were from PAA Laboratories (Dartmouth, NH, USA). Foetal bovine serum (FBS), trypsin-EDTA (0.25%) and TRIZOL reagent were from Gibco (Praisley, Scotland, UK). Triton X-100 was from Fisher Sciences (New Jersey, USA). CellTiter 96® Aqueous cell proliferation assay (MTS) was from Promega, (Madison, WI, USA). qPCR reagents (High capacity cDNA Transcription Kit, TaqMan Gene Expression Assays, TATAA PreAmp Grand Master Mix (2x), and TaqMan Universal PCR Master Mix) were from Applied Biosystems (Foster City, CA, USA). Anti-H2AX pS139 (130-107-586) and Anti-Ki-67-FITC (130-107-586) antibodies were from Miltenyi Biotec (Bergisch Gladbach, Germany).

2.2. Cell culture and spheroids formation

HepG2 cells (ATCC, HB-8065™) were cultured at 37°C and 5% CO_2 in MEME medium supplemented with 1% non-essential amino acids, 2.2 g/L NaHCO_3 , 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/mL penicillin/streptomycin and 10% FBS.

The spheroids were prepared by the forced floating method according to (Stampar et al., 2019) with minor modifications. Briefly, the cells (5000 cells/well) in growth media supplemented with 4% methylcellulose were centrifuged at 900 g for 1.5 h at 28°C in 96-well U-bottom low attachment plate to form spheroids. Subsequently they were cultured for 3 days at 37°C and 5% CO_2 prior treatment. Afterwards, the growth media was replaced with fresh media containing cylindrospermopsin (CYN) at concentrations 0.125, 0.25 and 0.5 $\mu\text{g/mL}$ and the spheroids were incubated for additional 72 h. In all experiments, untreated (growth media), solvent (0.05% methanol) and appropriate positive (PC) controls were included.

2.3. Monitoring of growth and morphology of spheroids

Five spheroids per treatment were monitored over 72 h of cultivation. The surface area of each spheroid in each treatment condition was measured every 24 h and the image of each spheroid was captured using NIS elements software 4.13 v (Nikon Instruments, Melville, NY, USA) connected with Ti Eclipse inverted microscope (Nikon, Japan). Spheroid growth was monitored in three independent parallels. Etoposide [1 µg/mL] was used as a PC. The difference between treated spheroids and solvent control was analysed by the Two-way ANOVA with Fisher's LSD test using GraphPad Prism V6 for Windows (GraphPad Software, California USA). $p < 0.05$ was considered statistically significant.

2.4. Cell viability test

The tetrazolium-based (MTS) assay was performed according to the manufacturer's protocol with minor modifications (Stampar et al., 2019). Briefly, after 72 h exposure to CYN, a freshly prepared MTS:PMS solution (20:1) was added to each well corresponding to one spheroid. After 3 h of incubation, the absorbance was measured at 490 nm using the spectrofluorimeter (Synergy MX, BioTek, USA). The experiment was repeated three times independently and each time eight spheroids per treatment were included. Etoposide [30 µg/mL] was used as a PC. The difference between treated groups and the solvent control was analysed by the One-way ANOVA using GraphPad Prism Software. $p < 0.05$ was considered statistically significant.

2.5. Confocal z-stack imaging of life/dead stained spheroids

After 72 h of CYN exposure, the culture media was replaced with FBS-free media with FDA stain [8 µg/mL] and incubated in darkness for 1 h. Afterwards, PI [20 µg/mL] was added and incubated for additional 5 min. Z-stack images of single spheroids were taken using the confocal microscope Leica SP8 TCS at 100x magnification. Etoposide [1 µg/mL] was used as a PC. The image analysis and the quantification of the ratio of dead cells was performed using the Image-Pro 10 software (Media Cybernetics, USA). At least 40 Z-stack images per spheroid were recorded through the entire volume of the spheroid using excitation and emission settings for simultaneous dual channel recordings and at least three spheroids per treatment point were included in the analysis. The difference between treated groups and the solvent control was analysed by the One-way ANOVA using GraphPad Software. $p < 0.05$ was considered statistically significant.

2.6. Flow cytometric analyses of cell cycle, cell proliferation and gamma-H2AX formation

For the analyses of the cell cycle, cell proliferation and gamma-H2AX formation single-cell suspension was prepared with enzymatic digestion and mechanical degradation according to (Stampar et al., 2019) with modifications. After the exposure, 30 spheroids per treatment were collected in a tube, washed in PBS and incubated in trypsin-EDTA (0.25%, Gibco) for 4 min. Next, the spheroids were disintegrated into a single-cell suspension using 200 µL pipet tips. The obtained single cells were washed in PBS, fixed in ethanol and stored at -20°C until analysis (for details see (Herczeg et al., 2019)). Fixed cells were washed in cold PBS and labelled for simultaneous detection of three endpoints. First, they were labelled with anti-H2AX pS139-APC and anti-Ki67-FITC (50-fold diluted antibodies in 1% BSA), washed with PBS, and subsequently stained with Hoechst 33342 dye (diluted in 0.1% Triton X-100 1:500). Flow cytometric analyses were carried out on a MACSQuant Analyzer 10

(Miltenyi Biotec, Germany). APC fluorescence, corresponding to DNA double strand breaks (DSBs), was detected in R1 (655–730 nm) channel; FITC fluorescence, corresponding to the proliferation marker Ki67+, was detected in the B1 (525/50 nm) channel and Hoechst fluorescence corresponding to DNA content for cell cycle analysis was detected in V1 (450/50 nm) channel. Unspecific binding of antibodies was excluded based on reagent-FITC and reagent-APC controls (Miltenyi Biotec, Germany). The experiment was repeated three times independently where each time 10^4 single cells per experimental point were recorded. Etoposide [1 µg/mL] was used as a PC.

Flow cytometry data were analysed and graphically presented in the FlowJo software V10 (Becton Dickinson, New Jersey USA). The comparison of the cell cycle distribution of cells in the solvent control and treated samples was done by two-way ANOVA with Fisher's LSD test, while the significance of Ki67 positive cells was tested by one-way ANOVA with Dunnett's multiple comparison test both using GraphPad Software. The statistical significance in the change of APC fluorescence was tested using exported.csv values in the R software with Linear and Nonlinear Mixed Effects Models (nlme) package (Pinheiro et al., 2007).

