Toxicon 154 (2018) 50–59

Contents lists available at ScienceDirect

Toxicon

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

Genotoxicity and cytotoxicity of three microcystin-LR containing cyanobacterial samples from Antioquia, Colombia

Natalia Herrera[a], Carolina Herrera[a], Isabel Ortíz[b], Luz Orozco[b], Sara Robledo[c], Diana Agudelo[d], Fernando Echeverri[a,*]

[a] Grupo de Química Organica de Productos Naturales, Instituto de Química, Universidad de Antioquia, Calle 67 No. 53-10, Medellín, 050010, Colombia
[b] Universidad Pontificia Bolivariana, Medellín, Colombia
[c] Programa de Estudio y Control de Enfermedades Tropicales, Instituto de Investigaciones Médicas, Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia
[d] Grupo de investigación en Gestión y Modelación Ambiental (GAIA), Universidad de Antioquia, Medellín, Colombia

ARTICLE INFO

Keywords:
Cyanobacteria
Microcystins
Genotoxic
Comet assay
Damage level

ABSTRACT

The presence of cyanobacterial blooms and cyanotoxins in water presents a global problem due to the deterioration of ecosystems and the possibility of poisoning in human and animals. Microcystin LR is the most widely distributed cyanotoxin and liver cells are its main target. In the present study, HepG2 cells were used to determine DNA damage of three crude extracts of cyanobacterial blooms containing MC-LR, through comet assay. The results show that all extracts at a concentration of 500 μg mL^{-1} caused low damage in hepatocytes exposed for 24 h, but produced total mortality even at low concentrations at 48 h. Moreover, balloons corresponding to cell apoptosis were found.

Through HPLC/MS, MC-LR was detected in all samples of cyanobacterial blooms at concentrations of (5,65 μg ml^{-1}) in sample 1, (1,24 μg ml^{-1}) in sample 2 and (57,29 μg ml^{-1}) in sample 3. In addition, in all samples high molecular weights peaks were detected, that may correspond to other microcystins.

Besides, the cytotoxic effect of a cyanobacterial bloom and some of its chromatographic fractions from the crude extracts were evaluated in U-937, J774, Hela and Vero cell lines, using the enzymatic micromethod (MTT). The highest toxicity was detected in U-937 cells (LC_{50} = 29.7 μg mL^{-1}) and Vero cells (LC_{50} = 39.7 μg mL^{-1}).

Based on these results, it is important to remark that genotoxic and cytotoxicity assays are valuable methods to predict potential biological risks in waters contaminated with blooms of cyanobacteria, since chemical analysis can only describe the presence of cyanotoxins, but not their biological effects.

1. Introduction

The increase in human population has brought a growing demand for water resources for human consumption but also a decrease in the volume and quality of water supply because of the environmental degradation of aquatic ecosystems. This situation has encouraged the development of research aimed to identify and prevent the causal factors of deterioration of water sources, as well as improving water purification techniques.

Cyanobacterial blooms are one of the factors with highest incidence in water quality degradation and are produced by numerous genera of Cyanobacteria Phylum. They are characterized by the production of several compounds (Carmichael, 1994), which are known as hepatotoxins and neurotoxins (Reynolds, 2006). Presence of blooms or cyanobacterial blooms and production of cyanotoxins affect water quality, making treatment processes more expensive (Garcia Nieto et al., 2011; Quesada et al., 2004) and restricting recreative water activities (Quesada et al., 2004) including fishing. Several cases of intoxication by these compounds have been reported worldwide in wild and domestic animals and human (Hillebrand, 1999; Reynolds, 2006).

Microcystins are a class of cyanotoxins produced by species of the genera *Microcystis*, *Anabaena*, *Oscillatoria* (*Planktothrix*), *Nostoc*, and *Anabaenopsis* (Sivonen and Jones, 1999); their ingestion by mammals may lead to increased liver weight, hepatic histological damage (Heinze, 1999), liver cancer (Hernández et al., 2009; Hu et al., 2008; Li et al., 2016) and renal damage (Milutinović et al., 2003). These cyanotoxins may affect the cytoskeleton of liver cells, triggering apoptosis, necrosis and internal hemorrhage that can lead to death due to acute hemorrhagic shock (Dawson, 1998). Microcystin LR is the most common toxin and long-term exposure to this compound is mainly

* Corresponding author. Universidad de Antioquia, Instituto de Quimica, CP, 050010, Colombia.
E-mail addresses: nahelo241980@udea.edu.co (N. Herrera), fernando.echeverri@udea.edu.co (F. Echeverri).

https://doi.org/10.1016/j.toxicon.2018.09.011
Received 28 May 2018; Received in revised form 19 September 2018; Accepted 25 September 2018
Available online 28 September 2018

associated with expression of proteins in the caspase pathway (Huang et al., 2016), increasing oxidative stress, and also affecting mitochondrial DNA, altering cytokine expression, and causing lung deterioration in mice (Li et al., 2016; Ma et al., 2017).

Nevertheless, the molecular mechanism by which MC-LR (microcystin LR) induces toxicity has not been fully elucidated. Therefore, a great diversity of biological models, techniques and experimental designs have been used for the evaluation of their toxicity. In addition, there are few reports on the cytotoxicity and genotoxicity of samples of cyanobacterial blooms with mixtures of MC-LR and other cyanotoxins. Also, these studies have not been well understood (Žegura et al., 2006).

One of the main problems related to the presence of cyanobacteria in water reservoirs is the risk of exposure to complex mixtures of toxins for consumers of contaminated fish and drinking water (Carneiro et al., 2017). This type of risk could be a problem of public environmental health and must be addressed from different points of view.

Studies carried out by our research group have shown this problem in Riogrande II and Porce II reservoirs used as multiproposite and for power generation respectively (Herrera et al., 2015). Additionally, the bioaccumulation results of these microcystins were reported in Cladocerans (Herrera et al., 2014) for this reason in this study, genotoxicity is evaluated as an indicator of possible chronic effects in human cell lines such as HepG2 and the cytotoxicity in several cell lines to show the effect on different target organs and would be an indicator of possible acute effects.

Due to the lack of knowledge about effects of crude toxin mixtures in mammal cells, the aim of this study was to analyze the DNA in HepG2 hepatocytes and cytotoxic effects induced by different samples of blooms in U-937 promonocyte cells, mouse J774 macrophages, human Hela endothelial cervix cells and monkey kidney Vero epithelial cell lines. These bioassays could be tools to predict the risk of exposure to the health of people and animals who are in contact with this type of samples (blooms) composed of mixtures of toxins and other types of compounds.

2. Materials and methods

2.1. Study area

Porce II and Riogrande II are two water reservoirs located in the department of Antioquia, Colombia. Porce II is used for power generation and artisanal fishing while Riogrande II has a multipurpose use. In the tributary basins of both reservoirs, take place intensive agricultural and industrial activities.

One sample was taken from the most eutrophic area of Riogrande II, the arm of Rio Chico located 15 km away from the water intake tower (sample 1). Other two samples were taken from a nearby point located in the main body water (sample 2) and the last one in the dam (sample 3) of Porce II reservoir (Fig. 1).

2.2. Sample processing

2.2.1. Lyophilization

The three cyanobacterial bloom samples were collected with a 20-μm mesh nylon net, stored in plastic bottles of five liters and maintained in darkness and refrigerated at 4 °C until their lyophilization in the laboratory, previously reported (Herrera et al., 2015). After that they were processed for the HPLC analyses. Fig. 2 shows the origin of each of the analyzed samples, fractions and subfractions.

For the genotoxicity and cytotoxicity test, only lyophilized samples were used without any further treatment, then, 20 mg were taken and resuspended in 1 ml of DMSO (dimethyl sulfoxide).

2.2.2. Subfractionation

In addition, the sample from the Riogrande II reservoir was fractionated by chromatography and tested for cytotoxicity. Thus, 40 g of the lyophilized sample was resuspended in methanol 90% and sonicated for 15 min with a Branson Ultrasonic (model 2510); afterwards, it was filtered through a glass fiber membrane of 47 mm (Advantec). The extract obtained (8.0 g) was separated by liquid chromatography in column with a mix of dichloromethane: methanol (4:1, v/v) until obtaining 24 fractions of 20 mL each. The last two fractions (***a*** and ***b***) were chosen to continue purifying them, because the presence of microcystins was detected by the ELISA test. The last fractions of this new purification process were eluted with 100% methanol, were selected and named (***a-1***, ***b-1*** **and** ***b-2***). For cytotoxicity assays, each sample was diluted in DMSO to determine the cytotoxic effect on four cell lines.

