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Chlorination and ozonation differentially reduced the microcystin content and tumour promoting activity of a complex cyanobacterial extract

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ABSTRACT

Despite intensive research and management efforts in the past decades, cyanobacterial blooms and their toxins, such as microcystins (MCs), continue to represent a major ecological and health problem in fresh waters throughout the world. Our objective was to compare the efficacy of two commonly used drinking water treatment technologies, chlorination and ozonation, in removing MCs and in reducing tumour promotion-related effects of cyanobacteria, such as inhibition of gap junctional intercellular communication (GJIC) and activation of mitogen activated protein kinases (MAPKs) in a rat liver epithelial stem-like cell line (WB-F344). This combined chemical and bioassay approach demonstrated that ozone effectively removed all MCs from an extract of a globally important bloom-forming cyanobacterium, *Microcystis* sp. Ozone also significantly reduced the overall tumour promotional potency of the cyanobacterial extract, as indicated by a substantial reduction in the ability of the extract to inhibit GJIC and activate extracellular receptor kinase 1/2 (ERK1/2). Although comparable reduction of total organic carbon was achieved by ozone and chlorine treatment, chlorination was much less effective in removing MCs and reducing the effects on GJIC. Chlorination had a biphasic effect with an observed decrease of extract-induced activation of ERK1/2 at the lower chlorine doses; whereas at high doses of chlorine the by-products of chlorination actually induced the activation of ERK1/2. The extracts induced p38 activation, and chlorination was not effective in reversing this effect, while ozone did reverse this effect, albeit not as much as the activation of ERK1/2. Thus, ozone was effective in reducing the toxicity of cyanobacterial extracts while chlorination was not only lacking efficacy, but at high doses of chlorination further produced by-products that were equally toxic as the untreated samples. Our study indicates the value of using an effect-based approach to assess the efficacy of water treatment systems in removing toxins, and more specifically demonstrates that ozone was more effective at reducing the toxic potential of cyanobacterial-contaminated water.

Key words: Cyanobacteria; water treatment; toxicity; chlorination; ozonation; microcystin.

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INTRODUCTION

Despite intensive research identifying causes, consequences and possible preventive measures of cyanobacterial mass proliferations, cyanobacterial blooms represent a major problem in fresh waters throughout the world. The adverse consequences include water quality degradation, accumulation and microbial decay of bloom biomass followed by lowering of oxygen content in water (Wiegand and Pflugmacher, 2005). In addition, a wide spectrum of toxins and secondary metabolites produced by cyanobacteria have been shown to adversely affect aquatic organisms (Codd *et al.*, 2005; Zanchett and Oliveira-Filho, 2013), livestock (McGorum *et al.*, 2015) and human health (Kuiper-Goodman *et al.*, 1999; Zanchett and Oliveira-Filho, 2013).

While different cyanobacterial taxons can contribute to the formation of dense cyanobacterial water blooms depending on geographical and ecological conditions, *Microcystis* sp. represents cosmopolitan and pervasive cyanobacterial genera, which is frequently reported to dominate water blooms in freshwaters of all continents except Antarctica (Harke *et al.*, 2016). *Microcystis* species (such as *Microcystis aeruginosa*) are also among the prominent producers of the most broadly studied cyanobacterial toxins microcystins (MCs) (Bláha *et al.*, 2009). MCs are known liver tumour promoters (Nishiwaki-Matsushima *et al.*, 1992), with the most common structural variant microcystin-LR (MC-LR) being classified by the International Agency for Research on Cancer as a possible human carcinogen (IARC2B) (Grosse *et al.*, 2006). MCs have been shown to act via inhibition of serine/threonine protein phos-

phatases (PPs) (Campos and Vasconcelos, 2010) and induction of oxidative stress followed by damage to cellular macromolecules (Mathe *et al.*, 2016). Chronic exposures to cyanobacteria and their toxins – *e.g.*, via contaminated drinking water – have been associated with increased occurrence of liver and colorectal cancer (Yu, 1995; Zhou *et al.*, 2002; Svircev *et al.*, 2009). Other (often yet unidentified) compounds produced by complex cyanobacterial blooms can also induce or contribute to different adverse effects (Oberemm *et al.*, 1997; Berry *et al.*, 2009) including tumour promotion (Bláha *et al.*, 2010).

Occurrence of toxic cyanobacteria in surface water presents a challenge to drinking water treatment facilities. Preventive measures as well as various water treatment technologies used to minimize human health risks caused by cyanobacteria and their toxins have been recently summarized and critically discussed (Westrick *et al.*, 2010; Merel *et al.*, 2013; Roegner *et al.*, 2014; Hiskia *et al*., 2016; Ibelings *et al*., 2016). The available approaches able to remove MCs to different extents include coagulation-flocculation-sedimentation, standard oxidation and disinfection by chlorine or permanganate, ozonation and UV disinfection, sorption by activated carbon, nano- and ultrafiltration, and advanced oxidation processes (AOP) (Westrick *et al.*, 2010; Merel *et al.*, 2013; Roegner *et al.*, 2014). Different drinking water treatment technologies are applied in different countries and contexts, and evaluation of their treatment efficiency should focus on i) removal of targeted priority pollutants (*e.g.*, MCs in case of cyanobacterial toxins) to comply with current treatment goals; as well as ii) removal of other potentially harmful and toxic components of the complex material which may not be fully chemically characterized; and iii) formation of new harmful metabolites/toxic by-products during the application of the water treatment technology (Upham *et al.*, 1994; Upham *et al.*, 1995; Prasse *et al.*, 2015).

To address these different aspects of drinking water treatment, complementary chemical and biological tools (*i.e*. instrumental analyses and bioassays) should be included in the monitoring plans (Maier *et al.*, 2015). This ongoing effort can be highlighted by current implementation of the effect-based tools into the monitoring guidelines for water quality assessment (Wernersson *et al.*, 2014), which combines several bioassays targeting different toxic modes of action (MoAs) to provide additional information to classical chemical analyses and thus a more integrative view.

Gap junctional intercellular communication (GJIC) plays a fundamental role in maintaining tissue homeostasis, and provides an excellent biological endpoint to assess potential adverse health effects of many anthropogenic toxicants and natural toxins (Vinken *et al.*, 2009). GJIC is a critical cellular process for the coordination of different intra-, extra-, and inter-cellular signalling pathways required for proper cell behaviour, tissue development, tissue function and maintenance of tissue homeostasis. Most chemical carcinogens and tumour promoters inhibit GJIC in *in vitro* assays, and demonstrated to be a representative marker of tumour promoting potency (Rosenkranz *et al.*, 1997). Recently cyanobacterial extracts and exudates were determined to be potent *in vitro* inhibitors of GJIC (Bláha *et al.*, 2010; Novakova *et al.*, 2011). Inhibition of GJIC by these extracts were independent of the well-recognized tumour promoting cyanotoxins, MCs or cylindrospermopsin, indicating the existence of not-yet-identified toxic compounds (Novakova *et al.*, 2013). Aquatic contaminants, such as polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls synergistically potentiated the inhibitory effects of cyanobacterial extracts on GJIC (Novakova *et al.*, 2012), which further highlights the need to bioassay these mixtures for adverse effects in the complex assessment of drinking water quality, and efficacy of treatment technologies.

In fact, *in vitro* assessment of GJIC has been successfully used along with chemical analysis as a principal bioassay to study tumour promoting activity of water chlorination by-products (Hakulinen *et al.*, 2004; Nishikawa *et al.*, 2006). Similarly, GJIC assay has been applied to evaluate the efficiency of removing different anthropogenic contaminants and their toxic by-products with ozone, such as polycyclic aromatic hydrocarbons (Upham *et al.*, 1994; Upham *et al.*, 1995; Herner *et al.*, 2001; Luster-Teasley *et al.*, 2002; Luster-Teasley *et al.*, 2005) and various pesticides (Upham *et al.*, 1997; Masten *et al.*, 2001; Wu *et al.*, 2007). The *in vitro* bioassays used in these studies to assess GJIC were based on the scrape-load dye transfer (SL-DT) technique (El-Fouly *et al.*, 1987). This SL-DT assay provides fast (minutes of exposure) and integrative responses, which reflect dysregulations of different cell processes and multiple signalling pathways controlling GJIC (Upham *et al.*, 2016). Dysregulation of GJIC is an epigenetic, phenotypic marker for determining tumour promotional activity, which contrasts and compliments the more commonly used genotoxic and specific nuclear receptor transactivation assays (Leusch and Snyder, 2015). GJIC can be assessed *in vitro* using diverse non-tumorigenic cells, with a rat liver epithelial cell line, WB-F344, being one of the most widely used GJIC model for the assessment of tumour promoting activity, as well as determining chemopreventive effects of chemicals (Upham *et al.*, 1998; Sovadinova *et al.*, 2015; Babica *et al.*, 2016b). To further validate tumorigenic activity, compounds that dysregulate GJIC are often tested for effects on signal transduction pathways implicated in neoplastic transformation, such as mitogen-activated protein kinases (MAPK-ERK1/2 and MAPK-p38) (Upham *et al.*, 2008; Osgood *et al.*, 2014; Babica *et al.*, 2016a).

Our objective was to evaluate and compare the effi-

cacy of two broadly used drinking water treatment oxidation technologies, namely chlorination and ozonation, on the removal of known cyanotoxin MC concentrations as well as on changes in biological effects that are independent of the MC content (*i.e*., removal of overall cytotoxicity and tumour promotional potency). We used a natural bloom sample that was dominated by the cosmopolitan and environmentally relevant bloom-forming cyanobacteria *M. aeruginosa*. Extracts were prepared and characterized for content of MC, total organic carbon (TOC) concentration, initial cytotoxicity, effect on GJIC and modulation of signalling kinases (MAPKs). These cyanobacterial extracts were treated by chlorine or ozone, and evaluated for the changes in the toxin content, TOC, and *in vitro* cytotoxicity and tumour promoting activity.