2.7. The qPCR analyses of the expression of selected genes

The expression of selected genes was determined in spheroids exposed to the highest concentration of CYN [0.5 µg/mL], solvent control and B(a)P [30 µM] as a PC. Total mRNA was isolated from a pool of 32 spheroids per treatment, using TRIzol reagent, according to manufacturer's instructions. The mRNA concentration and quality were spectrophotometrically determined using NanoDrop 1000 (Thermo Fischer Scientific, Wilmington, USA). 1 µg of total isolated mRNA per sample was reverse transcribed using The High capacity cDNA Transcription Kit. The experiment was repeated in three parallels with two technical repetitions. The BioMarkTM HD System to perform real-time quantitative PCR (qPCR) analysis on the 48.48 Dynamic Array™ was employed to analyse the level of expression of selected genes (Table 1) according to the manufacturer's instructions. The relative quantification of gene deregulation was performed with the QuantGenious web-based tool (Baebler et al., 2017) comparing the Ct values of reference (GAPDH and HPRT1) and target genes using the standard curve. Relative gene expression ≥ 1.5 -fold or ≤ 0.66 compared to control was considered as up- or down-regulation, respectively. The significance of the difference between treated groups and the solvent control was analysed by two tailed Student's t-test with Welch's correction using GraphPad Software.

3. Results and discussion

In the last decade, the toxicity and genotoxicity and potential carcinogenicity of CYN has been extensively studied using traditional monolayer (2D) cell culture models *in vitro*. However, 2D cultures are associated with inherent weaknesses, which reduce their reliability for predicting of the effects *in vivo* in whole organisms. Recently we developed *in vitro* 3D cell model from HepG2 cells by the forced floating method (Stampar et al., 2019) that was in the present study used for studying the cyto-/genotoxic effects and the underlying molecular mechanisms of toxin activity.

3.1. The effects of CYN on HepG2 spheroid morphology and cell viability

The exposure of HepG2 spheroids to CYN for 72 h caused dose-dependent decrease of spheroid's growth (Fig. 1C). At the end of exposure the average surface area of control spheroids cultivated in

Table 1
Genes included in the gene expression analyses.

	Gene symbol	Full Gene Name	Taqman Gene Expression Assay
Housekeeping gene	GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	Hs02758991.g1
	HPRT1	Hypoxanthine Phosphoribosyltransferase 1	Hs02800695.m1
Metabolism of xenobiotics	CYP1A1	Cytochrome P450 Family 1 Subfamily A Member 1	Hs01054797.g1
	CYP1A2	Cytochrome P450 Family 1 Subfamily A Member 2	Hs00167927.m1
	CYP3A4	Cytochrome P450 Family 3 Subfamily A Member 4	Hs02514989.s1
	ALDH3A1	Aldehyde Dehydrogenase 3 Family Member A1	Hs00964880.m1
	AHR	Aryl Hydrocarbon Receptor	Hs00169233.m1
	NAT1	N-Acetyltransferase 1	Hs02511243.s1
	NAT2	N-Acetyltransferase 2	Hs01854954.s1
	SULT1B1	Sulfotransferase Family 1 B Member 1	Hs00234899.m1
	SULT1C2	Sulfotransferase Family 1C Member 2	Hs00602560.m1
	UGT1A1	UDP Glucuronosyltransferase Family 1 Member A1	Hs02511055.s1
	UGT2B7	UDP Glucuronosyltransferase Family 2 Member B7	Hs00426592.m1
Oxidative stress	HIF-1 α	Hypoxia Inducible Factor 1 Subunit Alpha	Hs00153153.m1
DNA damage response and cell cycle arrest	CDKN1A	Cyclin Dependent Kinase Inhibitor 1 A	Hs00355782.m1
	GADD45 α	Growth Arrest And DNA Damage Inducible Alpha	Hs00169255.m1
	ERCC4	ERCC Excision Repair 4, Endonuclease Catalytic Subunit	Hs00193342.m1
	CND1	Cyclin D1	Hs00765553.m1
Proliferation	MKI67	Marker Of Proliferation Ki-67	Hs01032443.m1
	PCNA	Proliferating Cell Nuclear Antigen	Hs00427214.g1
	TOP2 α	DNA Topoisomerase II Alpha	Hs01032137.m1
Apoptosis	BBC3	BCL2 Binding Component 3	Hs00248075.m1

medium with solvent (0.05% methanol) was $0.451 \pm 0.040 \text{ mm}^2$, while the surface area of spheroids exposed to $0.5 \text{ }\mu\text{g/mL}$ CYN was $0.433 \pm 0.039 \text{ mm}^2$. When the spheroid growth was normalized to its size at the treatment point (time 0), the measurements revealed that after 72 h CYN inhibited spheroid growth for 8% on average when compared to the solvent control ($p = 0.0003$) (Fig. 1A and C). Etoposide, the positive control, after 72-h exposure decreased the spheroid area for 31% on average compared to solvent control ($p < 0.0001$). CYN affected the surface and the integrity of spheroids, which was less compact than that of control ones (Fig. 1B). The loosened and detached cells on the surface were viable (Fig. 2). Basu et al. (2018) described similar effects of CYN ($1.04 \text{ }\mu\text{g/mL}$) on the surface of spheroids developed from liver stem cells HLI-hT1. The surface of control spheroids was generally smooth, while in CYN treated spheroids it was ruffled and disintegrated. Previously, Straser et al. (2013b) reported CYN induced morphological changes in HepG2 cells cultured in a monolayer. In cells exposed to CYN, formation of a compact monolayer of cells was reduced and a less firm attachment of cells to the surface of the culture plates and to each other was reported, which is in line with our observations on the surface of the spheroids. There are several indications that CYN affects the cytoskeleton of cells (reviewed in Mathe et al., 2017), which results in changed cell morphology and intracellular bonds, which may explain the observed effects.

The exposure of the spheroids to CYN for 72 h did not significantly reduce the viability of cells. No difference between cells from CYN treated and control spheroids was observed with the tetrazolium-based (MTS) assay (Fig. 3A), while the live/dead staining of the whole spheroid showed a trend of dose dependent increase in the ratio of dead cells that was however not statistically significant (Fig. 3B). The positive control, etoposide significantly reduced cell viability (Fig. 3). Contrary to these results, under the same exposure conditions, in 2D HepG2 cultures, CYN reduced cell viability for approximately 50% (Straser et al., 2013b). Higher cell survival in 3D compared to 2D HepG2 cultures has been observed also after the exposure to two heterocyclic aromatic amines PhIP and IQ, a polycyclic aromatic hydrocarbon B(a)P, and a mycotoxin AFB1, (Stampar et al., 2019). Li (2008) reported that HepG2 cells cultured in 3D are more resistant to drug-induced apoptosis and the same phenomenon has been observed in several studies where cells cultured in 3D showed higher resistance to anticancer drugs

than cells cultured in 2D, irrespective of the compound's mechanism of action (Karlsson et al., 2012; Loessner et al., 2010; Patra et al., 2016). Similar pattern of chemoresistance as developed in 3D spheroids is observed also *in vivo* (Sodek et al., 2009). Lower sensitivity of 3D culture vs. 2D can result from the differences in i) physical and physiological characteristics (interactions between neighbouring cells and extracellular matrix influencing cellular decision-making process); ii) presence and spatial arrangement of membrane receptors; iii) gene expression levels; iv) cell stages and v) drug accessibility and local pH (reviewed in Edmondson et al., 2014). The difference in CYN cytotoxic effects obtained in 3D vs. 2D models is in line with higher resistance of 3D models towards cytotoxic compounds.