2.2.3. Cyanobacteria detection

To determine the presence of cyanobacteria, 250 mL of water sample was fixed with 1 mL of Lugol's solution (1%). For the analysis of *Microcystis* mucilage Chinese ink was used. For quantitative analysis, the samples were shaken 30 times and pelleted, following the method of Utermöhl (Rzóska and Margalef, 1979). One milliliter of the precipitate was placed in a counting chamber Sedgwick-Rafter and microscopic observations were performed (Herrera et al., 2015).

2.3. MC-LR detection by HPLC/MS

Two hundred mg of lyophilized material were resuspended in 80% methanol; then the sample was sonicated for 15 min in a Branson 2510 equipment and centrifuged at 3000 rpm for 5 min. Then, samples were dried on a rotary evaporator (Heidolph) at 35 °C and the obtained extract was filtered through C18 cartridges (CNWBOND HC-C18) eluted with methanol and analyzed by HPLC-MS.

Detection and confirmation of MC-LR was carried out by (HPLC-MS/MS) (Agilent HPLC 1200, AB SCIEX 3200 QTRAP), following the methodology by Herrera et al. (2015): Column, Kinetex 2.6 μm C18 100 Å, 50 × 2.1 mm; Mobile phase A, 5 mM ammonium acetate in

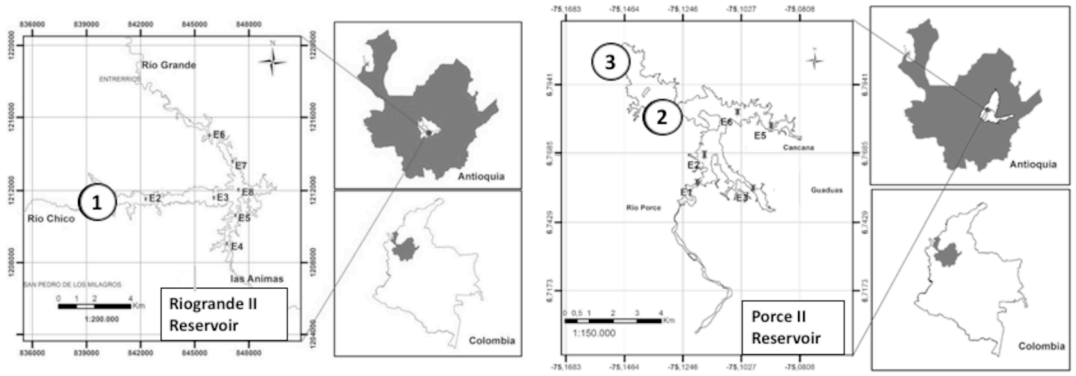


Fig. 1. Localization of the three cyanobacterial bloom samples collected. Three samples of cyanobacterial blooms were taken: two in Porce II dam (3), one in the main body (2), and the last one in the most eutrophic area of Riogrande II, the arm of Rio Chico (1).

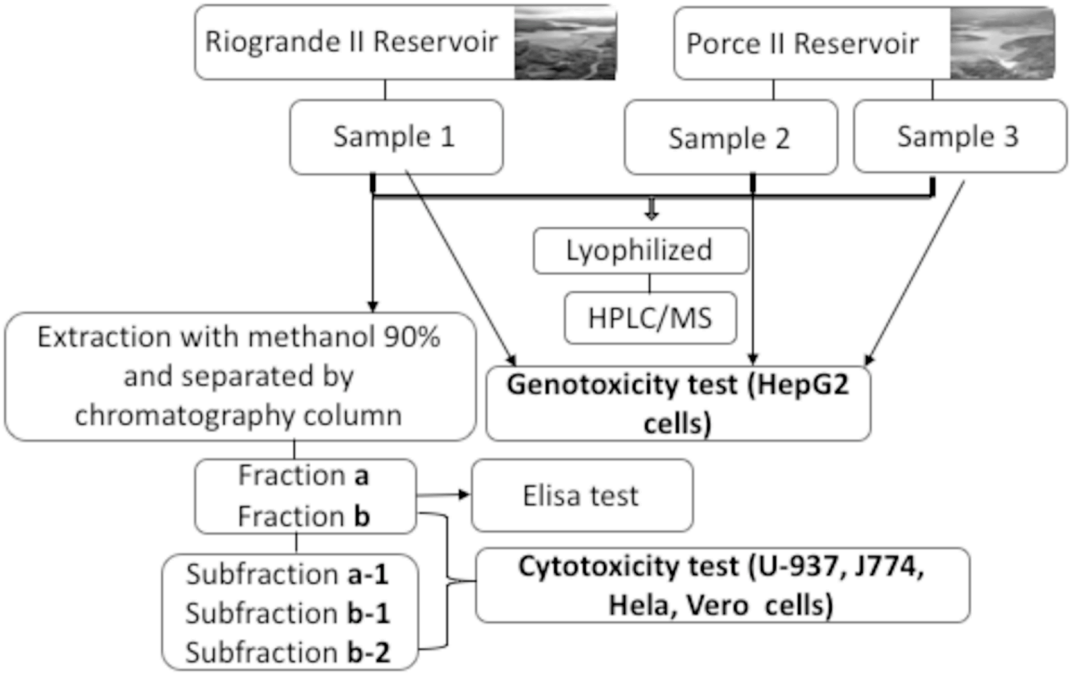


Fig. 2. Processing of samples used for the genotoxicity and cytotoxicity tests.

water and 0,1% formic acid; Mobile phase B, 5 mM ammonium acetate in acetonitrile and 0,1% formic acid; Flow rate, 450 µL min-1; Injection volume, 10 µL; gradient, 0–6 min 85% A and 15% B, 6–10 min 65% A, 10 min 10% A followed by a return to 85% A.

2.4. Analysis of microcystins by ELISA

The subtracted samples from Riogrande II reservoir were tested using Elisa a Microcystin Plate Kit (Envirologix Inc.) (detection limit 0.16) for the detection of microcystins (in cross-reactivity with variants LR, LA, RR, YR, and nodularin) was used to test the extract of sample 1 (Riogrande II). The optical density (O.D.) was measured with a microplate reader (ELx 800 NB, Bio-Tec Instruments Inc.). In each 8-well strip, four controls were used (negative, calibrator 1: 0.16 ppb, calibrator 2: 0.6 ppb and calibrator 3: 2.5 ppb). To determine the concentration of microcystin in the samples an equation derived from the calibration curve was used. This calibration curve was constructed from the microcystin concentration of the calibrators and the %Bo (O.D. of each calibrator/O.D. of the negative control x 100). % Bo is the maximum amount of Microcystin-enzyme conjugate that is bound by the antibody in the absence of any Microcystin in the sample (i.e. negative control). The Elisa technique was used because it is considered highly sensitive (LOD = 0.02–0.15 µg/L) and the samples require little preparation (Heussner et al., 2014).

2.5. Biological analysis

2.5.1. Viability assays

Cell viability was determined with the MTT exclusion method using OD_{570} in a Multiskan-go spectrophotometer (Senthilraja and Kathiresan, 2015). Twenty serial dilutions of all the samples were analyzed to evaluate the cellular viability by MTT. Measurements were performed by triplicate using 1% DMSO and DMEM as positive and negative controls, respectively.

Cell viability (%) = Mean OD treated cells/Mean Control OD x 100%

The concentration required for a 50% and 10% inhibition of viability (LC_{50} and LC_{10}) were established by Probit analysis; the LC_{10} was used to determine sublethal concentrations in the genotoxicity assays.

2.5.2. Genotoxicity of cyanobacterial extracts (comet assay)

HepG2 cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, incubated at 37 °C at an atmospheric humidity above 95% and 5% CO_2 (Valentin-Severin et al., 2003). When cells reached 80% confluence were exposed to the samples resuspended in 1% DMSO. All genotoxicity assays were performed by testing three sub-lethal concentrations ($\leq LC_{10}$) of all samples. Only concentrations that yield viability results above 80% were chosen to perform the genotoxicity assays with the purpose of avoiding false positive results (Platel et al., 2009). The exposure time was 24, 48 and 72 h; it was chosen 24 h because the viability of the culture was superior to 90%, since at 48 h there was total cytotoxicity.