METHODS

Cyanobacterial sample

The sample of toxic cyanobacterial water bloom was collected from a lake located within the campus of Michigan State University (East Lansing, MI, USA; 42°40'50.09"N, 84°29'14.27"W) in September 2008 using a 20 μm plankton net. The bloom was dominated by *Microcystis* species: *M. aeruginosa* (>50% of the cell counts) accompanied by *M. flos-aquae* (~20%) and *M. ichthyoblabe* (~20%). The biomass was freeze-dried and 38 g of dry weight (DW) was extracted by 20 min sonication (Fisher Sonic Dismembrator Model 300; Fisher Scientific, Pittsburgh, PA, USA) while stirring on ice with 566 mL of 50% methanol (*i.e*., 66.7 g DW L^{-1} equivalent). The samples were centrifuged at 31,000 × g and the supernatant fraction was collected and dried using a vacuum evaporator. The dry extract was dissolved in 47.2 mL of MilliQ water (MilliQ Synthesis A10; Millipore, Billerica, MA, USA) to obtain the final concentrated extract corresponding to 800 g DW of original biomass per one litter of water.

Chlorination and ozonation

The extract was aliquoted into 4 mL fractions to be treated by chlorination or ozonation as summarized in Tab. 1. Chlorination was carried out with sodium hypochlorite (NaOCl) in a phosphate buffer (0.5 M K_2HPO_4-0.293 M NaOH, pH 7.0±0.2) according to the Method 5710C (Clesceri *et al.*, 1998). The sample was treated for 30 min to 24 h with NaOCl at different concentrations of free chlorine (7 to 1000 mg L^{-1}) corresponding to contact time (CT) values 0.21×10^3, 7×10^3, 50×10^3 and 1440×10^3 mg min L^{-1}. The oxidation reaction was stopped by addition of 10% w/v of $NaHSO_3$. Vehicle controls were prepared from MilliQ water equally treated with chlorine and quenched. No residual chlorine was present in the vehicle controls after the quenching. To increase the weight ratio of chlorine to dry weight of the extract (or TOC or MC concentration), the concentration of the original biomass was also diluted four- and eight times (*i.e*., "¼" or "⅛") before the chlorination step with 500 mg L^{-1} of chlorine for 100 min.

The original extract dissolved in MilliQ water was ozonated for 30 min using a commercial ozone generator (OZ2PCS-V; Ozotech, Yreka, CA, USA) at the concentration of O_3 (gas)=5.0 mg L^{-1} with a gas flow rate of one L min^{-1} and temperature of 20.0±0.5°C to maximize ozone dissolution.

Microcystin analysis

The concentrations of MCs in the original biomass as well as in the samples after the chlorination and ozonation treatment were analysed by HPLC-UV/DAD following the procedure described earlier (Babica *et al.*, 2006). Toxins were identified based on their retention times and characteristic UV absorption spectra and quantified using the calibration curves of standards of MC-RR, -YR, -LR, -LW, -LF, and nodularin. An example of the HPLC chromatograph of the cyanobacterial extract recorded at 238 nm is in Supplementary Fig. 1.

TOC analysis

TOC of the extract before and after chlorination and ozonation was determined using LiquiTOC analyzer (Elementar Analysensysteme, Hanau, Germany) where measurements were made by high temperature oxidation of the carbon (850-900°C) and detection of CO_2 by an NDIR photometer.

Bioassays

WB-F344 rat liver non-tumorigenic stem-like cells (Tsao *et al.*, 1984) were cultured in D-media (Kao *et al.*, 1997) with 5% v/v of fetal bovine serum (Gibco, Life Technologies, Grand Island, NY, USA) at 37°C and 5% CO_2. Cells were cultured to full confluence for 48 h in 35 mm tissue culture dishes (Costar; Cambridge, MA, USA). These confluent cells were used for the various time and dose related experiments. The vehicle controls were water chlorinated or ozonated using the same conditions as applied for studied samples. A sample or vehicle was added directly to the cell culture medium in the dish and gently mixed. Non-treated cells were used as negative controls. The final concentrations of extracts in the bioassays were expressed as the original weight of dry biomass used for extract preparation per unit volume (g DW L^{-1}). Viability/cytotoxicity was tested after 30-min and 24-h exposures using the neutral red assay as reported before (Babica *et al.*, 2016b). The method determines viable cells capable of neutral red inclusion into lysosomes (Borenfreund and Puerner, 1985). Viability was expressed as the fraction of negative (non-treated) control (FOC).

Tab. 1. Conditions of chlorination and ozonation of the studied cyanobacterial extract.

Abbreviation	Treatment type and CT value (mg min L^{-1})	Dose of Cl or O_3 (mg L^{-1})	Treatment duration	Total MC (μg g^{-1} DW)°	MC-LR (μg g^{-1} DW)°	TOC (μg g^{-1} DW)°	24-h Cell viability IC_{50} (95%CI) LOEC (g DW L^{-1})	30-min GJIC IC_{50} (95%CI) LOEC (g DW L^{-1})	30-min ERK1/2 LOEC (g DW L^{-1})	30-min p38 LOEC (g DW L^{-1})
NT	no treatment	--	--	517 (0.52-12.41)§	411 (0.41-9.86)§	104,761 (105-2514)§	11.6# (10.2-13.1) *12*	7.8# (7.0 to 8.7) *8*	*8*	*4*
Cl(7-30)	Chlorination 0.21×10^3	7	30 min	559 (0.56-13.42)	443 (0.44-10.63)	114,761 (114-2739)	14.8 (12.6-17.3) *12*	10.0 (9.3-10.7) *8*	*8*	*4*
Cl(70-100)	Chlorination 7×10^3	70	100 min	447 (0.45-10.74)	352 (0.35-8.44)	88,355 (88-2121)	14.3 (12.2-16.5) *12*	10.7***^ (9.7-11.8) *8*	*16*	*4*
Cl(500-100)	Chlorination 50×10^3	500	100 min	467 (0.47-11.20)	371 (0.37-8.91)	86,895 (87-2085)	9.1 (6.9-12.2) *12*	12.6*** (11.2-14.1) *8*	*16*	*4*
¼ Cl(500-100)$	Chlorination 50×10^3	500	100 min	346 (0.35-8.31)	275 (0.28-6.60)	77,229 (77-1853)	10.4 (7.1-15.4) *12*	9.6 (8.4-11.0) *8*	*8*	*4*
⅛ Cl(500-100)$	Chlorination 50×10^3	500	100 min	412 (0.41-9.88)	331 (0.33-7.94)	81,193 (81-1949)	7.0 (7.0-15.5) *12*	8.1 (6.7-9.7) *8*	n.a.	n.a.
Cl(1000-24h)	Chlorination 1440×10^3	1000	24 h	367 (0.37-8.81)	295 (0.29-7.07)	78,754 (79-1890)	12.3 (9.7-15.7) *12*	12.7*** (10.7-15.0) *12*	*16*	*4*
O_3	Ozonation	5°°	30 min	n.d.	n.d.	70,622 (71-1695)	>24 *>24*	>24 *24*	*>16*	*4*

CT values: the concentration of free chlorine multiplied by the contact time with the sample being disinfected; it is expressed in units of mg min L^{-1}. °The concentration is calculated per g DW of original biomass before methanol extraction. #Plain text represents IC_{50} values with 95% confidence intervals in parentheses, italicized numbers show LOEC values. §Values in parentheses indicate the range of actual MC and TOC concentrations (mg L^{-1}) in the experiments (calculated for the range of tested concentrations of 1-24 g DW L^{-1}). ^Significantly different than IC_{50} value of NT extract (Student's *t*-test, $P<0.05$). $Before chlorination, the concentration of original biomass was diluted four (¼) or eight times (⅛). °°Gas flow rate of 1 L min^{-1} for 30 min; n.d., not detected: minimal detection limit (MDL) for an individual MC variant=1.3 μg g^{-1} DW; n.a., not analysed.

Tumour promotion assay based on GJIC assessment used only the concentrations that were determined to be noncytotoxic using the neutral red assay. Cells were exposed for 30 min to the tested samples or corresponding vehicle. Treatment with 1-methylanthracene (70 µM, 30 min) was used as a positive control inducing complete inhibition of GJIC. GJIC was assessed using modified SL-DT technique (El-Fouly *et al.*, 1987; Babica *et al.*, 2016c; Upham *et al.*, 2016) . The migration of the dye through gap junctions was visualized with a Nikon Eclipse TE3000 phase contrast/fluorescent microscope and the images digitally captured with Nikon EZ Cool Snap CCD camera (Nikon Instruments, Melville, NY, USA), where three representative images were acquired from each dish. The area of dye transfer was measured for each image using ImageJ (https://imagej.nih.gov/ij/). The measured areas were adjusted by subtracting an area of the dye transfer in the positive control with completely inhibited GJIC. Adjusted areas from each image were compared with an averaged adjusted area of the negative control and expressed as FOC.