3.2. The effects of CYN on cell cycle, cell proliferation and DNA damage

The effects of CYN on cell cycle, cell proliferation and DNA damage was determined by flow cytometry with simultaneous measurement of the fluorescent signals of Hoechst 33342 for cell cycle determination, FITC corresponding to proliferation marker Ki67 and APC corresponding to DNA DSBs. The effect of CYN on the cell cycle was observed at concentrations $\geq 0.25 \text{ }\mu\text{g/mL}$ (Fig. 4A). Compared to solvent control, the number of cells in G0/G1 phase was increased together with the reduction of the number of cells in S phase. The proliferation marker Ki67 was equally present in the cells of treated and control spheroids (Fig. 4B). However, the simultaneous staining with Hoechst and anti-Ki67 antibody enabled us to analyse the distribution of Ki67 positive cells across the cell cycle phases (Fig. 4D) and therefore to distinguish between cells in G0 and G1 phase (Kim and Sederstrom, 2015) since Ki67 is not present in G0 phase. The ratio of Ki67 positive cells in G0/G1 phase was similar in the control and CYN treated groups (approximately 55%) (Fig. 4C), which means that the increased number of cells in G0/G1 after CYN exposure was not due to the increased number of non-proliferating cells (G0), but represents G1 cell cycle arrest (Fig. 4A). In etoposide (PC) exposed spheroids 68% of cells in G0/G1 cells were Ki67 negative (Fig. 4C), meaning that less cells were dividing.

In G1 phase of the cell cycle, crucial decisions on DNA replication and completing the cell division are made in cells. Genotoxic stress

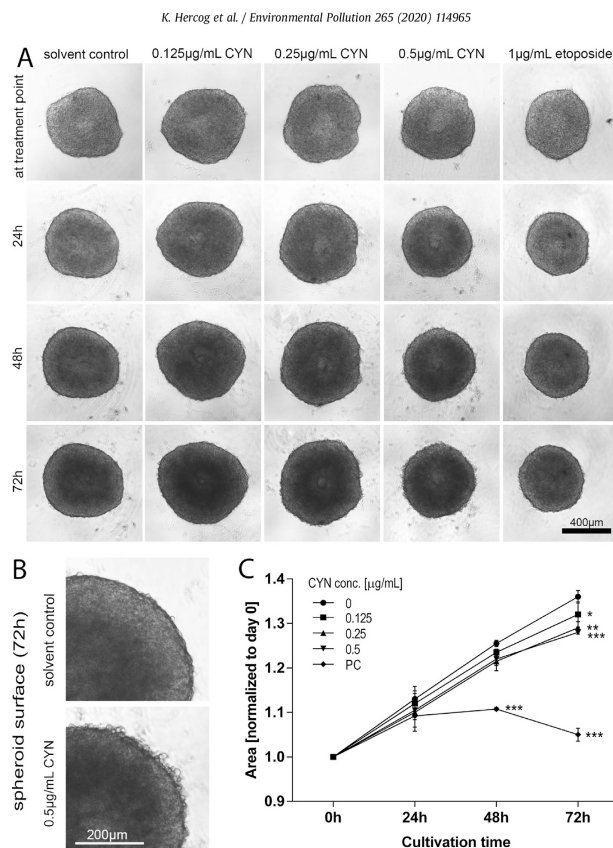


Fig. 1. A: Monitoring of the spheroids at different times of exposure to CYN (0.125, 0.25 and 0.5 µg/mL) and controls. For each treatment, a representative image sequence of the same spheroid is shown. The scale (400 µm) corresponds to all images; B: The surface of spheroid cultivated in the medium with solvent [0.05% methanol] and CYN [0.5 µg/mL] treated for 72 h; C: The area of spheroids at different times of cultivation and exposure to CYN and PC (ET [1 µg/mL]).

of various sources leads to activation of p53 protein which regulates the transcription of genes involved in G1/S cell-cycle arrest, including the cyclin-dependent kinase inhibitor 1 (p21) (Bartek and Lukas, 2001).

CYN [1 µg/mL] induced cell cycle arrest at G0/G1 phase was observed also in human lymphocytes (Poniedzialek et al., 2014). On the contrary, Straser et al. (2013b) reported that in the HepG2 monolayer culture 72 h exposure to CYN [0.5 µg/mL] decreased the number of cells in G0/G1 phase and caused S-phase cell cycle arrest. In the same study CYN induced DNA DSBs were observed (Straser et al., 2013b), while the results from the present study showed no increase of γH2AX positive cells corresponding to DNA DSBs in HepG2 spheroids (Fig. 4E). The S-phase DNA synthesis arrest is specifically related to DSBs via the p53-independent ATM pathway (Kastan and Lim, 2000). This explains why the cells in spheroids exposed to CYN were not arrested in S phase as has been previously

observed by Straser et al. (2013b). These results suggest differences in the mechanisms of action of CYN in 2D and 3D models. In HepaRG cells exposed to CYN, Huguet et al. (2019) reported cell cycle alternations with G2/M arrest and the induction of γH2AX, but at concentrations at which the cell viability was reduced to 50% [≥ 1.3 µg/mL] and 10% [5.2 µg/mL]. The DNA DSBs observed at such high cytotoxicity may not reflect genotoxic activity but correspond to degradation of DNA in the process of apoptosis or necrosis (Müller et al., 1999).