Single cell gel electrophoresis (SCGE) also known the Comet assay as is a powerful technique that permits the evaluation of single and double strand DNA breaks on individual cells. This methodology allowed us to evaluate DNA damage in HepG2 after treating them separately with three sub-lethal concentrations (500, 125 and 50 µg mL^{-1}) of cyanobacterial samples extract. The comet assay consists of separating and characterizing DNA that migrates out of the cell (Singh et al., 1988). This process involves the immobilization of cells by embedding them in agarose gel (GelBond® film) with low melting point agarose (LMPA), then the agarose gel is submerged in a lysing buffer to achieve cell membrane rupture, the DNA migrates to the anode when exposed to electric current during electrophoresis leaving a trail of ruptured DNA behind like a “comet tail.” Samples were stained with GelRed™ for better visualization of the comet tail. The results of the test were read by measuring the length of the tail. The comet assay has proven effective for the identification of strand breaks, alkaline-labile site damage and mismatch repair sites (Collins et al., 2014).

HepG2 cells were seeded in 6 well plates at a density of 1.0×10^5 and 1.0×10^4 respectively, under previously described conditions. Forty-eight hours after seeding, cells were treated with the three sublethal concentrations (500, 125 and 50 µg mL^{-1}), corresponding to either type of cyanobacterial samples extract, an exposed for 24 h. Post treatment cell viability was evaluated by MTT test. Cell suspensions were mixed with LMPA, embedded in the agarose gel film and placed in the lysing solution for 16–24 h. The lysis solution contained 100 mM

EDTA, 0.05% SDS, 2.5M NaCl, 1% Triton X-100, 10 mM Trizma-base and 10% DMSO added prior to its use, the pH was adjusted to 10 with NaOH. The high concentration of salt in the lysis solution helps to break down the cell membrane, remove histones and other soluble proteins (Karlsson, 2010). After the cells were lysed, the agarose film was placed in a horizontal electrophoresis chamber and covered with cold electrophoresis buffer pH > 13 (often containing 300 mM NaOH, 1 mM EDTA) for 40 min. After 40 min, the agarose film with the embedded cells was electrophoresed for 30 min at 25 mV and 300 mA. The agarose film was removed from the electrophoresis chamber, rinsed with neutralizing buffer and set aside in a draining plate, dehydration of the embedded LMPA cells was done with methanol prior to staining. Cells were stained with GelRed™ [0.01 ×] for approximately 10 s and rinsed with distilled water to remove excess dye.

The agarose film was cut and mounted on glass slides; samples were analyzed with a Nikon® Eclipse 55i fluorescence microscope (Tokyo, Japan) and a 40× objective. DMEM was used as a negative control and H_2O_2 (50 μM) was used as a positive control. The comet assay was done on two independent moments with two replicates each time it was performed; 160 cells were analyzed per treatment. DNA damage was measured based on the length (μm) of the DNA fragments that migrated out of the cell (comet tail), this was measured using an ocular micrometer (Platel et al., 2009). The comet assay was selected as an alternative to cytotoxicity assays since is a relatively fast, simple and sensitive technique when a small number of test compounds; besides it is useful to avoid false positives (Switalla et al., 2013). Finally, the cells were stained and observed under a DNA migration from a total of 160 nuclei per treatment Damage was quantified based on five categories (Mena-Huertas et al., 2011) considering the median of the negative control plus two standard deviations (X ± 2SD) (Song et al., 2007), since 2SD include 95% of the data. After this baseline value, the following damage categories were obtained: (Table 1).

2.5.3. Damage classification and induction factor (IF)

A classification of the damage was carried out with the data of comet length, which is directly related to the ability of the cyanobacterial bloom samples to rupture or fragment the DNA. DNA damage induced by the extracts of the cyanobacterial samples was classified into 5 categories as follows: “0” or undamaged DNA, “1” or lightly damaged DNA, “2” or moderately damaged DNA, “3” or highly damaged DNA and “4” or total damaged DNA. For each category, the percentage of cells was determined as indicative of the intensity of the treatment (Cadrazco et al., 2017; Rodriguez Ferreiro et al., 2002).

The frequency of damaged cells in each treatment was also taken into account for genotoxicity analyses. In order to avoid false positives, a treatment was considered to induce genotoxic activity when the cell viability was equal or greater than 70 ± 5% since genotoxic effects may be associated with null to moderate cytotoxicity (Platel et al., 2009).

The Induction Factor was also a criterion for genotoxicity analyzes, which refers to the times that a treatment exceeds the migration mean of the negative or solvent control. It is calculated with the following formula:

FI = X migration of the DNA of a treatment/X migration of the negative control

Table 1
Levels of DNA damage of HepG2 cells exposed to different concentrations of cyanobacterial blooms.

Level of DNA Damage	Type of Damage	Average comet tail length (μm)
0	Undamaged	0–26
1	Low	27–56
2	Medium	57–86
3	High	86–116
4	Total	> 117

Data reported in this table corresponds to average damage scored in 160 cells per treatment; cell culture media was used as negative control.

2.5.4. Culture condition (cytotoxicity)

The cytotoxic activity of cyanobacterial bloom was evaluated in different cell lines, which were cultured and treated in culture media according to each cell line, in the following way: Cells U-937 (human promonocytic cells) were cultured in suspension in RPMI 1640 complete medium with 10% fetal calf serum (FCS) in the presence of 5% CO2 at 37 °C. J774 cells (mouse monocytic cells) was cultured in suspension in Dulbecco's Modified Eagle's medium (DME) with 10% fetal calf serum (FCS) in the presence of 5% CO2 at 37 °C. Hela cells (human cervical cells) and Vero (African green monkey kidney cells) were cultured in suspension in Eagle's Minimum Essential medium with 10% fetal calf serum (FCS) in the presence of 5% CO2 at 37 °C. cellular media were renewed to intervals of 2 days, until acquiring a confluence of 80%.

2.5.5. In vitro cytotoxicity of cyanobacterial sample 1 (Riogrande II)

Each sample was diluted in DMSO; cytotoxicity was evaluated in cell lines U-937, J774, Hela and Vero using the MTT enzymatic micromethod. In short, cells were exposed to the corresponding concentrations (200, 50, 12.5 and 3.125 μg mL^{-1}) of cyanobacterial bloom samples and the drug used as controls for cytotoxicity. After 72 h of incubation at 37 °C with 5% CO_2 10 μL/well of MTT solution (0.5 mg/mL) was added to each well and plates were incubated at 37 °C for 3 h. The reaction was stopped by adding 100 μL/well of isopropanol solution at 50% with sodium dodecyl sulphate at 10% and incubating for 30 min. Cell viability was determined by the amount of formazan produced according to the color intensity (absorbance), recorded as the optical density (O.D) obtained at 570 nm in a Varioskan reader (ThermoFisher Scientific, MA, USA). Untreated cells were used as a viability control, and amphotericin B (200, 50, 12.5, and 3.25 μg mL^{-1}) was a control for cytotoxicity. Each sample was tested in triplicate, in at least two independent trials. The results were expressed as the half lethal concentration (LC_{50}) (Taylor-Harding et al., 2010).

2.6. Statistical analysis

The LC_{50} values were calculated with the Probit method. In order to establish statistically significant differences among comet assay measurements, a non-parametric Kruskal-Wallis analysis of variance was applied with a significance level of 0.05. When significant differences were found, a multiple ranges analysis was carried out in order to determine differences between levels of damage.

3. Results

3.1. Detection of microcystin-LR by HPLC-MS

Microcystis *aeruginosa* was the most abundant cyanobacterium; the HPLC-MS/MS analysis showed microcystin-LR in all samples (Table 2).

Table 2
The microcystin-LR concentrations by HPLC/MS in the total samples and samples for subsequent genotoxicity analysis.