Activation of regulatory kinases MAPK ERK1/2 and p38 after a 30 min exposure to the sample was determined by Western blotting. Western blot analyses were done as reported previously (Babica *et al.*, 2016a). Briefly, the proteins were extracted with 20% SDS solution containing inhibitors of proteases and phosphatases, and the protein concentration of the cell lysates was determined with DC assay kit (Bio-Rad, Hercules, CA, USA). The proteins (20 µg per lane) were separated on 12.5% SDS-PAGE (Laemmli, 1970) and then electrophoretically transferred to a 0.45 µm PVDF membrane (Millipore). To visualize activated, *i.e.* phosphorylated ERKs and p38, we used rabbit phospho-specific polyclonal antibodies directed to ERK-1 phosphorylated at Thr 202/Tyr204, and ERK-2 phosphorylated at Thr185/Tyr187 (Cell Signaling #9101S, Danvers, MA, USA) and directed to p38 phosporylated at Thr180/Tyr182 (Zymed #36-8500, San Francisco, CA, USA), and secondary donkey anti-rabbit IgG conjugated with horse radish peroxidase (Amersham Bioscience # NA934V, Life Science, Denver, CO, USA). Levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping protein, and determined with mouse anti-GAPDH antibodies (Chemicon #MAB374, Millipore) and secondary sheep anti-mouse IgG conjugated with horse radish peroxidase (Amersham Bioscience #NA931V; Life Science). The ERK, p38 and GAPDH protein bands were detected using the ECL SuperSignal West Pico Chemiluminesence detection kit (Pierce, Arlington Heights, IL, USA) and Bio-Rad Image Analyzer.

Data analyses and statistics

At least three independent experiments were done for each treatment (except for Western blot analyses), and the mean ± standard deviations from independent experiments were calculated. IC_{50} concentrations causing 50% inhibition of the studied effects and their 95% confidence intervals were derived using non-linear regression in GraphPad (GraphPad software Inc., La Jolla, CA, USA). One-way Analysis of Variance (ANOVA) followed by Dunnett's *post-hoc* test was used to identify treatments significantly different from the control. Differences between IC_{50} values were assessed by Student's *t*-test. P-values less than 0.05 were considered statistically significant.

RESULTS

The original biomass contained 517 µg of total MC per g of DW with MC-LR being the dominant variant (411 µg g^{-1} DW, 79%, Tab. 1). The other variants present included MC-RR (2%), MC-LW (2%), MC-LF (3%) and two other structurally unidentified MC variants (14%). The total MC concentration in the original extract (corresponding to 800 g biomass DW L^{-1}) was 414 mg L^{-1} (MC-LR concentration: 329 mg L^{-1}). TOC concentration in the non-treated extract of 800 g biomass DW L^{-1} was 83.81 g L^{-1}, which represents 105 mg of extractable total organic carbon per g of biomass DW.

Ozonation of cyanobacterial samples had direct effects on the concentrations of organic matter in the biomass. As shown in Tab. 1, the 30-min treatment with ozone caused complete degradation of MCs and 33% reduction of TOC compared to non-treated extract. The chlorination with low dose of chlorine (7 mg L^{-1}) for short time (30 min) had no effect on MC and TOC concentrations. The higher doses (70 to 1000 mg L^{-1}) of chlorine and longer treatments (100 min to 24 h) were more effective in reducing MC levels (by 10 to 33%), and this reduction was dependent on chlorine concentrations and treatment times. There was no observed shift in the proportion of individual MC congeners with the MC-LR being the dominant variant. TOC degradation by chlorine (by 16 to 25%) correlated well with MC degradation. The increase of the weight ratio of chlorine to original biomass by diluting non-treated extract before chlorination (Tab. 1, variants ¼ or ⅛ Cl(500-100)) slightly improved reduction of MC and TOC amount, when the results were adjusted by the corresponding dilution factor and then compared to the non-diluted extract chlorinated under the same conditions (Cl(500-100)).

The original extract (non-treated, NT) showed no significant cytotoxic effects on WB-F344 during 30-min exposure within the range of concentrations tested (up to 24 g DW L^{-1}; negative data not shown). After longer exposure time (24 h), the sample significantly decreased viability of WB-F344 cells (Fig. 1A) with the calculated IC_{50}=11.6 g DW L^{-1} (Tab. 1). The lowest observed effect concentration (LOEC) inducing statistically significant reduction of the cell viability was 12 g DW L^{-1} (Fig. 1A).

The effects of the NT extract on GJIC in WB-F344 cells after 30-min exposure are shown in Fig. 2A. The sample had pronounced inhibitory effects on GJIC, which were concentration-dependent with the calculated IC_{50}=7.8 g DW L^{-1} (Tab. 1). LOEC concentration of NT extract for inhibition of intercellular communication was 8 g DW L^{-1} (containing 4.1 mg L^{-1} MCs) (Fig. 2A). The tumour promotional activity of the NT sample indicated by its effect on GJIC was also confirmed by additional experiments assessing phosphorylation of signalling protein

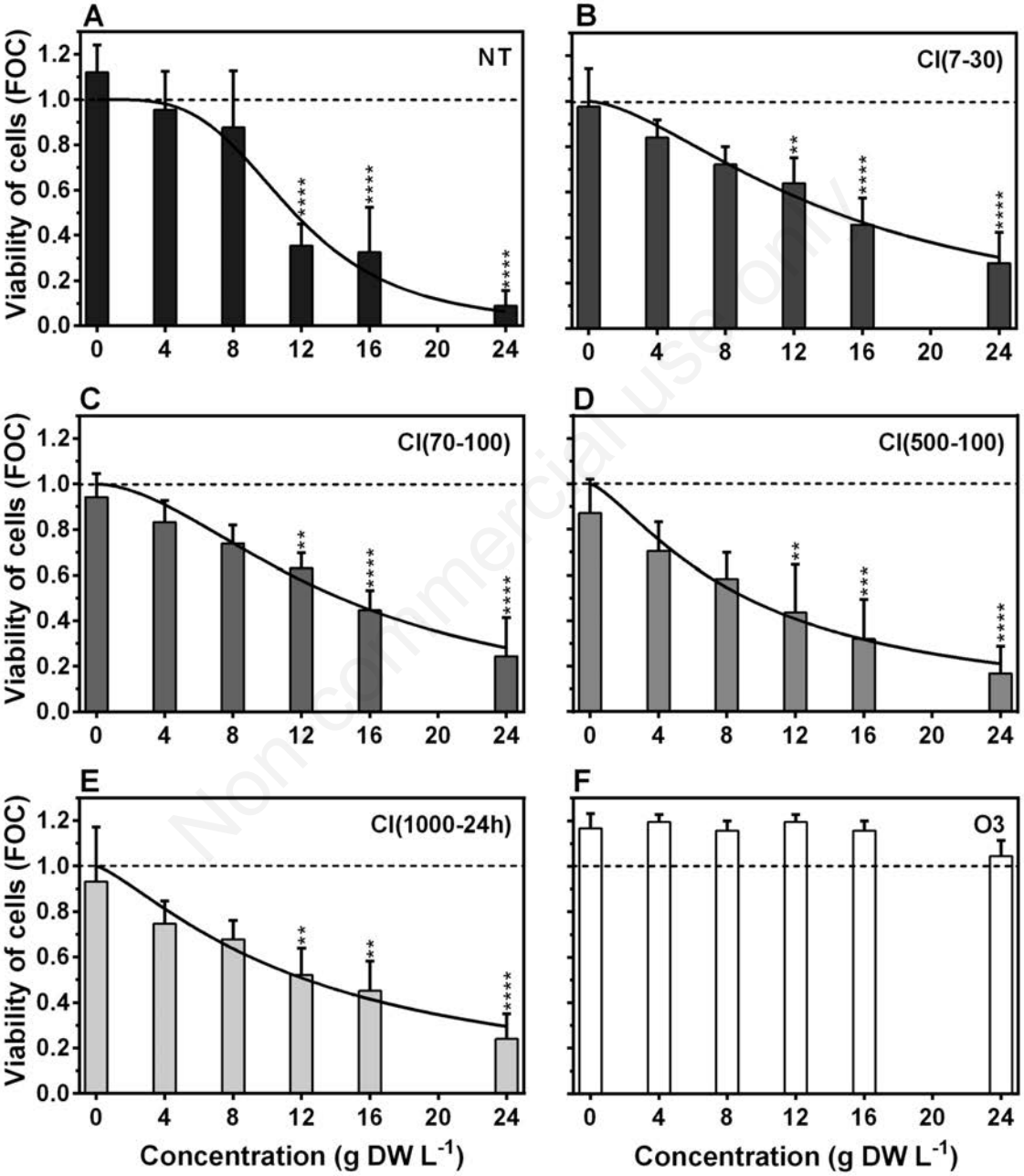

Fig. 1. The effect of chlorination (B-E) and ozonation (F) of the tested cyanobacterial extract on viability in WB-F344 cells after 24-h exposure to 6 different concentrations (4-24 g DW L^{-1}). Data are fractions of controls (FOC) as means ± standard deviations of independent repetitions of the experiment (n≥3). Significant differences from the vehicle control (A) are indicated by asterisks (one-way ANOVA followed by Dunnet's *post-hoc* test; *P≤0.05; **P≤0.01; ***P≤0.001; ****P≤0.0001). NT, original non-treated extract; Cl, chlorinated extracts (in parentheses - free Cl concentrations 7, 70, 500 and 1000 mg L^{-1}; treatment durations 30 min, 100 min, 24 h); O3, ozonated extract (30 min, the flow rate of 1 L min^{-1} with 5 g O_3 m^{-3}).

kinases in WB-F344 cells (MAPK-ERK 1/2 and p38, Fig. 3A). Rapid and clear concentration-dependent activation of both MAPKs was observed after 30-min exposure. The LOEC values for hyperphosphorylation of MAPK- ERK 1/2 and p38 were 8 or 4 g DW L^{-1} of the NT extract, respectively, with maximal effects observed at the highest tested concentration of 16 g DW L^{-1} (Fig. 3A).