3.3. The effects of CYN on the expression of selected genes

To elucidate the molecular mechanisms involved in CYN induced toxicity and genotoxicity we analysed changes in the transcription of selected genes involved in metabolic transformation, oxidative stress, DNA damage response, proliferation

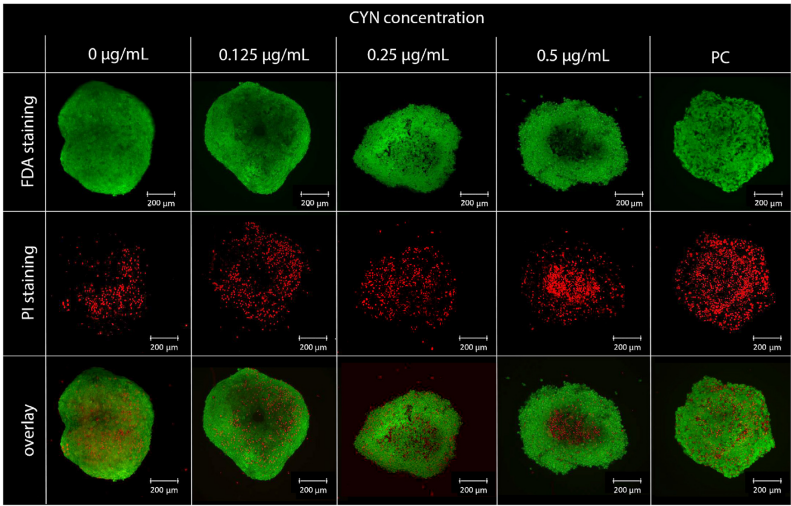


Fig. 2. Images of control and CYN (0.125, 0.25 and 0.5 µg/mL) treated spheroids stained with FDA (green, live cells) and PI (red, dead cells). Etoposide (1 µg/mL) was used as a positive control. The images were obtained using a confocal microscope at 100 $\times$ -magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

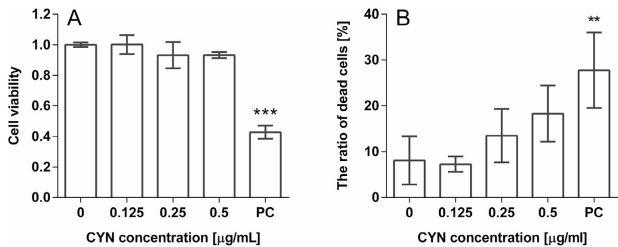


Fig. 3. The effect of CYN (0.125, 0.25 and 0.5 µg/mL) on the viability of HepG2 cells in spheroids after 72 h exposure. A: Viability determined with the MTS assay. B: The ratio of dead cells determined with live/dead staining of whole spheroid (confocal microscopy). PC = 30 µg/mL etoposide. Significant difference between treated spheroids and solvent control (0) (one-way ANOVA) is indicated by ** $p < 0.01$ and *** $p < 0.001$.

and apoptosis (Table 2). Nowadays, CYN is generally recognized as pro-genotoxic compound, which requires metabolic activation by cytochrome P-450 (CYP450) enzymes to cause genotoxic effects (Straser et al., 2011). The cytochrome P450 (CYP) enzymes are membrane-bound hemoproteins that play a pivotal role in the detoxification of xenobiotics, cellular metabolism and homeostasis. Induction or inhibition of CYP enzymes is a principal mechanism for metabolism-based drug-drug interactions. CYP enzymes can be transcriptionally activated by various xenobiotics and endogenous substrates through receptor-dependent mechanisms and consequently CYP-mediated biotransformation may result in metabolic activation of environmental chemicals to reactive carcinogenic products (Manikandan and Nagini, 2017). In metabolically incompetent *in vitro* models, CYN does not induce genotoxic effects

(Fessard and Bernard, 2003; Lankoff et al., 2007; Puerto et al., 2018), while in metabolically active models CYP450 inhibitors reduce toxic effects of CYN *in vitro* (Bazin et al., 2010; Humpage et al., 2005; Kittler et al., 2016) and *in vivo* (Frosio et al., 2003). The HepG2 spheroids used in the present study have been shown to be metabolically competent and sensitive for detecting the effects of indirect genotoxic compounds (Stampar et al., 2019). CYN upregulated the expression of *CYP1A1* (13.2-fold) and *CYP3A4* (2.45-fold), suggesting their role in the activation of the toxin, while the transcription of *CYP1A2* was not significantly affected. Another phase I enzyme *ALDH3A1*, a member of aldehyde dehydrogenase 3 family, which oxidizes various aldehydes to the corresponding acids (Lindahl, 1992), was also strongly upregulated (30.7-fold). Moreover, the transcription of *AHR*, the main nuclear factor in the

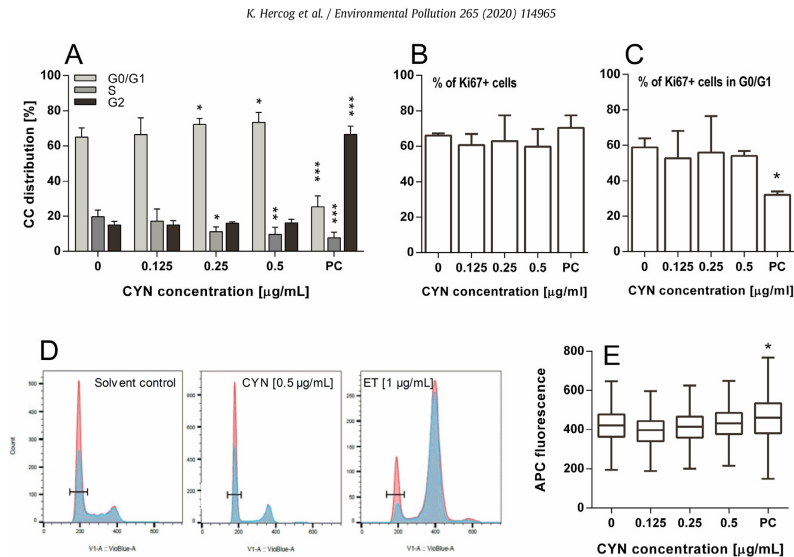


Fig. 4. A: Distribution of cells across different phases of cell cycle (CC) after exposure to CYN; B: Percentage of Ki67 positive cells after exposure to CYN; C: Percentage of Ki67 positive cells within G0/G1 phase of cell cycle after exposure to CYN; D: Representative overlays of simultaneous staining with Hoechst for cell cycle (red) and anti-Ki67 antibody (blue); E: relative values of APC fluorescence corresponding to anti-γH2AX labelled sites after exposure to CYN. PC = 1 µg/mL etoposide. Significant difference between treated sample and solvent control (0) for cell cycle (two-way ANOVA with Fisher's LSD test), Ki67 (one-way ANOVA with Dunnett's multiple comparison) and γH2AX (R nlme package) is indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Relative expression of selected genes after the exposure of HepG2 spheroids to CYN [0.5 µg/mL] for 72 h. BaP [10 µM] treatment was included as PC.