Sample	Concentration MC-LR total (μg ml^{-1})	Concentration MC-LR for genotoxicity test (μg ml^{-1})		
		500 μg ml^{-1}	125 μg ml^{-1}	50 μg ml^{-1}
1 Riogrande	5651	1,41	0,35	0,14
2 Porce Main body	1241	0,31	0,07	0,03
3 Porce Dam	57,296	14,32	3,58	1,43

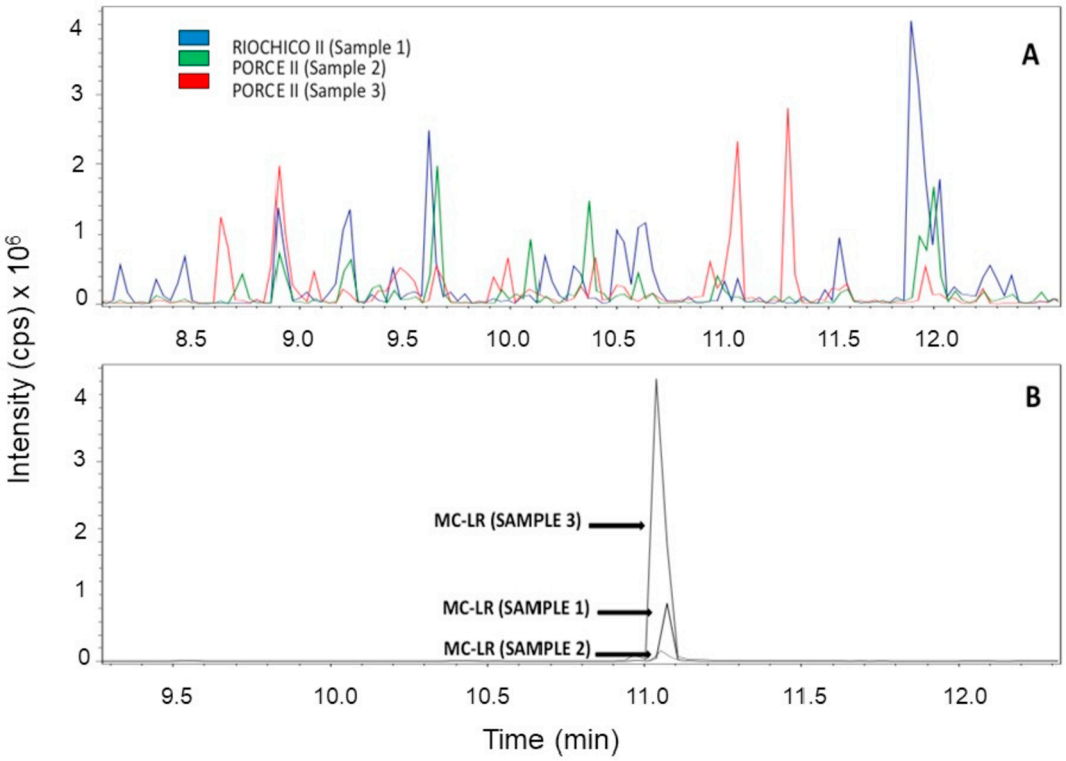


Fig. 3. Profile of compounds by liquid chromatography–mass spectrometry HPLC/MS (UHR-QqTOF Scan Mode: Auto MS/MS) of the three cyanobacterial bloom samples (A), and detection of MC-LR (m/z 995) (B).

The highest toxin concentrations were detected in samples **1** and **3** from Riogrande II and Porce II and the lowest in sample **2** from Porce II (Fig. 3-B). All samples were analyzed by HPLC/MS and molecular weights between 900 and 1300 were determined, and possibly corresponding to other microcystins (Fig. 3-A). The microcystin-LR concentrations in the samples for subsequent genotoxicity analysis are shown in Table 2.

The Riogrande II sample containing 5651 μg/mL^{-1} of the toxin was subfractionated for cytotoxicity assays; microcystins were detected by ELISA test.

3.2. Genotoxicity in HepG2 cells

All three samples induced significant genotoxicity, with induction factors (IF) up to 2,01. Fig. 4 shows the damage induction factor (IF) for three different concentrations in each one of the samples. In samples 1 and 3 there was not a significant different in the IF for different concentrations while in sample 2 there was an increment in the IF with the increased of the concentration. The lowest IF (0,94) was found in sample 2 for the lowest concentration (50 μg ml-1) while the highest IF (2,01) was found in sample 3 for the highest concentration (500 μg ml-1). According to the results of Fig. 4, sample 2 presented the least migration length of the DNA and the samples 3 the highest migration length at 24 h of exposure.

The correlation results between Damage Induction Factor (IF) and concentration of MC-LR for each cyanobacterial bloom samples to 500 μg mL^{-1} are consistent and correlate with the high toxin concentration detected by HPLC/MS. The linear regression model between the IF and the concentration of MC-LR is statistically significant (P value. 0.0109) with an adjusted $R^2 = 0.99$ indicates that the higher IF values, the higher the cyanotoxin concentration (Fig. 5). However, for the other samples of cyanobacterial blooms that were evaluated at concentrations of 125 and 50 μg mL $^{-1}$, a $R^2 = 0,65$ was obtained.

Fig. 6 shows the frequency of genotoxic effects of the three samples on HepG2 cells; thus, when exposed to bloom, samples 1 and 3 caused the greatest damage at all concentrations evaluated compared to the control (medium damage, Table 1). A low level of damage was observed in hepatocytes exposed to samples of cyanobacterial bloom at a concentration of 500 μg mL^{-1} for 24 h, in contrast with the negative control that did not displayed any damage. However, about 15% of cells treated with samples 2 and 3, with highest MC-LR content, showed moderate damage. Ninety percent of cells treated with sample 1 (Riogrande II) and 3 (Porce II) at a concentration of 125 μg mL^{-1} had a lightly damaged of DNA. In addition, when cells were treated with the lowest concentration of sample 1 (50 μg ml^{-1}), 99.3% of them had low DNA damage. In general, cells exposed to sample 3 had greater damage only when exposed to the highest concentration (500 μg ml^{-1}).

On the other hand, exposure of HepG2 cells to all concentrations of each samples of cyanobacterial bloom for 48 h caused total damage of DNA in all cells. Statistically significant differences $p < 0.05$ were observed between the samples and the negative control with respect to the level of damage.

The frequency of individuals per damage indicates a low-level damage. In addition, no cells showed total damage within 24 h. The extension of the genotoxicity is displayed in Fig. 7; moreover, balloons corresponding to cell apoptosis were found.

3.3. Cytotoxicity

The sample **1** was initial analyzed against U937 and Hep G2, however, only cytoxicity was observed in U-937 cells with LC_{50} of 78.2 ± 3.8 μg mL^1. No cytotoxicity was observed for HepG2 cells with $LC_{50} > 200$ μg mL^1.

Chromatographic fraction ***a*** was cytotoxic to U-937 and Vero cell lines, with LC_{50} of 41.2 ± 6.2 μg mL^{-1} and 39.7 ± 8.0 μg mL^{-1}, respectively, whereas the cytotoxicity was lower for J774 and Hela (cells