Ozonation and chlorination of the original sample had a pronounced effect on the biological activity. The cytotoxic effect was completely eliminated by the ozonation of the extract (Fig. 1F). At the same time, none of the chlorination experimental protocols had any significant effect on cytotoxicity of the original NT extract (Figs. 1B-E), with the estimated IC_{50} values ranging between 9.1 and 14.8 g DW L^{-1} and not being significantly different ($P<0.05$) from the IC_{50} value for the NT extract (Tab. 1). Also, the LOEC values for viability of cells exposed to chlorinated extracts remained at 12 g DW L^{-1}. Interestingly, slight but non-significant increase of IC_{50} values was associated with lower rather than higher chlorine doses. The increase of weight ratio of chlorine to the original biomass by diluting non-treated extract before chlorination did not decrease the cytotoxicity with the IC_{50} values of 10.4 g DW L^{-1} for ¼ Cl(500-100) and 7.0 g DW L^{-1} for ⅛ Cl(500-100) (Tab. 1, Supplementary Fig. 2). The corresponding vehicle controls for these chlorination conditions caused 20 to 30% significant decrease in WB-F344 cell viability when compared to non-treated control (Supplementary Fig. 2).

The ozone application was also highly potent in eliminating tumour promotional activity of the original NT extract, as shown in Fig. 2F. After 30-min ozonation, the LOEC value for GJIC inhibition was 24 g DW L^{-1}, *i.e.* three times higher than in NT extract. GJIC was reduced only by 30% at 24 g DW L^{-1} concentration (Fig. 2F), while nearly 100% inhibition of GJIC was observed at 16 to 24 g DW L^{-1} of NT extract (Fig. 2A).

In contrast, the chlorination treatments had much less pronounced effects on the inhibition of GJIC, with the IC_{50} values of the chlorinated extracts ranging between 10.0-12.7 g DW L^{-1}. Although these IC_{50} values were relatively similar to the IC_{50} estimated for NT extract, they were significantly higher except for the lowest free chlorine doses and shortest treatment ($P<0.05$), and their increase depended on the chlorine dose- and treatment duration (Tab. 1). When the weight-ratio of chlorine to the original biomass was increased by diluting the non-treated extract before chlorination (Supplementary Fig. 3), there was no observable decrease in the dysregulation of GJIC induced as compared to the original NT extract, with the IC_{50} values after chlorination being 8.1 to 9.6 g DW L^{-1} (Tab. 1, Supplementary Fig. 2). Both ozone and chlorine treatments significantly attenuated the activation of MAPK ERK1/2 induced by NT extract (Fig. 3). Ozonation was more effective, when no activations of the ERK1/2 kinase were observed at concentrations between 4 to 16 g DW L^{-1}. Chlorination also resulted in reduced levels of MAPK ERK1/2 activation (Fig. 3, Tab. 1). The protective effects had biphasic character, when initially increased but then became less apparent with an increase in chlorine dose and chlorination time (Fig. 3, Tab. 1). The effects of both ozone and chlorine treatments on the activation of p38 MAPK were much less pronounced when compared to MAPK ERK1/2, and activation of p38 was still apparent for the ozonated extract as well as most of the chlorinated extracts even at the lowest experimental concentration of 4 g DW L^{-1} (Fig. 3).

DISCUSSION

Adverse effects of toxic cyanobacteria on human health remain a major issue for both researchers and water managers. Our study confirmed that rapid inhibitions of GJIC and activations of MAPK-ERK1/2 might be a common effect induced by bloom-forming cyanobacteria. The effective concentration for 30-min GJIC inhibition (IC_{50}=8 g DW L^{-1}) was similar to previously reported values for other extracts from natural blooms dominated by *Microcystis* sp. (IC_{50}=4 or 6 g DW L^{-1}, respectively) (Bláha *et al.*, 2010; Novakova *et al.*, 2011). Modulation of these cellular events by chemicals *in vitro* are considered to be relevant biomarkers of tumour promoting potency *in vivo*, as was demonstrated *e.g.* for tumour promoting phorbol esters like TPA (Madhukar *et al.*, 1996), organochlorine pesticides (Trosko *et al.*, 1987), PCBs (Kang *et al.*, 1996), low molecular weight PAHs (Bláha *et al.*, 2002), clofibrate, phenobarbital, perfluorooctanoic acid, or organic peroxides (Upham *et al.*, 2007; Upham *et al.*, 2009; Vinken *et al.*, 2009). MCs and other cyanotoxins like cylindrospermopsin have been shown to modulate development of tumours (Nishiwaki-Matsushima *et al.*, 1992; Falconer and Humpage, 2005; Svircev *et al.*, 2010; Zegura *et al.*, 2011; de la Cruz *et al.*, 2013). However, neither MC-LR nor cylindrospermopsin had any direct effect on rapid inhibition of GJIC or activation of MAPK ERK 1/2 indicating that other metabolites in cyanobacteria might be responsible for their GJIC-dependent tumour promoting activity (Bláha *et al.*, 2010; Novakova *et al.*, 2011). However, these metabolites have not been identified yet.

In addition, our present study demonstrates, for the first time, a rapid activation of another MAPK-p38 by cyanobacterial environmental extract in the cell line WB-F344, which possesses characteristics of liver progenitor cells (Babica *et al.*, 2016a, b). MAPK-p38 is a critical participant in cellular stress responses and has a key role in inflammation, as well as in tissue homeostasis, by controlling cell proliferation, differentiation, death, survival and the

migration of specific cell types (DiDonato *et al.*, 2012). In contrast to MAPK-ERK1/2, which are activated by mitogens or growth factors, MAPK-p38 is activated by environmental and genotoxic stresses including hypoxia, UV, ROS, hyperosmolarity, and heat shock, and its activation has been linked to protein phosphatase 2 (PP2A) (Nebreda and Porras, 2000; Wagner and Nebreda, 2009). Indeed, MC-LR, a known PP2A inhibitor, has been previously reported to activate MAPKs ERK1/2, p38 or JNK in rodent liver *in vivo* and also in experiments with various cell lines

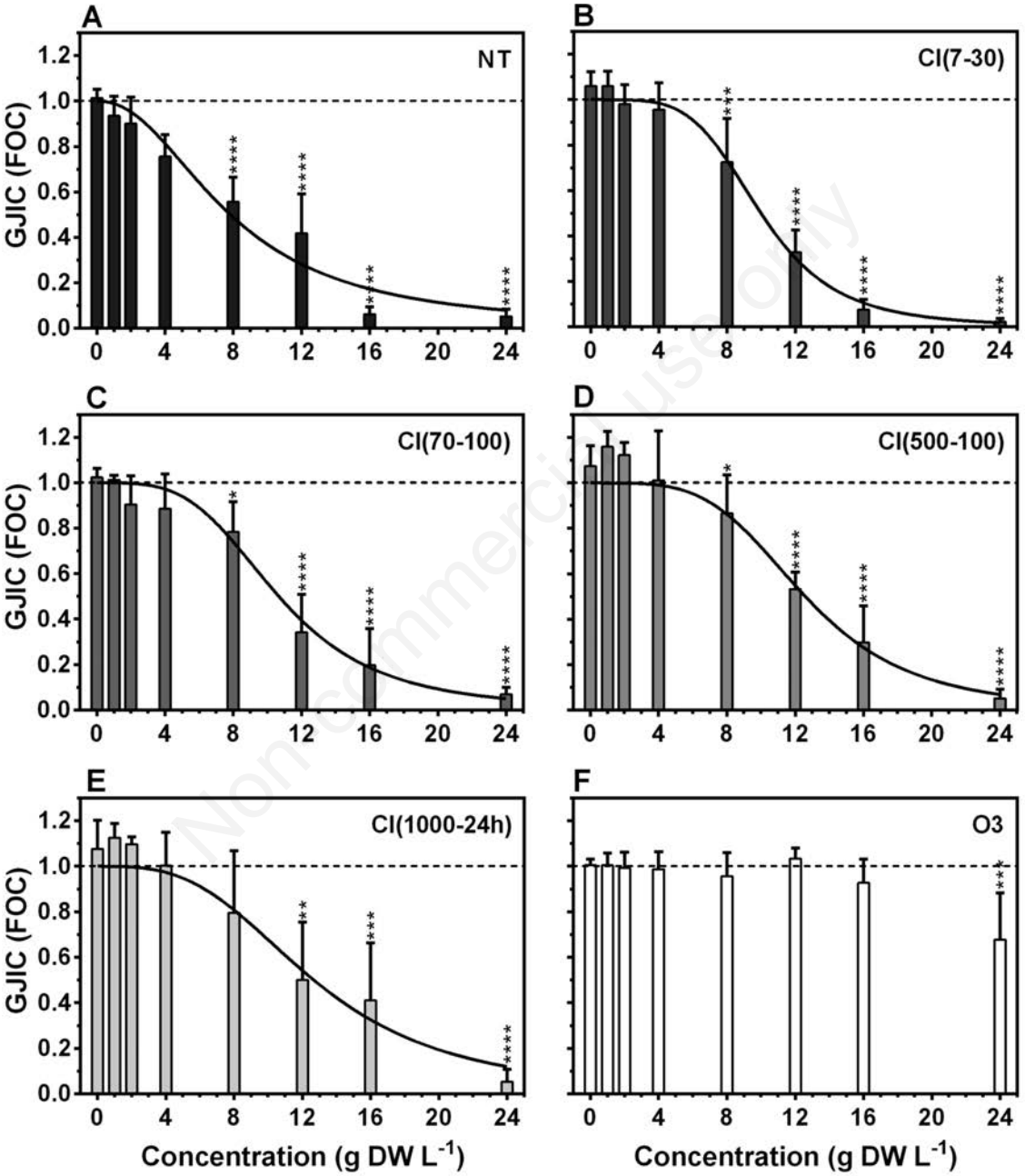

Fig. 2. The effect of chlorination (B-E and ozonation (F) of the tested cyanobacterial extract on gap-junctional intercellular communication (GJIC) in WB-F344 cells after 30-min exposure to 6 different concentrations (1-24 g DW L^{-1}). Data are fractions of controls (FOC) as means ± standard deviations of independent repetitions of the experiment (n≥3). Significant differences from the NT extract (A) are indicated by asterisks (one-way ANOVA followed by Dunnet's post hoc test; *P≤0.05; **P≤0.01; ***P≤0.001; ****P≤0.0001). NT, original non-treated extract; Cl, chlorinated extracts (in parentheses - free Cl concentrations 7, 70, 500 and 1000 mg L^{-1}; treatment durations 30 min, 100 min, 24 h); O3, ozonated extract (30 min, the flow rate of 1 L min^{-1} with 5 g O_3 m^{-3}).