function	gene	CYN [0.5 µg/mL]			BaP [10 µM]				
		Average	SD	p-value	average	SD	p-value		
metabolism of xenobiotics	CYP1A1	13.16	2.24	0.00155	**	340.91	21.51	0.00002	***
	CYP1A2	0.86	0.03	0.00421	**	11.93	1.74	0.00089	***
	CYP3A4	2.45	0.69	0.04072	*	2.00	0.46	0.03709	*
	ALDH3A1	30.74	9.50	0.01144	*	270.28	103.16	0.02099	*
	AHR	4.85	0.38	0.00014	***	2.15	0.18	0.00076	***
	NAT1	5.36	0.41	0.00011	***	2.85	0.10	0.00001	***
	NAT2	0.61	0.33	0.168972		4.69	2.68	0.12311	*
	SULT1B1	2.56	1.91	0.31259		2.65	1.63	0.22543	
	SULT1C2	2.32	0.02	0.00000	***	1.51	0.12	0.00412	**
	UGT1A1	12.69	1.97	0.00111	**	61.73	4.81	0.00006	***
oxidative stress	UGT2B7	2.43	0.09	0.00003	***	11.82	0.64	0.00002	***
	HIF1α	2.13	0.26	0.00331	**	1.88	0.08	0.00011	***
DNA damage response and cell cycle arrest	CDKN1A	10.58	0.54	0.00001	***	22.02	3.17	0.00072	***
	GADD45α	32.74	5.11	0.00093	***	4.76	0.59	0.00082	***
	ERCC4	5.27	0.31	0.00004	***	1.90	0.11	0.00033	***
	CCND1	1.46	0.08	0.00119	**	1.96	0.21	0.00303	**
proliferation	PCNA	0.47	0.03	0.00001	***	1.82	0.08	0.00016	***
	TOP2α	0.40	0.03	0.00001	***	0.49	0.06	0.00024	***
	MKI67	0.73	0.08	0.01061	*	0.42	0.05	0.00006	***
apoptosis	BBC3	4.75	0.85	0.00334	**	3.34	0.33	0.00055	***

regulation of CYPs and ALDHs (Omiecinski et al., 2011), was upregulated (4.85-fold), indicating that aryl hydrocarbon receptor (AHR) mediated induction of the transcription of phase I enzymes. CYN upregulated the mRNA level of all studied phase II enzymes, with the exception of NAT2, which was downregulated (−1.64-fold). The highest relative expression was observed for UGT1A1 (12.7-fold) and NAT1 (5.4-fold), while SULT1A1, SULT1C2, and

UGT2B7 were approximately 2.4-fold upregulated. The results are in line with previously reported deregulation of the expression of genes encoding metabolic enzymes in HepG2 monolayer culture exposed to 0.5 µg/mL CYN for up to 24-h (Hercog et al., 2017; Straser et al., 2013c, 2011). CYN upregulated the expression of CYP1A1 also in human peripheral blood lymphocytes (Zegura et al., 2011a, 2011b). Recently, strong downregulation of genes encoding

enzymes involved in the metabolism of xenobiotics was detected in the differentiated HepaRG monolayer culture exposed to CYN [$\geq 0.2 \mu\text{g/mL}$] for 24 h together with reduced activity of cytochromes P450, including CYP3A4 [$\geq 0.66 \mu\text{g/mL}$] (Huguet et al., 2019). These data are contrary to our results; however, also to other published data (Bazin et al., 2010; Frosio et al., 2003; Hercog et al., 2017; Humpage et al., 2005; Kittler et al., 2016; Straser et al., 2013c, 2011; Zegura et al., 2011a, 2011b). Up to date, there are no literature data describing deregulation of the enzymes SUL1B1, SUL1C2, and UGT2B7 following cell exposure to CYN. Our study demonstrated potential involvement of enzymes encoded by these genes in CYN detoxification. From the obtained data we can conclude that in the 3D HepG2 cells model CYN upregulated the expression of metabolic genes especially those involved in detoxification (phase II) to a higher extent than previously reported in 2D cultures, further supporting the evidence that there are differences in metabolic pathways between 2D and 3D cell cultures. More efficient metabolism of CYN in 3D spheroids not only through the activation phase but also through the detoxification is in alignment with its reduced cytotoxic and genotoxic effects as well as lower effects on proliferation in 3D spheroids compared to 2D cultures, which is described above.

One of the CYN mechanisms of action is generation of reactive oxygen species (ROS) and induction of oxidative stress (Evans et al., 2019; Liebel et al., 2011; Poniedzialek et al., 2015; Runnegar et al., 1994, 1995), which corresponds to the upregulation of *HIF1A* (2.13-fold) in the present study. The *HIF1A* gene is constitutively expressed at low levels; however, its expression is often significantly upregulated in response to hypoxia and may also be upregulated by ROS (Yeo, 2019).

Despite no increase in γH2AX positive cells was observed under the conditions applied in the present study, the genes involved in DNA damage response were highly upregulated, showing genotoxic activity of CYN in HepG2 spheroids. The up-regulation of DNA damage responsive genes *CDKN1A*, *GADD45a*, *MDM2*, and *ERCC4* upon CYN exposure has already been described in HepG2 monolayer cultures (Bain et al., 2007; Hercog et al., 2017; Straser et al., 2013c, 2011), human peripheral blood lymphocytes (Zegura et al., 2011a, 2011b) and human dermal fibroblasts (Bain et al., 2007).

DNA damage is followed by rapid induction of the *GADD45* protein family members resulting in cell cycle arrest. Afterwards, the cell undergo apoptosis or DNA repair mechanisms in which *GADD45* proteins play an active role (Tamura et al., 2012). The *GADD45*-mediated cell cycle arrest is induced primary via the *CDK1/Cyclin B1* complex, which is responsible for the G2/M checkpoint. However, through interaction with p21 (encoded by *CDKN1A*), *GADD45* proteins modulate G1/S transition (reviewed in (Tamura et al., 2012)), which was also observed in the present study. Likewise, *ERCC4* is also induced by genotoxic stress, especially chemicals causing covalent helix-distorting adducts. *ERCC4* acts as an essential element of DNA nucleotide excision repair (NER) pathway (Manandhar et al., 2015). The upregulation of *GADD45a* (32.7-fold), *CDKN1A* (10.6-fold) and *ERCC4* (5.27-fold) together with cell-cycle arrest discussed above is an indication of DNA damaging effects of CYN in HepG2 spheroids. The positive control, benzo(a)pyrene, a well-known indirectly acting genotoxic compound at concentration of $10 \mu\text{M}$ induced similar deregulation of DNA damage responsive genes as CYN that was, used at 8.3-times lower concentrations ($0.5 \mu\text{g/mL}$ corresponds to $1.2 \mu\text{M}$) (Table 2).