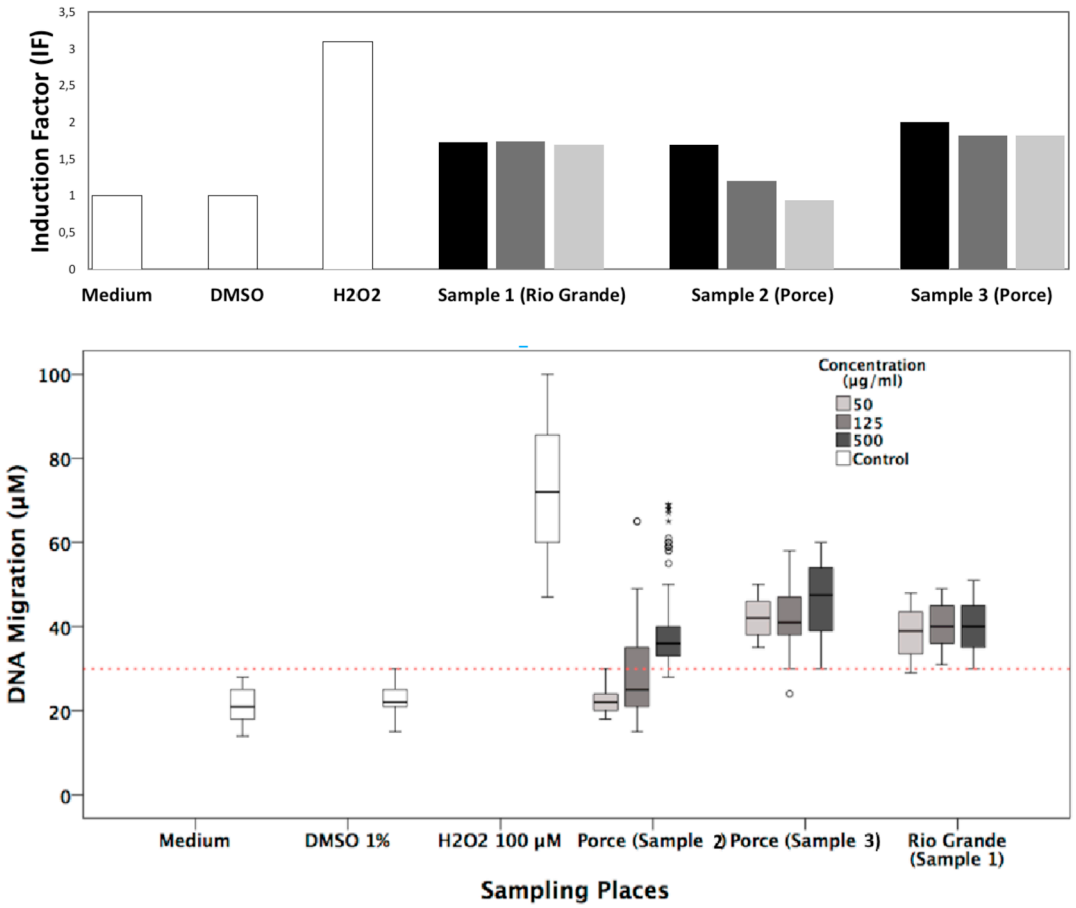


Fig. 4. Damage induction factor of each sample in HepG2 cells (top) and Extent of HepG2 damage level (bottom) with different concentrations of cyanobacterial bloom samples at 24 h of exposure. The dotted line indicates the migration length of the DNA from which the cell is considered to be damaged (average of solvent + 2SD) (Table 1).

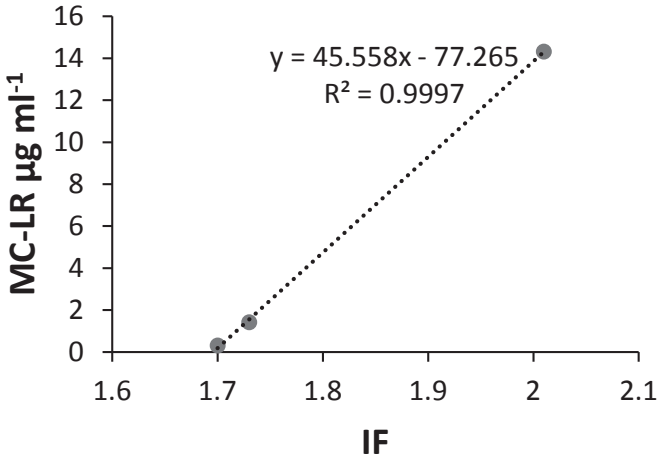


Fig. 5. The correlation between Damage Induction Factor (IF) and the concentration of MC-LR for each of the cyanobacterial bloom samples to 500 µg mL^{-1}.

human cervical) cells, with LC_{50} of 74.8 ± 6.6 µg mL^{-1} and 109.5 ± 1.6 µg mL^{-1}, respectively. Fraction ***b*** did not show cytotoxicity for any of the cell lines evaluated (Table 3).

On the other hand, subfraction ***a-1*** presented lower toxicities for the same cell lines than the original fraction ***a*** (84.8 + 2.8 µg mL^{-1} and 51.3 + 6.3 µg mL^{-1}) for Vero cells and for U-937, respectively. In contrast, subfractions ***b-1*** and ***b-2*** showed a high toxicity for U-937 cells (29.7 ± 0.34 µg mL^{-1} and 16.8 ± 2.0 µg mL^{-1}) but were potentially non-toxic for Vero cells (Table 1). The microcystins present in the initial fractions (**a** and **b**) were detected by the ELISA test in concentrations of 770 µg mL-1 and 742 µg mL-1 respectively.

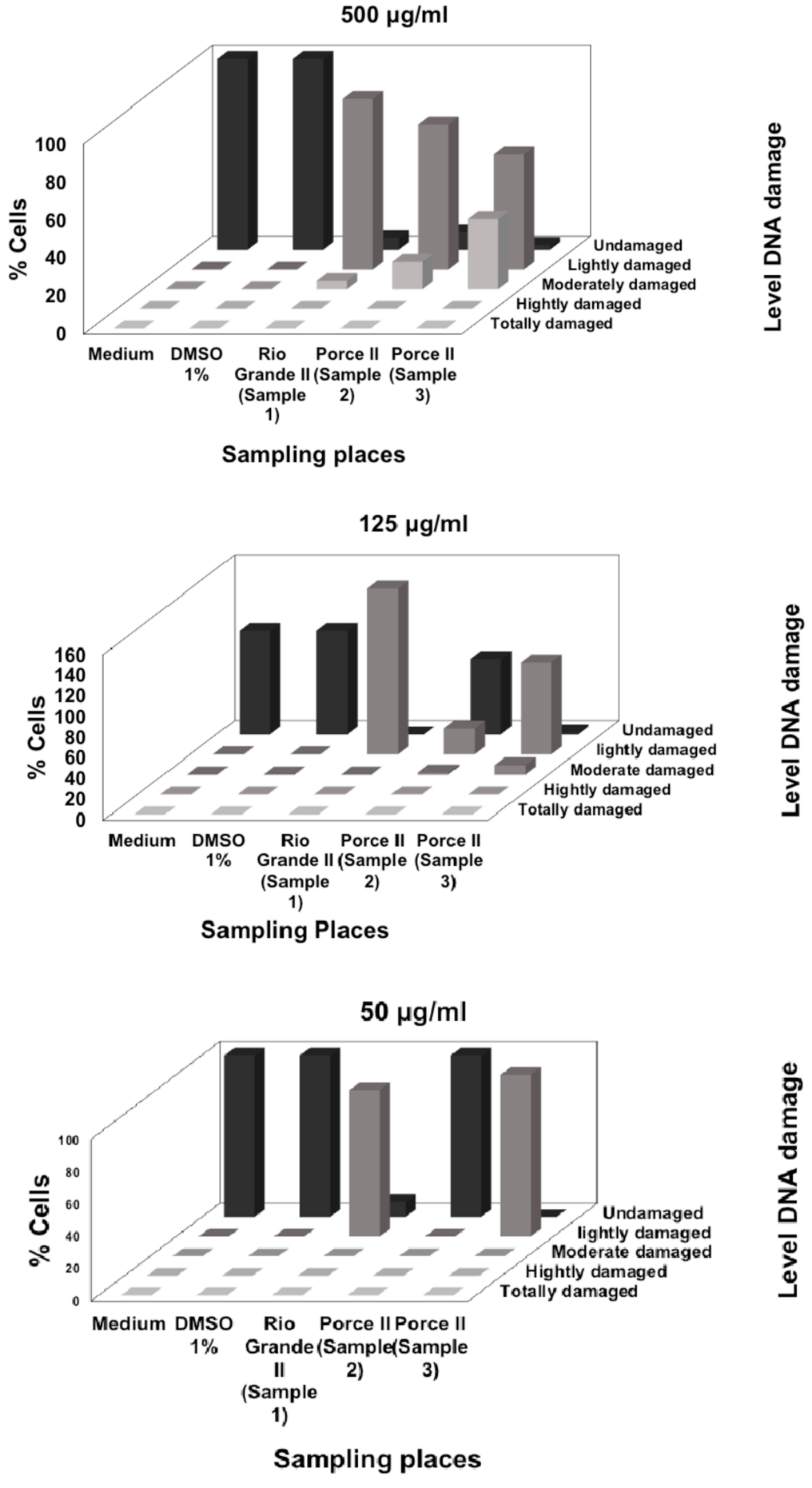
4. Discussion

One of the main problems related to the presence of cyanobacteria in reservoirs of waters is the high risk of exposure to complex mixtures of toxins for consumers of fish and drinking water (Carneiro et al., 2017). This risk becomes an environmental public health problem that must be approached from different perspectives. One of these approaches is the determination of the biological effects of blooms, since chemical analysis do not show the real effect of the mixtures of toxins in the target organisms.