in vitro. However, MC-LR effects on MAPKs seem to be dependent on the kinase type, a cell type, and probably also exposure times and concentrations. Depending on the study, MC-LR was found to activate ERK1/2 but not p38 (Dias *et al.*, 2010; Zhang *et al.*, 2013; Adamovsky *et al.*, 2015), p38 but not ERK1/2 (Meng *et al.*, 2011; Lezcano *et al.*, 2012), and both ERK1/2 and p38 (Komatsu *et al.*, 2007; Daily *et al.*, 2010; Sun *et al.*, 2011; Chen *et al.*, 2012; Liu *et al.*, 2016; Wang *et al.*, 2017). The effective concentrations of MC-LR in these studies were typically between 1 and 10 μM (~1-10 mg L^{-1}).

In the present study, cytotoxicity, GJIC and MAPKs were affected by the cyanobacterial extract diluted to 4-12 g DW L^{-1} containing MCs with concentrations between 2 and 6 mg L^{-1}, which corresponds to 2 to 6 μM range and is quite comparable with the other *in vitro* studies reporting MAPK activation by MCs. However, we previously demonstrated that tumour promoting events, such as rapid GJIC inhibition and MAPK ERK1/2 activation in rat liver progenitor cells, are induced by other cyanobacterial metabolites but not MC-LR or cylindrospermopsin (Bláha *et al.*, 2010). Our results suggest that MAPK p38 can also be activated by transformation or degradation products of MCs, other compounds of cyanobacterial origin and/or their transformation or degradation products, since p38 was activated not only by MC-containing non-treated cyanobacterial extract, but also by ozonated extract without detectable levels of MCs. These findings suggest that progenitor cells, in comparison with differentiated hepatocytes, might be less prone to the effects of MCs, possibly due to limited expression of key proteins involved in MCs uptake and metabolism, such as organic-anion transporting polypeptides (OATPs). Nevertheless, progenitor cells can be apparently a target of other compounds present in cyanobacterial biomass, which are capable to induce toxic and tumour promoting effects in this specific population of liver cells (Bláha *et al.*, 2010) known to play a critical role in the maintenance of liver tissue homeostasis, liver regeneration and hepatocarcinogenesis (Canovas-Jorda *et al.*, 2014).

The evidence supporting the existence of other components of cyanobacterial biomass contributing to the tumour promoting and toxic effects of complex cyanobacterial samples emphasize the need for effect-based evaluation of the efficacy of water treatment technologies in addition to chemical analyses. Different physicochemical purification processes employed in DWTPs may have different efficacies in removing the target contaminant, such as well recognized toxicants (such as MCs) *vs*. elimination of the overall toxicity. Our results demonstrate that ozone effectively and rapidly removes the MC fraction of the complex cyanobacterial samples. Although the highest chlorine dose resulted in a decrease of TOC that was comparable to the ozone treatment, reactive chlorine was not as effective compared to ozone in removal of MCs. Similarly, removal of cytotoxicity and overall epigenetic toxicity of the studied sample by ozonation appeared to be much more effective than chlorination, although ozone had a less pronounced effect in decreasing p38 activation as compared to ERK1/2 and GJIC.

With regard to chlorine the literature demonstrate that its application removes MCs but the efficiency of removal decreases with increases in pH and dissolved organic material along with formation of less effective oxidant ClO^- (Merel *et al.*, 2010). Several studies agreed that 0.5 mg of residual chlorine per liter should efficiently remove pure MCs in distilled water during 5-30 min (depending on MC concentration) at pH lower than 8 (Nicholson *et al.*, 1994; Newcombe and Nicholson, 2004; Acero *et al.*, 2005). Similar scenarios are also expected at DWTP at environmentally relevant concentrations of MCs (Merel *et al.*, 2009).

Several previous studies using PP-inhibition as a biomarker of toxicity as well as other bioassays reported a decrease in MC concentration and toxicity after chlorination of cyanobacterial samples (Nicholson *et al.*, 1994; Tsuji *et al.*, 1997; Rodriguez *et al.*, 2008; Merel *et al.*, 2010). For example, the chlorination of *M. aeruginosa* extract (16 mg DW L^{-1}) with the dose of 1 mg L^{-1} chlorine and contact time of 30 min (CT value=30) effectively removed 95% of MCs (initial concentration: 192 μg L^{-1}) and completely eliminated the acute toxicity of this extract in a mouse bioassay (Nicholson *et al.*, 1994). Interestingly, our study showed that chlorination of cyanobacterial extract was less effective. The dose of 7 mg of chlorine per liter for 30 min at pH 7.2 did not decrease MCs nor TOC concentrations. Higher doses and longer treatment times removed up to 10-30% of the original MCs and TOC levels, and caused only moderate reduction of toxicity (cytotoxicity, GJIC inhibition and MAPK activations). Chlorination apparently removed some compounds responsible for the inhibition of GJIC and activation of ERK1/2 as reflected by the slight, yet statistically significant, increase in respective IC_{50} or LOEC values, and had only a minor effect on cytotoxicity and p38 activation. The lower effectiveness of chlorination observed in our study could be explained by the interactions of chlorine with the relatively higher concentrations of organic matter, which might have reduced the effectiveness of the oxidation process due to the competition between the toxins and the dissolved organic carbon reacting with the oxidant (Rodriguez *et al.*, 2008).

An important problem associated with the chlorine application is the formation of by-products such as halogenated organic compounds, especially in the presence of high amounts of organic matter. These by-products can have toxic or potential carcinogenic potencies (Neale *et al.*, 2012); and also for MCs, chlorine was shown to cause substitutions and modifications of the toxic Adda moiety