The *CCND1* encodes the cyclin D1 protein, which binds to cyclin-dependent kinases 4 and 6 and regulates progression from G1 into S phase of the cell cycle (reviewed in Bertoli et al., 2015). Elevated expression of cyclin D1 has been reported in many human tumours and correlates to increased cell proliferation and differentiation due to shortened G1/S transition (Snoj et al., 2009). In our study,

upregulation of the expression of *CCND1*, although statistically significant was less than 1.5-fold that is considered as biologically irrelevant. The lack of upregulation of the expression of *CCND1* is in line with the observed G1 cell cycle arrest. Although no significant reduction of the proliferation marker Ki67 measured using flow cytometry and qPCR (at the mRNA level) was observed, down-regulation of *PCNA* (~ 2.13 -fold) and *TOP2a* (~ 2.5 -fold) by CYN indicated the toxin's negative impact on the cell proliferation. Proliferating Cell Nuclear Antigen (PCNA) is a protein associated with DNA polymerase alpha; thus it plays a critical role in DNA synthesis and is highly abundant in cells during the G1/S phase of the cell cycle, while in quiescent and senescent cells the levels of PCNA mRNA and protein are low (Kelman, 1997). Topoisomerase II alpha (*TOP2a*) is a protein essential for DNA condensation before its replication and the segregation of chromosomes in mitotic cells (Colozza et al., 2005). The expression of *TOP2a* is cell cycle-dependent and its elevated protein as well as mRNA levels are recognized as a marker of cell proliferation (Neubauer et al., 2016). Several authors reported reduced cell proliferation upon exposure to CYN. It significantly decreased the mitotic index and cell proliferation in CHO-K1 cells (Lankoff et al., 2007), proliferation of T-lymphocytes was significantly reduced after exposure to CYN at $1 \mu\text{g/mL}$ (Poniedzialek et al., 2014) and in 2D HepG2 culture CYN ($0.5 \mu\text{g/mL}$, ≥ 24 h) reduced proliferation measured as the ratio of Ki67 positive cells by immunostaining (Straser et al., 2013b). Moreover, negative impact of CYN on proliferation was observed at the gene transcription level in HepG2 (Straser et al., 2013c) as well as in HepaRG (Huguet et al., 2019) monolayer cultures. Therefore, it can be concluded that CYN is inhibiting cell proliferation in variety of 2D *in vitro* models as well as in the 3D HepG2 model as shown by the present study.

Finally, we analysed the influence of CYN on the apoptosis by measuring the expression of *BBC3*. The *BBC3* gene encodes the Bcl-2-binding component 3 also known as 'p53 upregulated modulator of apoptosis (PUMA). This pro-apoptotic protein interacts with anti-apoptotic Bcl-2 family members resulting in mitochondria induced apoptosis and cell death through the caspase cascade. The expression of *BBC3* is regulated on the transcription level by p53 and not by post-translational modifications (Nakano and Vousden, 2001). In the present study, *BBC3* was upregulated (4.75-fold) after 72 h exposure to $0.5 \mu\text{g/mL}$ CYN, suggesting that in HepG2 spheroids one of the mechanisms of CYN action is the induction of apoptosis. There are several reports that CYN induces apoptosis in CHO-K1 cells (Fessard and Bernard, 2003; Gácsi et al., 2009; Lankoff et al., 2007), primary human lymphocytes (Pichardo et al., 2017; Poniedzialek et al., 2014) and primary rodent hepatocytes (López-Alonso et al., 2013). However, other authors are reporting less conclusive results (Straser et al., 2013c) or even no apoptotic effects in hepatic HepG2 (Straser et al., 2013a) and HepaRG cells (Huguet et al., 2019) nor in human lymphocytes (Bojana Zegura et al., 2011a, 2011b).

The results represent relative mRNA expression normalized to solvent control.

4. Conclusions

In experimental toxicology, a lot of effort has recently been focused on the development and use of advanced *in vitro* models to reduce, replace and refine animal experiments. Compared to the classical 2D cultures, 3D cell models *in vitro* resemble cell organization of tissues and organs and thus better mimic *in vivo* micro-environment, which is an important aspect in cellular responses and cell survival after exposure to various compounds. In the present study, the advanced 3D cell model *in vitro* (spheroids) from HepG2 cells was used for studying the adverse effects of

cyanobacterial toxin cylindrospermopsin for the first time. The toxin affected the growth and the shape of HepG2 cells in spheroids. The formation of γ H2AX positive cells corresponding to DNA double strand breaks was not detected; however, at the same time, strong up-regulation of DNA damage responsive genes *CDKN1A*, *GADD45a*, and *ERCC4* was observed confirming the genotoxic activity of CYN. The cell cycle status was altered after the exposure to CYN and several genes involved in cell cycle regulation were deregulated, indicating that the TP53-pathway was importantly involved in cell cycle arrest induced by CYN. Further, phase I and phase II metabolic enzymes that are transcriptionally induced by the exposure to indirect-acting genotoxic compounds were up-regulated, which confirmed that metabolism plays an important role in CYN activation and its toxic and genotoxic activity, where genes such as *CYP1A1*, *CYP3A4*, *ALDH3A1* as well as their main transcription factor *AHR* play a significant role. Even more importantly, results on the mRNA induction of phase II enzymes *NAT1*, *SULT1B1*, *SULT1C2*, *UGT1A1*, and *UGT2B7* suggested their involvement in CYN detoxification pathways. The developed 3D HepG2 cell model can contribute to more reliable genotoxicity assessment of chemical compounds, including cyanobacterial toxins, due to its higher physiological relevance for human exposure.

Author contribution

Klara Hercog: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization; Martina Stampar: Methodology, Software, Validation, Formal analysis, Investigation, Writing - Review & Editing, Visualization; Alja Stern: Methodology, Software, Validation, Formal analysis, Investigation, Writing - Review & Editing, Visualization; Metka Filipić: Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition; Bojana Žegura: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - Original Draft, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 3

Conclusions

Climate change and water eutrophication resulted in increased occurrence and expansion of cyanobacterial blooms. Cyanobacteria are producing a wide variety of toxic secondary metabolites – cyanotoxins, which are consequently present in the blooming water. In the same period, the production of man-made chemicals and their release into the environment expanded, among which plastics and their ingredients are of top concern. Despite their different sources and functions, after ending up in the common water environment, they represent the constituents of the mixture of pollutants to which humans and other organisms are simultaneously exposed. Our research focused on two distinct groups of ubiquitous pollutants – naturally produced cyanotoxins and anthropogenic bisphenols, identifying their individual as well as combined cytotoxic and genotoxic effects and mechanisms of action.

A non-protein amino acid β -N-methylamino-L-alanine (L-BMAA) is a globally present cyanobacterial product with known neurotoxic properties, while other toxicity data are scarce. Our study was the first addressing its genotoxic potential and has shown that L-BMAA is not a bacterial mutagen. This information is essential for the risk assessment; however, recently, there are some indications that L-BMAA exhibit genotoxic effects in eukaryotic cells. Therefore, for proper human risk assessment, it needs to be further elucidated whether the neurotoxin L-BMAA could also induce adverse effects like DNA damage in cells other than neurons and what are the mechanisms involved in its genotoxic activity.