Hepatocytes are considered the main target of microcystins, especially MC-LR; for this reason, the use of HepG2 cell line such as has proved to be an efficient model for genotoxicity assessment (Da Silva et al., 2011; Mankiewicz et al., 2002), and toxicity associated with liver damage also has been demonstrated (Azevedo et al., 2002; Ma et al., 2018).

In this work, the damage caused to the DNA by crude extracts of cyanobacteria bloom was demonstrated. In addition, genotoxicity and cytotoxicity tests were carried out as a method to predict a possible biological risk of damage.

The sample 1 (Riogrande) was selected for cytotoxic assays because is a multipurpose dam (fishery, recreational, energy); samples 2 and 3 were obtained from an energy generation reservoir (Porce). Thus, a high risk is evident for many people and animals directly exposed to this type of water, moreover containing concentrations as low as almost



(caption on next page)

Fig. 6. Frequency of damage on HepG2 cells exposed to different concentrations of each cyanobacterial bloom sample after 24 h of exposure.

one tenth of the reservoir richest in MC-LR (5.6 vs 57.2 μgmL^{-1}) (Table 2).

Although no cell exhibited high damage at 24 h, cytotoxicity at 48 h shows the extent of the toxicological effects these substances can have over time in a prolonged exposure. Moreover, the results of this study, which reports that high concentrations of MC-LR in the bloom samples directly correlate with DNA damage (Induction factor), are consistent with dates given for (Li et al., 2017) who reported that when HepG2 cells were exposed to low concentrations of MC-LR (less than 1.0–5.5 μM) there were no damage effects; however, they can promote the expressions of some genes involved in the formation of liver cancer.

The three samples of cyanobacterial blooms tested for genotoxicity were lyophilized to preserve the original content, maintaining the natural conditions. Sample **3** containing the highest microcystin concentration had the highest genotoxic effect, with a low level of damage in 85% and moderate damage in 15% of the exposed cells. The other two samples showed some damage, but to a lesser degree.

While the mechanisms related to its genotoxicity are not well understood, MC-LR induces alterations at level of intracellular reduced glutathione and DNA damage, suggesting that MC-LR may induce biochemical disturbance and oxidative stress in HepG2 cells (Ma et al., 2017; Žegura et al., 2006). However, this effect cannot be attributed only to MC-LR toxin, since HPLC/MS analyzes detect the presence of other substances with molecular weights similar to those of the of microcystins.

These results indicate the possibility that MC-LR was not directly responsible for the genotoxicity observed for the analyzed samples, as there may be some synergistic interactions with other compounds and other types of microcystins detected by HPLC, that remain unidentified (Fig. 3A).

Additionally, some studies have highlight the toxicity of mixtures of bloom compounds on different organisms; for example, the significant decrease in cytosolic glutathione transferases activity in *Venerupis philippinarum* (Carneiro et al., 2017), and severe decreased survival and extreme reduction in heart rate in fish, *Oryzias latipes* (Saraf et al., 2018).

The unfavorable effect of these blooms has been reported on in aquatic microcrustaceans such as cladocerans, mainly at the level of survival and reproduction (Herrera et al., 2015) and their ecophysiological parameters (Herrera et al., 2014). Nevertheless, other organisms such as zebrafish have also shown pathological changes mainly in the liver (Liu et al., 2014). All these results show that cyanobacterial blooms at the level of different organisms and different cell lines exert a broad-spectrum effect that consequently acts negatively on human and animal health. In this study the importance of considering total crude extracts of blooms is highlighted, due to the direct exposure of some organisms to this type of samples.

On the other hand, after 48 h of exposure of the HepG2 cells there was complete mortality with all samples of blooms at all concentrations evaluated. Therefore, it was not possible to detect DNA damage. However, balloons corresponding to cell apoptosis were found. Signs of apoptosis in cells exposed to microcystins have already been reported (Ma et al., 2017; Valério et al., 2014).

Frequently, people are exposed to low concentrations of mixtures of non-lethal substances for cells, but they may induce DNA damage, generating accumulation of mutations (Poirier and Beland, 1992). Thus, carcinogenesis may occur (Kakizoe, 2003) or epigenetic processes may be affected (Allis et al., 2015).

The highest cytotoxicity of sample **a** was observed for monkey kidney cells (Vero) and human promonocytic cells (U-937) with a LC_{50} of 39.7 ± 8.0 μg mL^{-1} and 41.2 ± 6.2 μg mL^{-1}, respectively, indicating the effects may occur in other tissues. Nevertheless, some authors have shown that prolonged exposure of rats to MC-LR leads to accumulation and causes serious damage to organs such as the spleen and affecting immunological mechanisms, oxidative stress, energy metabolism and cytoskeleton (Dong et al., 2012; Li et al., 2017). According to Huang et al. (2016), ratios of mass of free MC-LR in serum to the mass of MC-LR in a dose given to mice ranging from 3.1 to 25.0 μg kg^{-1} day^{-1} were 3.8% and 4.5%, while the total MC-LR ratios in liver was 34.4 and 38.5%. Although most studies indicate that microcystins accumulate mainly in the liver (Kotak et al., 1996; Soares et al., 2004; Wang et al., 2008), it has been shown that microcystins can cross the blood-brain barrier in animals and may induce cognitive dysfunction (Wang et al., 2013).

The anti-proliferative effect caused by modification of cell cycle progression instead of a cytotoxic effect, using flow cytometry and specific molecular markers, including caspases and annexin should be further studied.

Furthermore, the more polar fractions (**a** y **b**) obtained with 100% methanol were used for the cytotoxicity assays. It has also been reported that higher concentrations of microcystins could be obtained with polar solvents due to the properties of the molecule (Meriluoto and Spoof, 2008). Only these fractions were positive for microcystins by the Elisa test, detecting concentrations of 770 μg mL-1 and 742 μg mL-1, respectively. Accordingly, the cytotoxicity of fraction ***a*** was higher than subfraction (a-1). Some results with bacterial cells showed that purified cyanotoxins at a concentration of 10 μg mL^{-1} were not mutagenic or cytotoxic; however, toxic effects were detected after bacterial exposure to a mix of purified cyanotoxins (Sieroslawska, 2013). In contrast, subfractions ***b-1*** and ***b-2*** showed higher cytotoxic effect for U-937 cells than fraction ***b.*** As expected, the toxicity increased when the microcystin is further purified; in addition, there was a balance between accumulation and metabolism (Ito et al., 2002).

The discrepancies in toxicity levels between crude extracts of cyanobacteria and their fractions are probably a result of a possible degradation of sensitive molecules during elution with methanol and the consequent loss of biological effect (Da Silva et al., 2011; Sieroslawska, 2013). But it is also likely that the components of the crude extract have a toxic synergistic effect.

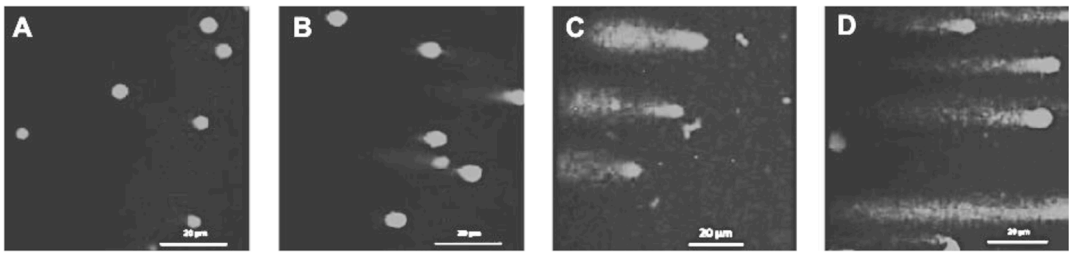


Fig. 7. DNA damage observed in HepG2 cells exposed to cyanobacterial bloom sample 2 (500 μg mL^{-1}) from Porce II reservoir (A and B: undamaged DNA; C: lightly damaged DNA; D moderately damaged DNA) at 24 h of exposure.

Table 3
LC_{50} of the fractions obtained from sample 1 for different cell lines.