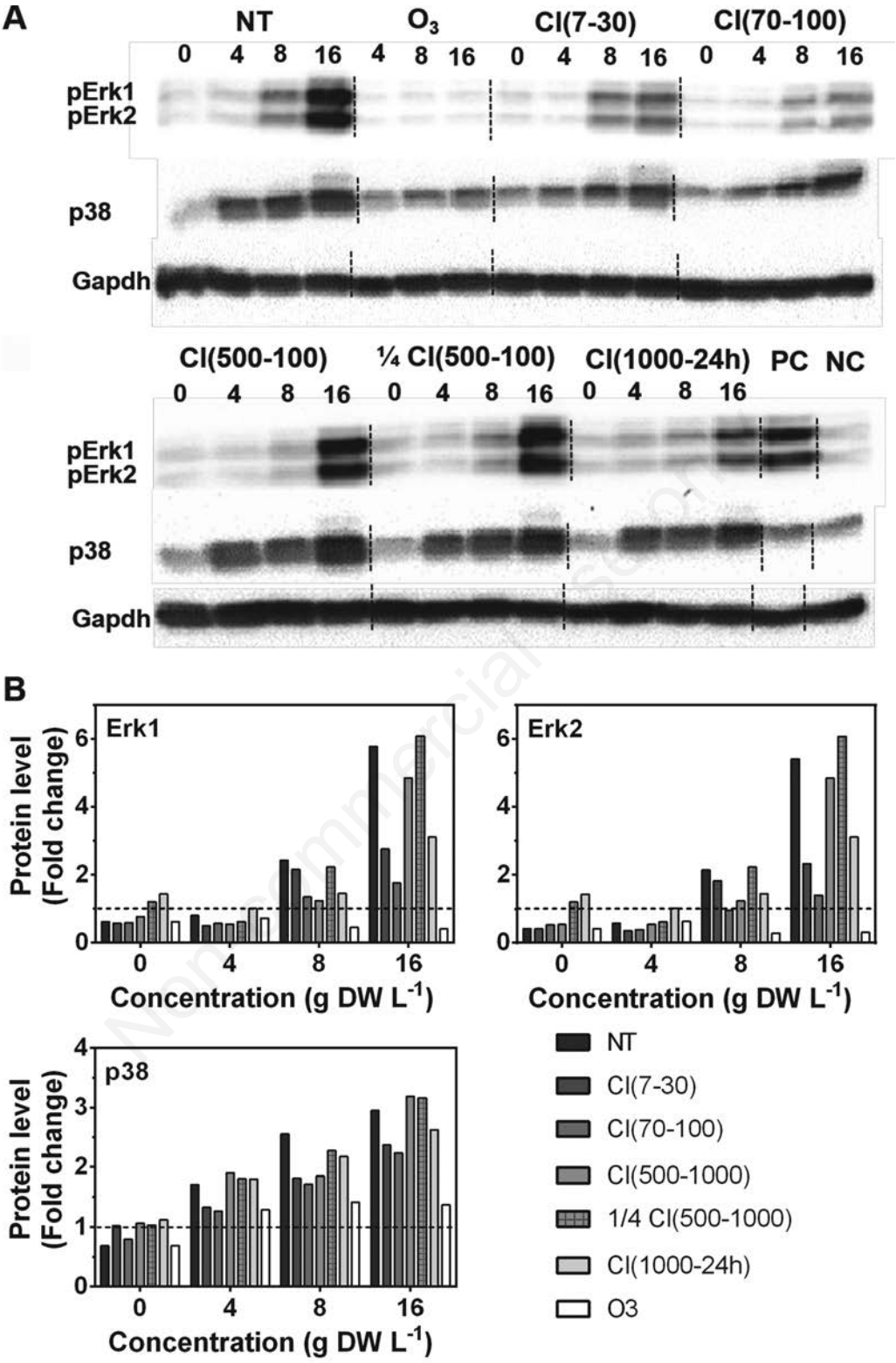

Fig. 3. Activation of mitogen-activated protein kinases (MAPKs) by studied samples after 30-min exposure to 3 different concentrations (4-16 g DW L^{-1}). Phosphorylation of extracellular receptor kinases 1 and 2 (ERK1/2) and p38 was determined by Western blotting (A). The bar graphs (B) show values from the densitometric image analysis normalized to negative control (NC=1). NT, original non-treated extract; Cl, chlorinated extracts (in parentheses - free Cl concentrations 7, 70, 500 and 1000 mg L^{-1}; treatment durations 30 min, 100 min, 24 h), O3 – ozonated extract (30 min, the flow rate of 1 L min^{-1} with 5 g O_3 m^{-3}); NC, negative control (no treatment of the cells); PC, positive control for ERK1/2 activation (12-O-tetradecanoyl phorbol-13-acetate, 10 nM, 30 min).

(Tsuji *et al.*, 1997; Merel *et al.*, 2009). In addition, *de novo* formation of chlorinated by-products with potencies to affect GJIC and activate intracellular signalling (Hakulinen *et al.*, 2004; Nishikawa *et al.*, 2006) should also be considered, and could be related to the weak efficiency in removal of GJIC inhibitions and MAPK activations during the chlorination as observed in the present study. Possible toxicity of chlorinated by-products could also explain the observed biphasic effect, when lower doses of chlorine were slightly more effective in the elimination of cytotoxicity and MAPKs activation than the higher doses, although concentrations of TOC and MCs were slightly but progressively reduced with increasing chlorine dose and treatment time.

We demonstrated that ozonation completely removed MCs, substantial fractions of TOC and protected against cytotoxicity, GJIC inhibition or activation of ERK1/2. These findings are in agreement with similar studies, which documented complete MC removal (5 mg L^{-1}) by 2 mg L^{-1} O_3 within 2 min (Al Momani and Jarrah, 2010). Further improvements in kinetics could be achieved by increased O_3 doses and temperature, and decreased pH (Al Momani and Jarrah, 2010; Shawwa and Smith, 2001). Naturally, organic matter negatively reduces the efficiency of ozonation, but under realistic DWTP situations of levels as low as 0.05 mg L^{-1} of residual O_3 assures MC removal (Newcombe and Nicholson, 2004; Brooke *et al.*, 2006). Despite a high amount of organic material in our sample that also competes with toxins for ozone, ozonation was highly effective in MC reduction and elimination of toxicity even after short treatment.

A high efficiency of oxidation of MC is known to be mediated by hydroxyl radicals attacking conjugated diene structure in MC followed by the cleavage of the Adda side chain (responsible for PPase inhibition) and ultimately opening of the peptide ring (Al Momani and Jarrah, 2010; Miao *et al.*, 2010). Biological assessments using PP-inhibition assay or mouse test confirmed elimination of the toxicity along with the described structural changes of MC (Brooke *et al.*, 2006; Miao *et al.*, 2010). Although ozone was quite efficient in removal of cytotoxic, GJIC inhibiting and ERK1/2 activating compounds in our study, it had only a partial effect on the removal of p38 activating components. This might indicate that p38 is not involved in GJIC inhibition and its activation was caused by metabolites with different modes of actions (Wagner and Nebreda, 2009). With respect to the critical role of p38 in cellular responses to different types of stress and also in controlling the proliferation, differentiation, survival, migration and inflammatory responses of specific cell types, further research should address interactions of cyanobacterial metabolites with this signalling pathway and evaluate its relevance as a biomarker of environmental and genotoxic stress induced by cyanobacteria. Interestingly, activation of p38 was found to be the most sensitive endpoint in this study, where the increased levels of p38 phosphorylation were observed after 30-min exposure to the non-treated extract at concentration of 4 g DW L^{-1}, whereas significant inhibition of GJIC and activation of ERK1/2 occurred at concentrations 8 g DW L^{-1} and higher, and significant reduction of cell viability required 24-h exposure to 12 g DW L^{-1}. Inhibition of GJIC and activation of MAPKs was induced by lower concentrations and after shorter exposures than the cytotoxic effects, which indicates that these cell signalling events were altered via rapid non-genotoxic and non-cytotoxic mechanisms. *In vitro* evaluation of GJIC and MAPKs thus represent a simple and sensitive bioassay for assessment of 'epigenetic toxicity' and tumour promoting potential of complex cyanobacterial extracts, which is also suitable for effect-based studies focusing on the elimination of these hazardous properties of contaminated water.

CONCLUSIONS

Ozonation of an extract of a *Microcystis* water bloom sample was shown to be a very effective method in the complete removal of MCs, as well as the substantial elimination of the overall cytotoxicity and tumour promotional potency. On the contrary, chlorination experiments, despite high doses and long exposures, were much less effective, and potentially led to the formation of by-products, which could add to the observed toxic effects. Our study also demonstrated strong activations of p38 MAPK by cyanobacterial samples, which were not effectively removed by chlorination and only partially by ozonation. With respect to the role of p38 in inflammation as well as maintenance of tissue homeostasis, further research should address interactions of cyanobacterial samples with this biomarker of cellular stress and evaluate its environmental relevance. In agreement with several recent reports, the study also demonstrates the need to enforce effect-based (bioassay) tools into the assessment of water quality and monitoring the efficacy of water treatment systems.

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REFERENCES

Acero JL, Rodriguez E, Meriluoto J, 2005. Kinetics of reactions between chlorine and the cyanobacterial toxins microcystins. Water Res. 39:1628-1638.

Adamovsky O, Moosova Z, Pekarova M, Basu A, Babica P, Svihalkova Sindlerova L, Kubala L, Blaha L, 2015. Immunomodulatory potency of microcystin, an important water-polluting cyanobacterial toxin. Environ. Sci. Technol. 49:12457-12464.

Al Momani FA, Jarrah N, 2010. Treatment and kinetic study of cyanobacterial toxin by ozone. J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng. 45:719-731.

Babica P, Kohoutek J, Blaha L, Adamovsky O, Marsalek B, 2006. Evaluation of extraction approaches linked to ELISA and HPLC for analyses of microcystin-LR, -RR and -YR in freshwater sediments with different organic material contents. Anal. Bioanal. Chem. 385:1545-1551.

Babica P, Zurabian R, Kumar ER, Chopra R, Mianecki MJ, Park JS, Jasa L, Trosko JE, Upham BL, 2016a. Methoxychlor and vinclozolin induce rapid changes in intercellular and intracellular signaling in liver progenitor cells. Toxicol. Sci. 153: 174-185.

Babica P, Ctverackova L, Lencesova Z, Trosko JE, Upham BL, 2016b. Chemopreventive agents attenuate rapid inhibition of gap junctional intercellular communication induced by environmental toxicants. Nutr. Cancer 68: 827-837.

Babica P, Sovadinova I, Upham BL, 2016c. Scrape loading / dye transfer, p. 133-144. In: M. Vinken and S. Johnstone (eds.), Methods in molecular biology. 1437. Gap Junction Protocols. Springer.

Berry JP, Gibbs PDL, Schmale MC, Saker ML, 2009. Toxicity of cylindrospermopsin, and other apparent metabolites from *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*, to the zebrafish (*Danio rerio*) embryo. Toxicon 53 289-299.

Bláha L, Kapplová P, Vondráček J, Upham B, Machala M, 2002. Inhibition of gap-junctional intercellular communication by environmentally occurring polycyclic aromatic hydrocarbons. Toxicol. Sci. 6543-51.

Bláha L, Babica P, Maršálek B, 2009. Toxins produced in cyanobacterial water blooms – toxicity and risks. Interdiscip. Toxicol. 2:36-41.

Bláha L, Babica P, Hilscherová K, Upham BL, 2010. Inhibition of gap-junctional intercellular communication and activation of mitogen-activated protein kinases by cyanobacterial extracts - indications of novel tumor promoting cyanotoxins? Toxicon 55:126-134.

Borenfreund E, Puerner JA, 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol. Lett. 24:119-124.

Brooke S, Newcombe G, Nicholson B, Klass G, 2006. Decrease in toxicity of microcystins LA and LR in drinking water by ozonation. Toxicon 48:1054-1059.

Campos A, Vasconcelos V, 2010. Molecular mechanisms of microcystin toxicity in nnimal cells. Int. J. Mol. Sci. 11:268-287.