Microginins (MGs) are another group of emerging cyanotoxins with no data on genetic toxicity. We identified various microginin congeners in cyanobacterial extract isolated from an environmental sample of eutrophic Lake Balaton and revealed their DNA-damaging potential. It can be concluded that MGs represent a genotoxic hazard for aquatic organisms and humans, which has not been recognised before, and it confirms our hypothesis. The cyanobacterial extract containing the mixture of microginins induced a higher amount of DNA strand breaks than the individual congeners. However, microginin GH787 and cyanostatin B were more potent in inducing genomic instability than the cyanobacterial extract. Therefore, the second part of the hypothesis cannot be confirmed as some MGs exhibited stronger genotoxic potential than the extract B-14-01. Moreover, despite their structural similarities, combined exposure to MGs cannot be ascribed neither to the concentration addition nor the independent action concept; thus, the underlying interactions between simultaneously occurring congeners need to be further examined.

MCLR and CYN are hepatotoxic cyanotoxins, which both exhibit genotoxic effects, but their mechanisms of toxicity differ. They often occur together in cyanobacterial blooms, thus combined exposure is plausible. The binary mixture of MCLR and CYN induced DNA strand breaks in HepG2 cells after 4 and 24 h exposure. The levels of DNA strand breaks induced by the mixture were lower from those induced by a single toxin, indicating an antagonistic interaction, whereas the induction of genomic instability by combined exposure was not significantly different from that induced by CYN alone. Also, the pattern of the alterations in the expression of genes involved in xenobiotic metabolism, immediate or early response/signalling and DNA damage responsive genes induced by the mixture

was the same as that induced by CYN alone. Altogether, MCLR/CYN combined effect was comparable to the effect of CYN alone. Therefore, our hypothesis expecting that the MCLR/CYN mixture will exhibit more pronounced genotoxic effects than the individual toxins was rejected. MCLR is a well-known cyanotoxin characterised as a possible human carcinogen; however, from our results, it can be concluded that CYN evinces even stronger genotoxic activity.

The use of bisphenol A and its analogues F, S and AF in the industry is increasing, and consequentially their release into the environment is rising. The comparison of their (geno)toxic effects emphasized BPAF as the most potent, while BPS showed the weakest genotoxic potential. At concentrations detected in environmental water samples or human body liquids, bisphenols and their combinations caused no DNA damage or changes in the transcription of selected genes involved in DNA damage response. However, the genes encoding xenobiotic metabolic enzymes were upregulated by BPA, BPAF and combinations even at very low concentrations, which demonstrates that at current exposure conditions they are capable of affecting the metabolism of xenobiotics, which may affect their disposition. Our results support the hypothesis that the DNA damaging potential of selected BPA analogues differs and that the transcriptomic analysis is the most sensitive method to identify the effects of bisphenols at low concentrations. The effects of combined and individual exposure cannot be compared while no induction of DNA damage was detected at tested concentrations.

Combined exposure to environmental pollutants from different sources closer represents the real-life situation than the exposure to one individual chemical. Continental eutrophic waters are the juncture of cyanotoxins and bisphenols, which result in probable co-exposure of organisms. At the same time, consuming contaminated food and water may lead to a similar effect. The exposure of HepG2 cells to cyanotoxin CYN alone and its combinations with most abundant bisphenols (BPA, BPF, BPS and BPAF) significantly reduced the viability of HepG2 cells and increased the formation of DNA DSBs. The BPs alone did not affect cell viability and had no effect on DNA DSB induction at applied concentrations. Generally, in co-exposure, the effect of CYN prevailed, which has also been observed in combination with MCLR. However, CYN/BPA combination induced slightly less DNA DSB compared to the induction by CYN alone, indicating an antagonistic effect. At the same time, in CYN/BPA treated cells, the expression of *CYP1A1* was increased in synergistic manner; therefore, an interaction at the level of metabolic activation of CYN may be expected. The results suggest that CYN is a toxin with a predominant genotoxic effect; thus, the hypothesis expecting combined exposure to exhibit stronger genotoxic potential than exposure to a single compound cannot be accepted. On the other hand, hypothesizing that gene expression analysis can elucidate the potential levels of interactions between CYN and the BPs was proven correct; however, the details of underlying molecular mechanisms remain unknown.

To better elucidate the molecular mechanisms of CYN toxicity, it was investigated in an advanced *in vitro* 3D cell model. Metabolic transformation and bile acid transmembrane transport play an important role in CYN bioactivity. 3D spheroids resemble these functions better than cells cultured in monolayer; therefore, they represent a more suitable model for studying its toxic effects. CYN abated the growth of spheroids, while the viability of cells was not significantly affected. Although no induction of DNA double-strand breaks was observed, cell cycle arrest and gene expression analysis demonstrated DNA damaging activity accompanied by increased markers of apoptosis and reduced proliferation. CYN upregulated the expression of several phase I as well as phase II metabolic enzymes, indicating their potential involvement in the biotransformation of CYN. These results confirmed our hypotheses. The study importantly contributes to the data on CYN toxicity,

and at the same time represents an example of the application of the 3D model in toxicological research demonstrating the proof of concept.

The results of this thesis provide missing toxicity and genotoxicity data needed for reliable environmental and human health risk assessment of newly identified and emerging cyanobacterial toxins as well as BPA analogues that are generally considered as less harmful than BPA. Special attention has been devoted to the studies of their combined effects in mixtures, which are relevant for environmental and human exposure through the contaminated water and have not been studied so far. Moreover, the methodological approach applied in the doctoral dissertation is novel to the field of genetic toxicology and indicates the potential possibilities of further development of 3D *in vitro* models in assessment of genotoxic effects and the underlying mechanisms of action.

The results related to this doctoral dissertation were presented in six scientific papers published in SCI international journals and in 10 national and international scientific conference contributions.