Sample name	Product type	LC $_{50}$ (μg/mL) X ± SD			
		U-937	Vero	J774	Hela
a	Fraction	41.2 ± 6.2	39.7 ± 8.0	74.8 ± 6.6	109.5 ± 1.6
b	Fraction	> 200.0	> 200.0	> 200.0	> 200.0
a-1	Subfraction	84.8 ± 2.8	51.3 ± 6.3	128.2 ± 22.6	170.2 ± 15.5
b-1	Subfraction	29.7 ± 0.34	> 200.0	> 200.0	> 200.0
b-2	Subfraction	16.8 ± 2.0	> 200.0	> 200.0	> 200.0
Amphotericin B	Drug	35.9 ± 5.5	61.5 ± 4.0	12.7 ± 1.5	53.5 ± 0.1

5. Conclusions

The results of this study indicate that *Microcystis aeruginosa* blooms with a mixture of toxins, including MC-LR, can exert genotoxic effects in HepG2 hepatocytes. It should be highlighted that a continuous exposure to those kinds of materials or water containing them could have a long-term harmful effect due to the genotoxicity and cytotoxicity detected at 48 h of exposure.

Thus, cytotoxicity and genotoxicity assays are valuable methods to predict potential biological risks in waters contaminated with blooms of cyanobacteria, since chemical analysis and ELISA test can only take into account the presence of cyanotoxins, but not their biological effects. This approach is also very relatively realistic, because it correctly describes the situation in a bloom. Usually, the effects of the pure toxins are analyzed, but they are produced as a complex mixture of substances, each of which has a biological profile and a specific target tissue.

Conflict of interest

Empresas Públicas de Medellín co-financed this work but was not involved in the final decision about submitting this paper to publication.

Acknowledgments

This work was financed by COLCIENCIAS (Colombia), grant FP44842-049-2016 and Empresas Públicas de Medellín (Colombia), grant 29990832845; NH thanks to Drs. Wayne Carmichael and Linda Lawton for their contributions during this research.

References

Allis, D., Caparros, M.L., Jenuwein, T., R.D, 2015. In: EPIGENETICS, second ed. Cold Spring Harbor, New York, NY Cold Spring Harbor.

Azevedo, S.M.F.O., Carmichael, W.W., Jochimsen, E.M., Rinehart, K.L., Lau, S., Shaw, G.R., Eaglesham, G.K., 2002. Human intoxication by microcystins during renal dialysis treatment in Caruaru - Brazil. Toxicology 181–182, 441–446. https://doi.org/10.1016/S0300-483X(02)00491-2.

Cadrazco, M., Agudelo, J.R., Orozco, L.Y., Estrada, V., 2017. Genotoxicity of diesel particulate matter emitted by port-injection of hydrous ethanol and N-butanol. J. Energy Resour. Technol. 139, 1–10. https://doi.org/10.1115/ICEF2016-9409.

Carmichael, W.W., 1994. The toxins of cyanobacteria. Sci. Am. 270, 78–86. https://doi.org/10.1038/scientificamerican0194-78.

Carneiro, M., Antas, P., Reis, B., Azevedo, J., Osório, H., Campos, A., Vasconcelos, V., Martins, J.C., 2017. Modulation of hepatic glutathione transferases isoenzymes in three bivalve species exposed to purified microcystin-LR and Microcystis extracts. Toxicon 137, 150–157. https://doi.org/10.1016/j.toxicon.2017.07.003.

Collins, A.R., El Yamani, N., Lorenzo, Y., Shaposhnikov, S., Brunborg, G., Azqueta, A., 2014. Controlling variation in the comet assay. Front. Genet. 5, 1–7. https://doi.org/10.3389/fgene.2014.00359.

Da Silva, R.R.P., Pires, O.R., Grisolia, C.K., 2011. Genotoxicity in Oreochromis niloticus (Cichlidae) induced by Microcystis spp bloom extract containing microcystins. Toxicon 58, 259–264. https://doi.org/10.1016/j.toxicon.2011.06.005.

Dawson, R.M., 1998. The toxicology of microcystins. Toxicon 36, 953–962. https://doi.org/10.1016/S0041-0101(97)00102-5.

Dong, L., Lv, L.-B., Lai, R., 2012. Molecular cloning of Tupaia belangeri chinensis neuropeptide Y and homology comparison with other analogues from primates. Zool. Res. https://doi.org/10.3724/SP.J.1141.2012.01075.

Garcia Nieto, P.J., Sánchez Lasheras, F., de Cos Juez, F.J., Alonso Fernández, J.R., 2011. Study of cyanotoxins presence from experimental cyanobacteria concentrations using a new data mining methodology based on multivariate adaptive regression splines in Trasona reservoir (Northern Spain). J. Hazard. Mater. 195, 414–421. https://doi.org/10.1016/j.jhazmat.2011.08.061.

Heinze, R., 1999. Toxicity of the cyanobacterial toxin microcystin-LR to rats after 28 days intake with the drinking water. Environ. Toxicol. 14, 57–60. https://doi.org/10.1002/(SICI)1522-7278(199902)14:1<57::AID-TOX9>3.0.CO;2-J.

Hernández, J.M., López-Rodas, V., Costas, E., 2009. Microcystins from tap water could be a risk factor for liver and colorectal cancer: a risk intensified by global change. Med. Hypotheses 72, 539–540. https://doi.org/10.1016/j.mehy.2008.11.041.

Herrera, N., Palacio, J., Echeverri, F., Ferrão-Filho, A., 2014. Effects of a cyanobacterial bloom sample containing microcystin-LR on the ecophysiology of Daphnia similis. Toxicol. Rep. 1, 909–914. https://doi.org/10.1016/j.toxrep.2014.10.017.

Herrera, N.A., Echeverri, L.F., Ferrão-Filho, A.S., 2015. Effects of phytoplankton extracts containing the toxin microcystin-LR on the survival and reproduction of cladocerans. Toxicon 95, 38–45. https://doi.org/10.1016/j.toxicon.2014.12.016.

Heussner, A.H., Winter, I., Altaner, S., Kamp, L., Rubio, F., Dietrich, D.R., 2014. Comparison of two ELISA-based methods for the detection of microcystins in blood serum. Chem. Biol. Interact. 223, 10–17. https://doi.org/10.1016/j.cbi.2014.08.014.

Hillebrand, H., 1999. Biovolume calculation for palagic and benthic microalgae. J. Phycol. 424, 403–424.

Hu, C., Gan, N., He, Z., Song, L., 2008. A novel chemiluminescent immunoassay for microcystin (MC) detection based on gold nanoparticles label and its application to MC analysis in aquatic environmental samples. Int. J. Environ. Anal. Chem. 88, 267–277. https://doi.org/10.1080/03067310701657543.

Huang, H., Liu, C., Fu, X., Zhang, S., Xin, Y., Li, Y., Xue, L., Cheng, X., Zhang, H., 2016. Microcystin-LR induced apoptosis in rat sertoli cells via the mitochondrial caspase-dependent pathway: role of reactive oxygen species. Front. Physiol. 7, 1–10. https://doi.org/10.3389/fphys.2016.00397.

Ito, E., Takai, A., Kondo, F., Masui, H., Imanishi, S., Harada, K.I., 2002. Comparison of protein phosphatase inhibitory activity and apparent toxicity of microcystins and related compounds. Toxicon 40, 1017–1025. https://doi.org/10.1016/S0041-0101(02)00099-5.

Kakizoe, T., 2003. Chemoprevention of cancer - focusing on clinical trials. Jpn. J. Clin. Oncol. 33, 421–442. https://doi.org/10.1093/jjco/hyg090.

Karlsson, H.L., 2010. The comet assay in nanotoxicology research. Anal. Bioanal. Chem. 398, 651–666. https://doi.org/10.1007/s00216-010-3977-0.

Kotak, B.G., Semalulu, S., Fritz, D.L., Prepas, E.E., Hrudey, S.E., Coppock, R.W., 1996. Hepatic and renal pathology of intraperitoneally administered microcystin-LR in rainbow trout (*Oncorhynchus mykiss*). Toxicon 34, 517–525. https://doi.org/10.1016/0041-0101(96)00009-8.

Li, X., Xu, L., Zhou, W., Zhao, Q., Wang, Y., 2016. Chronic exposure to microcystin-LR affected mitochondrial DNA maintenance and caused pathological changes of lung tissue in mice. Environ. Pollut. 210, 48–56. https://doi.org/10.1016/j.envpol.2015.12.001.