Canovas-Jorda D, Louisse J, Pistollato F, Zagoura D, Bremer S, 2014. Regenerative toxicology: the role of stem cells in the development of chronic toxicities. Expert Opin. Drug Metab. Toxicol. 10:39-50.

Chen DN, Zeng J, Wang F, Zheng W, Tu WW, Zhao JS, Xu J, 2012. Hyperphosphorylation of intermediate filament proteins is involved in microcystin-LR-induced toxicity in HL7702 cells. Toxicol. Lett. 214:192-199.

Clesceri LS, Greenberg AE, Eaton AD. 1998. Standard methods for examinations of water and wastewater. 20th edition, American Public Health Association, Washington DC: 1496 pp.

Codd GA, Lindsay J, Young FM, Morrison LF, Metcalf JS. 2005. Cyanobacterial toxins, p. 1-23. In: J. Huisman, H. C. P. Matthijs and P. M. Visser (eds.), Harmful cyanobacteria. Springer.

Daily A, Monks NR, Leggas M, Moscow JA, 2010. Abrogation of microcystin cytotoxicity by MAP kinase inhibitors and N-acetyl cysteine is confounded by OATPIB1 uptake activity inhibition. Toxicon 55:827-837.

de la Cruz AA, Hiskia A, Kaloudis T, Chernoff N, Hill D, Antoniou MG, He X, Loftin K, O'Shea K, Zhao C, Pelaez M, Han C, Lynch TJ, Dionysiou DD, 2013. A review on cylindrospermopsin: the global occurrence, detection, toxicity and degradation of a potent cyanotoxin. Environ. Sci. Proc. Imp. 15:1979-2003.

Dias E, Matos P, Pereira P, Batoreu MC, Silva MJ, Jordan P, 2010. Microcystin-LR activates the ERK1/2 kinases and stimulates the proliferation of the monkey kidney-derived cell line Vero-E6. Toxicol. In Vitro 24:1689-1695.

DiDonato JA, Mercurio F, Karin M, 2012. NF-kappaB and the link between inflammation and cancer. Immunol. Rev. 246:379-400.

El-Fouly MH, Trosko JE, Chang C-C, 1987. Scrape-loading and dye transfer. Exp. Cell Res. 168:422-430.

Falconer IR, Humpage AR, 2005. Health risk assessment of cyanobacterial (blue-green algal) toxins in drinking water. Int. J. Environ. Res. Public Health. 2:43-50.

Grosse Y, Baan R, Straif K, Secretan B, El Ghissassi F, Cogliano V, 2006. Carcinogenicity of nitrate, nitrite, and cyanobacterial peptide toxins. Lancet Oncol. 7:628-629.

Hakulinen P, Maki-Paakkanen J, Naarala J, Kronberg L, Komulainen H, 2004. Potent inhibition of gap junctional intercellular communication by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) in BALB/c 3T3 cells. Toxicol. Lett. 151:439-449.

Harke MJ, Steffen MM, Gobler CJ, Otten TG, Wilhelm SW, Wood SA, Paerl HW, 2016. A review of the global ecology, genomics, and biogeography of the toxic cyanobacterium, *Microcystis* spp. Harmful Algae 54:4-20.

Herner HA, Trosko JE, Masten SJ, 2001. The epigenetic toxicity of pyrene and related ozonation byproducts containing an aldehyde functional group. Environ. Sci. Technol. 35: 3576-3583.

Hiskia A, Dionysiou DD, Antoniou M, Kaloudis T, 2016. Water Treatment for purification from cyanobacteria and cyanotoxins. 1st ed. Wiley-Blackwell: 272 pp.

Ibelings BW, Bormans M, Fastner J, Visser PM, 2016. CYANOCOST special issue on cyanobacterial blooms: synopsis - a critical review of the management options for their prevention, control and mitigation. Aquat. Ecol. 50:595-605.

Kang KS, Wilson MR, Hayashi T, Chang CC, Trosko JE, 1996. Inhibition of gap junctional intercellular communication in normal human breast epithelial cells after treatment with pesticides, PCBs, and PBBs, alone or in mixtures. Environ. Health Perspect. 104:192-200.

Kao CY, Oakley CS, Welsch CW, Chang CC, 1997. Growth requirements and neoplastic transformation of two types of normal human breast epithelial cells derived from reduction mammoplasty. In vitro cellular & developmental biology. Animal 33:282-288.

Komatsu M, Furukawa T, Ikeda R, Takumi S, Nong Q, Aoyama K, Akiyama S, Keppler D, Takeuchi T, 2007. Involvement of mitogen-activated protein kinase signaling pathways in microcystin-LR-induced apoptosis after its selective uptake mediated by OATP1B1 and OATP1B3. Toxicol. Sci. 97: 407-416.

Kuiper-Goodman T, Falconer I, Fitzgerald J. 1999. Human Health Aspects, p. 113-153. In: I. Chorus and J. Bartram (eds.), Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. World Health Organization, Geneva, Switzerland.

Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Leusch FDL, Snyder SA, 2015. Bioanalytical tools: half a century of application for potable reuse. Environmental Science: Water Res. Technol. 1:606-621.

Lezcano N, Sedan D, Lucotti I, Giannuzzi L, Vittone L, Andrinolo D, Mundina-Weilenmann C, 2012. Subchronic microcystin-LR exposure increased hepatic apoptosis and induced compensatory mechanisms in mice. J. Biochem. Mol. Toxic. 26:131-138.

Liu J, Wang B, Huang P, Wang H, Xu K, Wang X, Xu L, Guo Z, 2016. Microcystin-LR promotes cell proliferation in the mice liver by activating Akt and p38/ERK/JNK cascades. Chemosphere 163:14-21.

Luster-Teasley SL, Yao JJ, Herner HH, Trosko JE, Masten SJ, 2002. Ozonation of chrysene: evaluation of byproduct mixtures and identification of toxic constituent. Environ. Sci. Technol. 36:869-876.

Luster-Teasley SL, Ganey PE, DiOrio M, Ward JS, Maleczka RE, Jr., Trosko JE, Masten SJ, 2005. Effect of byproducts from the ozonation of pyrene: biphenyl-2,2',6,6'-tetracarbaldehyde and biphenyl-2,2',6,6'-tetracarboxylic acid on gap junction intercellular communication and neutrophil function. Environ. Toxicol. Chem. 24:733-740.

Madhukar BV, de Feijter Rupp HL, Trosko JE, 1996. Pulse treatment with the tumor promoter TPA delays the onset of desensitization response and prolongs the inhibitory effect on gap junctional intercellular communication of a rat liver epithelial cell line WB F-344. Cancer Lett. 106:117-123.

Maier D, Blaha L, Giesy JP, Henneberg A, Kohler HR, Kuch B, Osterauer R, Peschke K, Richter D, Scheurer M, Triebskorn R, 2015. Biological plausibility as a tool to associate analytical data for micropollutants and effect potentials in wastewater, surface water, and sediments with effects in fishes. Water Res. 72:127-144.

Masten SJ, Tian M, Upham BL, Trosko JE, 2001. Effect of selected pesticides and their ozonation by-products on gap junctional intercellular communication using rat liver epithelial cell lines. Chemosphere 44:457-465.

Máthé C, Beyer D, M-Hamvas M, Vasas G, 2016. The effects of microcystins (cyanobacterial heptapeptides) on the eukaryotic cytoskeletal system. Mini Rev. Med. Chem. 16:1063-1077.

McGorum BC, Pirie RS, Glendinning L, McLachlan G, Metcalf JS, Banack SA, Cox PA, Codd GA, 2015. Grazing livestock are exposed to terrestrial cyanobacteria. Vet. Res. 46:1-10.

Meng G, Sun Y, Fu W, Guo Z, Xu L, 2011. Microcystin-LR induces cytoskeleton system reorganization through hyperphosphorylation of tau and HSP27 via PP2A inhibition and subsequent activation of the p38 MAPK signaling pathway in neuroendocrine (PC12) cells. Toxicology 290:218-229.

Merel S, LeBot B, Clément M, Seux R, Thomas O, 2009. Ms identification of microcystin-LR chlorination by-products. Chemosphere 74:832-839.

Merel S, Clément M, Thomas O, 2010. State of the art on cyanotoxins in water and their behaviour towards chlorine. Toxicon 55:677-691.

Merel S, Walker D, Chicana R, Snyder S, Baurès E, Thomas O, 2013. State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environ. Int. 59:303-327.

Miao H-F, Qin F, Tao G-J, Tao W-Y, Ruan W-Q, 2010. Detoxification and degradation of microcystin-LR and -RR by ozonation. Chemosphere 79:355-361.

Moreira C, Azevedo J, Antunes A, Vasconcelos V, 2013. Cylindrospermopsin: occurrence, methods of detection and toxicology. J. Appl. Microbiol. 114:605-620.

Mowe MAD, Mitrovic SM, Lim RP, Furey A, Yeo DCJ, 2014. Tropical cyanobacterial blooms: a review of prevalence, problem taxa, toxins and influencing environmental factors. J. Limnol. 74:1005.

Neale PA, Antony A, Bartkow ME, Farre MJ, Heitz A, Kristiana I, Tang JY, Escher BI, 2012. Bioanalytical assessment of the formation of disinfection byproducts in a drinking water treatment plant. Environ. Sci. Technol. 46:10317-10325.

Nebreda AR, Porras A, 2000. p38 MAP kinases: beyond the stress response. Trends Biochem. Sci. 25:257-260.