Appendix A

Supplementary Material of Included Publications

A.1 Supplementary Material of Publication 2.4

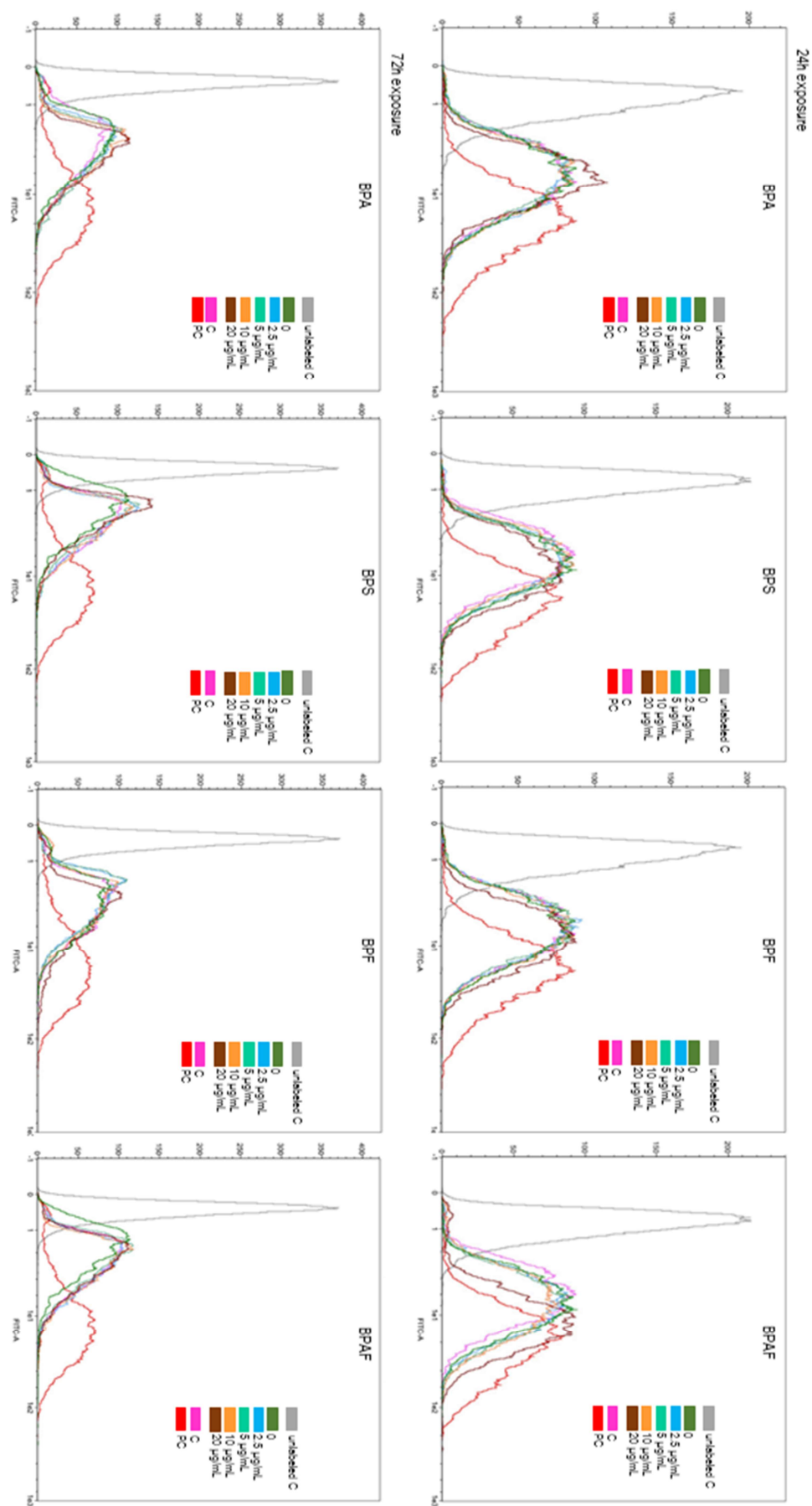


Fig 4. The induction of DSB by BPA, BPS, BPF and BPAF after 24h (above) and 72h (below) exposure time. The presence of DSB was analysed by flow cytometry of anti-H2AX PS139 stained cells. The intensity of FITC fluorescence was measured in 10⁴ cells per parallel and one representative parallel per treatment is presented as overlaid histogram. 0 = solvent control, 0.08% DMSO; C = cells in pure media; PC = positive control, 1 µg/mL ET.

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Other Publications

- Bolognesi C., Knasmueller S., Nersesyan, A., Roggieri P., Ceppi M., Bruzzzone M., Blaszczyk E., Mielzynska-Svach D., Milic M., Bonassi S., Benedetti D., Da Silva J., Toledo R., Salvadori D.M., Groot de Restrepo H., Filipic M., Hercog K., Aktas A., Burgaz S., Kundi M., Grummt T., Thomas P., Hor M., Escudero-Fung M., Holland N., Fenech M. (2016). Inter-laboratory consistency and variability in the buccal micronucleus cytome assay depends on biomarker scored and laboratory experience: results from the HUMNxl international inter-laboratory scoring exercise. *Mutagenesis*, 32(2), gew047. <https://doi.org/10.1093/mutage/gew047>
- Maisanaba, S., Hercog, K., Filipic, M., Jos, Á., & Zegura, B. (2016). Genotoxic potential of montmorillonite clay mineral and alteration in the expression of genes involved in toxicity mechanisms in the human hepatoma cell line HepG2. *Journal of Hazardous Materials*, 304, 425–433. <https://doi.org/10.1016/j.jhazmat.2015.10.018>
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Biography

The author of this thesis, Klara Hercog, was born on 13th of November 1987 in Celje. She attended Primary school Frana Kranjca, Celje, and High school Gimnazija Celje-Center. In 2006, she enrolled in the Biology study programme (EQF level 7) at the Biotechnical Faculty, University of Ljubljana. She defended her graduation thesis on bat ecology entitled Summer roosts of lesser horseshoe bat *Rhinolophus hipposideros* (Bechstein, 1800) in cultural heritage buildings in central Slovenia in March 2013. The thesis supervised by Assist. Prof. Maja Zagmajster was a part of Life+ project Life at night.

In October 2013, Klara Hercog enrolled in the PhD program Nanosciences and Nanotechnologies at the Jožef Stefan International Postgraduate School and started her PhD research as a young researcher at the Department of Genetic Toxicology and Cancer Biology at the National Institute of Biology under the supervision of Prof. Metka Filipič and co-supervision of Assist. Prof. Bojana Žegura. She has been involved in international collaborations with the University of Seville, Spain; University of Debrecen, Hungary; Faculty of Medical Research in Zagreb, Croatia, and Institute of Pharmaceutical Botany in Zagreb, Croatia, which resulted in joint publications. She also collaborated in the HUMNxl exercise – an international inter-laboratory validation of consistency and variability scoring in the buccal micronucleus cytome assay. She presented her work at several international conferences and meetings, such as International Conference on Toxic Cyanobacteria, Eurotox Conference, Conference on Translational Oncology, CellFit Workshop and annual meeting.

The PhD candidate attended workshops and educations related to laboratory techniques qPCR, CBMN assay, 3D models, and other related topics such as scientific writing, communication and promotion of science, protection of intellectual property with patent, basics of selling and negotiation. She was regularly involved in promotion activities of the institute and presentations of scientific work to children and the general public.