Li, X., Zhang, X., Xie, W., Zhou, C., Li, Y., Zhang, X., 2017. Alterations in transcription and protein expressions of HCC-related genes in HepG2 cells caused by microcystin-LR. Toxicol. Vitro 40, 115–123. https://doi.org/10.1016/j.tiv.2016.12.016.

Liu, W., Qiao, Q., Chen, Y., Wu, K., Zhang, X., 2014. Microcystin-LR exposure to adult zebrafish (Danio rerio) leads to growth inhibition and immune dysfunction in F1 offspring, a parental transmission effect of toxicity. Aquat. Toxicol. 155, 360–367. https://doi.org/10.1016/j.aquatox.2014.07.011.

Ma, J., Feng, Y., Jiang, S., Li, X., 2017. Altered cellular metabolism of HepG2 cells caused by microcystin-LR. Environ. Pollut. 225, 610–619. https://doi.org/10.1016/j.envpol.2017.03.029.

Ma, J., Li, Y., Wu, M., Li, X., 2018. Oxidative stress-mediated p53/p21WAF1/CIP1pathway may be involved in microcystin-LR-induced cytotoxicity in HepG2 cells. Chemosphere 194, 773–783. https://doi.org/10.1016/j.chemosphere.2017.12.051.

Mankiewicz, J., Walter, Z., Tarczynska, M., Palyvoda, O., Wojtysiak-Staniaszczyk, M., Zalewski, M., 2002. Genotoxicity of cyanobacterial extracts containing microcystins from polish water reservoirs as determined by SOS chromotest and comet assay. Environ. Toxicol. 17, 341–350. https://doi.org/10.1002/tox.10061.

Mena-Huertas, J., Embus-Córdoba, C., Rosero-Ruiz, V.L., Navarro-Yépez, J., Ortiz-Trujillo, I.C., Yépez-Chamorro, M.C., Resumen, 2011. Ausencia de efecto citotóxico, mutagénico y genotoxicode extracto acuoso y aceite esencial de Carica candamarcensis hook (Plantae:Caricacease). Actual. Biol. 33, 153–164.

Meriluoto, J. a O., Spoof, L.E.M., 2008. Cyanotoxins: sampling, sample processing and toxin uptake. Adv. Exp. Med. Biol. 619, 483–499. https://doi.org/10.1007/978-0-387-75865-7_21.

Milutinović, A., Živin, M., Zorc-Pleskovič, R., Sedmak, B., Šuput, D., 2003. Nephrotoxic effects of chronic administration of microcystins -LR and -YR. Toxicon 42, 281–288. https://doi.org/10.1016/S0041-0101(03)00143-0.

Platel, A., Nesslany, F., Gervais, V., Marzin, D., 2009. Study of oxidative DNA damage in TK6 human lymphoblastoid cells by use of the in vitro micronucleus test: determination of no-observed-effect levels. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 678, 30–37. https://doi.org/10.1016/j.mrgentox.2009.06.006.

Poirier, M.C., Beland, F.A., 1992. DNA adduct measurements and tumor incidence during chronic carcinogen exposure in animal models: implications for DNA adduct-based human cancer risk assessment. Chem. Res. Toxicol. 5, 749–755. https://doi.org/10.1021/tx00030a003.

Quesada, A., Sanchis, D., Carrasco, D., 2004. Cyanobacteria in Spanish reservoirs. How frequently are they toxic? Limnética 23, 109–118.

Reynolds, C.S., 2006. In: The Ecology of Phytoplankton, Cambridge. Cambridge University Press, Cambridge. https://doi.org/10.1017/CBO9780511542145.

Rodriguez Ferreiro, G., Cancino Badóas, L., Lopez-Nigro, M., Palermo, A., Mudry, M., González Elio, P., Carballo, M.A., 2002. DNA single strand breaks in peripheral blood lymphocytes induced by three nitroimidazole derivatives. Toxicol. Lett. 132, 109–115. https://doi.org/10.1016/S0378-4274(02)00039-5.

Rzóska, J., Margalef, R., 1979. Prácticas de Ecología. In: Ediciones Omega, S. (Ed.), Ecología, pp. 1100–1102 Barcelona España.

Saraf, S.R., Frenkel, A., Harke, M.J., Jankowiak, J.G., Gobler, C.J., McElroy, A.E., 2018. Effects of Microcystis on development of early life stage Japanese medaka (Oryzias latipes): comparative toxicity of natural blooms, cultured Microcystis and microcystin-LR. Aquat. Toxicol. 194, 18–26. https://doi.org/10.1016/j.aquatox.2017.10.026.

Senthilraja, P., Kathiresan, K., 2015. In vitro cytotoxicity MTT assay in vero, HepG2 and MCF-7 cell lines study of marine yeast. J. Appl. Pharm. Sci. 5, 80–84. https://doi.org/10.7324/JAPS.2015.50313.

Sieroslawska, A., 2013. Assessment of the mutagenic potential of cyanobacterial extracts and pure cyanotoxins. Toxicon 74, 76–82. https://doi.org/10.1016/j.toxicon.2013.07.029.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191. https://doi.org/10.1016/0014-4827(88)90265-0.

Sivonen, K., Jones, G.J., 1999. Cyanobacterial toxins. Toxic Cyanobacteria Water 41–111. https://doi.org/10.1016/B978-012373944-5.00005-5.

Soares, R.M., Magalhães, V.F., Azevedo, S.M.F.O., 2004. Accumulation and depuration of microcystins (cyanobacteria hepatotoxins) in Tilapia rendalli (Cichlidae) under laboratory conditions. Aquat. Toxicol. 70, 1–10. https://doi.org/10.1016/j.aquatox.2004.06.013.

Song, C.L., Zhou, Y.C., Huang, R.J., Wang, Y.Q., Huang, Q.F., Lü, G., Liu, K.M., 2007. Influence of ethanol-diesel blended fuels on diesel exhaust emissions and mutagenic and genotoxic activities of particulate extracts. J. Hazard. Mater. 149, 355–363. https://doi.org/10.1016/j.jhazmat.2007.03.088.

Switalla, S., Knebel, J., Ritter, D., Dasenbrock, C., Krug, N., Braun, A., Sewald, K., 2013. Determination of genotoxicity by the Comet assay applied to murine precision-cut lung slices. Toxicol. Vitro 27, 798–803. https://doi.org/10.1016/j.tiv.2012.12.015.

Taylor-Harding, B., Orsulic, S., Karlan, B.Y., Li, A.J., 2010. Fluvastatin and cisplatin demonstrate synergistic cytotoxicity in epithelial ovarian cancer cells. Gynecol. Oncol. 119, 549–556. https://doi.org/10.1016/j.ygyno.2010.08.017.

Valentin-Severin, I., Le Hegarat, L., Lhuguenot, J.C., Le Bon, A.M., Chagnon, M.C., 2003. Use of HepG2 cell line for direct or indirect mutagens screening: comparative investigation between comet and micronucleus assays. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 536, 79–90. https://doi.org/10.1016/S1383-5718(03)00031-7.

Valério, E., Vilares, A., Campos, A., Pereira, P., Vasconcelos, V., 2014. Effects of microcystin-LR on saccharomyces cerevisiae growth, oxidative stress and apoptosis. Toxicon 90, 191–198. https://doi.org/10.1016/j.toxicon.2014.08.059.

Wang, J., Lin, F., Cai, F., Yan, W., Zhou, Q., Xie, L., 2013. Microcystin-LR inhibited hippocampal long-term potential via regulation of the glycogen synthase kinase-3β pathway. Chemosphere. https://doi.org/10.1016/j.chemosphere.2013.04.069.

Wang, Q., Xie, P., Chen, J., Liang, G., 2008. Distribution of microcystins in various organs (heart, liver, intestine, gonad, brain, kidney and lung) of Wistar rat via intravenous injection. Toxicon 52, 721–727. https://doi.org/10.1016/j.toxicon.2008.08.004.

Žegura, B., Lah, T.T., Filipič, M., 2006. Alteration of intracellular GSH levels and its role in microcystin-LR-induced DNA damage in human hepatoma HepG2 cells. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 611, 25–33. https://doi.org/10.1016/j.mrgentox.2006.06.038.