Newcombe G, Nicholson B, 2004. Water treatment options for dissolved cyanotoxins. J. Water Supply Res. Technol. 53:227-239.

Nicholson BC, Rositano J, Burch MD, 1994. Destruction of cyanobacterial peptide hepatotoxins by chlorine and chloramine. Water Res. 28:1297-1303.

Nishikawa A, Sai K, Okazaki K, Son HY, Kanki K, Nakajima M, Kinae N, Nohmi T, Trosko JE, Inoue T, Hirose M, 2006. MX, a by-product of water chlorination, lacks in vivo genotoxicity in gpt delta mice but inhibits gap junctional intercellular communication in rat WB cells. Environ. Mol. Mutagen. 47:48-55.

Nishiwaki-Matsushima R, Ohta T, Nishiwaki S, Suganuma M, Kohyama K, Ishikawa T, Carmichael WW, Fujiki H, 1992. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J. Cancer Res. Clin. Oncol. 118:420-424.

Novakova K, Babica P, Adamovsky O, Blaha L, 2011. Modulation of gap-junctional intercellular communication by a series of cyanobacterial samples from nature and laboratory cultures. Toxicon 58:76-84.

Novakova K, Blaha L, Babica P, 2012. Tumor promoting effects of cyanobacterial extracts are potentiated by anthropogenic contaminants-evidence from in vitro study. Chemosphere 89:30-37.

Novakova K, Kohoutek J, Adamovsky O, Brack W, Krauss M, Blaha L, 2013. Novel metabolites in cyanobacterium *Cylindrospermopsis raciborskii* with potencies to inhibit gap junctional intercellular communication. J. Hazard. Mater. 262:571-579.

Oberemm A, Fastner J, Steinberg CEW, 1997. Effects of microcystin-LR and cyanobacterial crude extracts on embryo-larval development of zebrafish (*Danio rerio*). Water Res. 31:2918-2921.

Osgood RS, Upham BL, Hill T, Helms KL, Velmurugan K, Babica P, Bauer AK, 2014. Polycyclic aromatic hydrocarbon-induced signaling events relevant to inflammation and tumorigenesis in lung cells are dependent on molecular structure. PloS One 8:e65150.

Prasse C, Stalter D, Schulte-Oehlmann U, Oehlmann J, Ternes TA, 2015. Spoilt for choice: A critical review on the chemical and biological assessment of current wastewater treatment technologies. Water Res. 87:237-270.

Rodriguez EM, Acero JL, Spoof L, Meriluoto J, 2008. Oxidation of MC-LR and -RR with chlorine and potassium permanganate: Toxicity of the reaction products. Water Res. 42: 1744-1752.

Roegner AF, Brena B, González-Sapienza G, Puschner B, 2014. Microcystins in potable surface waters: toxic effects and removal strategies. J. Appl. Toxicol. 34:441-457.

Rosenkranz M, Rosenkranz HS, Klopman G, 1997. Intercellular communication, tumor promotion and non-genotoxic carcinogenesis: relationships based upon structural considerations. Mutat. Res. 381:171-188.

Shawwa AR, Smith DW, 2001. Kinetics of microcystin-LR oxidation by ozone. Ozone Sci. Eng. 23:161-170.

Sovadinova I, Babica P, Boke H, Kumar E, Wilke A, Park JS, Trosko JE, Upham BL, 2015. Phosphatidylcholine specific PLC-induced dysregulation of gap junctions, a robust cellular response to environmental toxicants, and prevention by resveratrol in a rat liver cell model. PloS One 10:e0124454.

Sun Y, Meng GM, Guo ZL, Xu LH, 2011. Regulation of heat shock protein 27 phosphorylation during microcystin-LR-induced cytoskeletal reorganization in a human liver cell line. Toxicol. Lett. 207:270-277.

Svircev Z, Krstic S, Miladinov-Mikov M, Baltic V, Vidovic M, 2009. Freshwater cyanobacterial blooms and primary liver cancer epidemiological studies in Serbia. J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev. 27:36-55.

Svircev Z, Baltic V, Gantar M, Jukovic M, Stojanovic D, Baltic M, 2010. Molecular aspects of microcystin-induced hepatotoxicity and hepatocarcinogenesis. J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev. 28:39-59.

Trosko JE, Jone C, Chang CC, 1987. Inhibition of gap junctional-mediated intercellular communication in vitro by aldrin, dieldrin and toxaphene: a possible cellular mechanism for their tumor-promoting and neurotoxic effects. Mol. Toxicol. 1:83-94.

Tsao MS, Smith JD, Nelson KG, Grisham JW, 1984. A diploid epithelial cell line from normal adult rat liver with phenotypic properties of 'oval' cells. Exp. Cell Res. 154:38-52.

Tsuji K, Watanuki T, Kondo F, Watanabe MF, Nakazawa H, Suzuki M, Uchida H, Harada K-I, 1997. Stability of Microcystins from cyanobacteria - iv. effect of chlorination on decomposition. Toxicon 35:1033-1041.

Upham B, Masten SJ, Lockwood BR, Trosko JE, 1994. Nongenotoxic effects of polycyclic aromatic hydrocarbons and their ozonation by-products on the intercellular communication of rat liver epithelial cells. Fundam. Appl. Toxicol. 23 470-475.

Upham BL, Yao JJ, Trosko JE, Masten SJ, 1995. Determination of the efficacy of ozone treatment systems using a gap junction intercellular communication bioassay. Environ. Sci. Technol. 29:2923-2928.

Upham BL, Boddy B, Xing X, Trosko JE, Masten SJ, 1997. Non-genotoxic effects of selected pesticides and their disinfection by-products on gap junctional intercellular communication. Ozone: Sci. Eng. 19:351-369.

Upham BL, Weis LM, Trosko JE, 1998. Modulated gap junctional intercellular communication as a biomarker of PAH epigenetic toxicity: structure-function relationship. Environ. Health Perspect. 106(Suppl 4):975-981.

Upham BL, Guzvic M, Scott J, Carbone JM, Blaha L, Coe C, Li LL, Rummel AM, Trosko JE, 2007. Inhibition of gap junctional intercellular communication and activation of mitogen-activated protein kinase by tumor-promoting organic peroxides and protection by resveratrol. Nutr. Cancer 57:38-47.

Upham BL, Blaha L, Babica P, Park JS, Sovadinova I, Pudrith C, Rummel AM, Weis LM, Sai K, Tithof PK, Guzvic M, Vondracek J, Machala M, Trosko JE, 2008. Tumor promoting properties of a cigarette smoke prevalent polycyclic aromatic hydrocarbon as indicated by the inhibition of gap junctional intercellular communication via phosphatidylcholine-specific phospholipase C. Cancer Sci. 99:696-705.

Upham BL, Park JS, Babica P, Sovadinova I, Rummel AM, Trosko JE, Hirose A, Hasegawa R, Kanno J, Sai K, 2009. Structure-activity-dependent regulation of cell communication by perfluorinated fatty acids using in vivo and in vitro model systems. Environ. Health Perspect. 117:545-551.

Upham BL, Sovadinova I, Babica P, 2016. Gap junctional intercellular communication: a functional biomarker to assess adverse effects of toxicants and toxins, and health benefits of natural products. J. Vis. Exp. 118:e54281.

Vinken M, Doktorova T, Decrock E, Leybaert L, Vanhaecke T, Rogiers V, 2009. Gap junctional intercellular communication as a target for liver toxicity and carcinogenicity. Crit. Rev. Biochem. Mol. Biol. 44:201-222.

Wagner EF, Nebreda AR, 2009. Signal integration by JNK and p38 MAPK pathways in cancer development. Nat. Rev. Cancer 9:537-549.

Wang B, Liu J, Huang P, Xu K, Wang H, Wang X, Guo Z, Xu L, 2017. Protein phosphatase 2A inhibition and subsequent cytoskeleton reorganization contributes to cell migration caused by microcystin-LR in human laryngeal epithelial cells (Hep-2). Environ. Toxicol. 32:890-903.

Wernersson A, Maggi C, Carere M, 2014. Technical report on aquatic effect-based monitoring tools. Office for Official Publications of the European Communities.

Westrick JA, Szlag DC, Southwell BJ, Sinclair J, 2010. A review of cyanobacteria and cyanotoxins removal/inactivation in drinking water treatment. Anal. Bioanal. Chem. 397:705-1714.

Wiegand C, Pflugmacher S, 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. Toxicol. Appl. Pharmacol. 203:201-218.

Wu J, Lin L, Luan T, Chan Gilbert YS, Lan C, 2007. Effects of organophosphorus pesticides and their ozonation byproducts on gap junctional intercellular communication in rat liver cell line. Food Chem.Toxicol. 45:2057-2063.

Yu SZ, 1995. Primary prevention of hepatocellular carcinoma. J. Gastroenterol. Hepatol. 10:674-682.

Zanchett G, Oliveira-Filho E, 2013. Cyanobacteria and cyanotoxins: From impacts on aquatic ecosystems and human health to anticarcinogenic effects. Toxins 5:1896.

Zegura B, Straser A, Filipic M, 2011. Genotoxicity and potential carcinogenicity of cyanobacterial toxins - a review. Mutat. Res. 727:16-41.

Zhang J, Chen J, Xia Z, 2013. Microcystin-LR exhibits immunomodulatory role in mouse primary hepatocytes through activation of the NF-kappaB and MAPK signaling pathways. Toxicol. Sci. 136:86-96.

Zhou L, Yu H, Chen K, 2002. Relationship between microcystin in drinking water and colorectal cancer. Biomed. Environ. Sci. 15 166-171.