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Biomonitoring of Microcystin and Aflatoxin Co-Occurrence in Aquaculture Using Immunohistochemistry and Genotoxicity Assays

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ABSTRACT

*This work investigated the effects of co-occurring aflatoxin B_1 (AFB_1) and microcystin (MC) in aquaculture, using immunohistochemistry and genotoxicity methods. Tilapia (*Oreochromis niloticus*) were exposed to AFB_1 by intraperitoneal and MC (cell extract of* Microcystis aeruginosa*) by intraperitoneal and immersion routes. The interaction of MC-AFB_1 was evaluated co-exposing the intraperitoneal doses. Blood samples were collected after 8, 24, and 48h to analyze the micronucleus frequency and comet score. The interaction of MC-AFB_1 showed a synergic mutagenic response by higher micronucleus frequency of co-exposed group. A slight genotoxic synergism was also observed in the comet score. Immunohistochemistry detected MC in al lthe fish liver tissues exposed to MC by intraperitoneal route, and only the immersed group with the highest dose of MC showed a positive response. Although MC was non-detectable in the edible muscle, the combination of immunohistochemistry with genotoxicity assay was an attractive biomonitoring tool in aquaculture, where the animals were frequently exposed to co-occurring synergic hazards.*

Key words: *Microcystis aeruginosa*, mycotoxin and tilapia

INTRODUCTION

Microcystins are hepatotoxic cyclic heptapeptides produced by the phytoplanktonic communities of cyanobacteria blooms in eutrophic fresh water (Carmichael 2001). Microcystin-LR (MCLR) is one of the most toxic in the group, where a single intraperitoneal dose (ip) can cause hemorrhagic and coagulative necrosis and emboli in the liver. MCLR inhibits the activity of protein phosphatase by covalent binding, and leads to hyperphosphorylation of cytoskeletal proteins, rearrangement of intermediate acting filaments, and microtubules with alteration of cell structure. The rat's liver loses its architecture and develops severe internal injuries. The loss of contact

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between the cells creates internal spaces that are filled by the blood that flows to those locations of the capillaries, causing severe bleeding intrahepatic (Hooser et al. 1991).

Aquaculture is a rapidly developing in the Brazilian agribusiness, where inadequate farming management has generated a chemical balance favorable for the cyanobacteria proliferation, which has resulted cyanotoxins exposure for the fishes. The hazard associated with the intake of such fish meat is unknown. The monitoring of fishponds in the Northern Parana state, Brazil from October 2002 to October 2003 showed eutrophication with cyanobacteria bloom, wherein microcystin was detected in 95% of the water samples at low concentrations (mean of 0.275µg/L) by ELISA assay (Envirologix Inc., ME, USA). Transient peak times with levels >1µg/L, i.e., above the guideline for the drinking water recommended by the World Health Organization (WHO) (1998), occurred in December 2002 and January 2003 (Kamogae et al. 2006).

Further risk to fish ponds can result by using mycotoxin-susceptible grain as a main ingredient in fish feeds. Aflatoxin B_1 (AFB_1) has been a topic of concern, and has been classified as a group 1 carcinogen (International Agency for Research on Cancer 1993). Previous surveys of 42 commercial fish feeds in the Northern Parana state detected aflatoxin concentrations from 0 to 15.6 µg/kg, with 62% showing <4 µg/kg levels (Hashimoto et al. 2003). The Brazilian aflatoxin guideline is 20 µg/kg (ANVISA - Brazilian Sanitary Vigilance Agency 2002).

Aquaculture is a common commercial agribusiness in the tropical countries, wherein the same climatic conditions favor the fungal growth and mycotoxin production. Inadequate management due to overfeeding exposes fish simultaneously to the carcinogen aflatoxin (Eaton and Groopman 1994) and the tumor promoting microcystin (Nishiwaki-Matsushima et al. 2002). In order to assess the possible combined synergistic effect of co-occurring toxins, micronucleus and comet assays could be useful tools to detect the genotoxic status in nucleated fish erythrocytes. Micronuclei result from lesions/adducts at the DNA or chromosomal level, or at the level of proteins directly or indirectly involved in chromosome segregation. The formation of micronuclei originating from the chromosome fragments or chromosome loss-events requires a mitotic or meiotic division (Kirsch-Volders et al. 2003). Micronuclei have been scored in fish erythrocyte as a measure of clastogenic activity, i.e., formation of condensed small membrane-involved chromosomal fragments that are unable to migrate, following the mitotic spindle (Al-Sabit and Metcalfe 1995). The comet assay, also called as single-cell gel electrophoresis, measures the broken DNA strand removed from the nucleus (Devaux et al. 1997). These genotoxicity assays, in combination with the direct detection of microcystin in defined tissue components by immunohistochemistry (Gimeno et al. 1997) can demonstrate the real significance of cumulative hazards in the food chain. Thus, in this work, microcystin bioaccumulation was evaluated by immunohistochemistry, and microcystin-AFB_1 synergism was monitored by the micronucleus and comet assay in Nile Tilapia (*Oreochromis niloticus*).

MATERIAL AND METHODS

Toxins, reagents and *M. aeruginosa* crude cell extract

Aflatoxin (AFB_1, 1mg), purchased from Sigma-USA (98% purity), was dissolved in methanol and divided into vials containing 100 µg each. The AFB_1 concentration in methanol was verified using UV-VIS with an excitation wavelength of 360 nm (extinction coefficient of 21,800, UV-VIS Cintra 20, GMB, Melborne, Australia), and thin layer chromatography (Soares and Rodrigues-Amaya, 1989). The working AFB_1 solution was diluted in sterile oil to a final concentration of 25 µg/mL.

Microcystin standards were MCLR and 7desmethyl-MCLR (7D-MCLR) obtained from the lyophilized cyanobacterial cells collected from the Laguna Bay, Philippines and Lake Suwa, Japan, respectively. These toxins were purified (>95% of purity, analyzed by the LC/IT-MS^2, liquid chromatography/ion trap mass spectrometer, Agilent 1100 HPLC system, Palo Alto, CA, USA) in the Laboratory of Environmental Science of Meijo University Japan. The peak of MCs was detected at 238 nm and MS/MS fragmentation the patterns of MCs were compared to identify the detected peak, according to Harada et al. (1996) and Mayumi et al. (2006).

M. aeruginosa BCCUSP strain 262 (Brazilian Cyanobacteria Collection of the University of Sao

Paulo) was isolated from the Garças Lagoon, Sao Paulo, Brazil, located in a region with intensive aquaculture. The strain BCCUSP 262 was characterized as a microcystin high-producer and then this strain was selected for the experiment. The strain was cultivated in a BG-11 medium (Rippka et al. 1979) at 21 to 24 ^{o}C in a 14:10 h light:dark photoperiod (irradiance 30-40 µmol/m^2/s) for 2 to 3 months. The cells density in the Fuchs-Rosental chamber was recorded in hexaplicate (Nikon E200, Melville, NY, USA), and centrifuged. Crude cell extract (MC) was obtained by disrupting the cells using eight cycles of freeze-thawing and a 10 min sonication process, and then lyophilized. The microcystin profile of this material was analyzed by the LC/IT-MS^2 (liquid chromatography/ ion trap mass spectrometer, Agilent 1100 HPLC system, Palo Alto, CA, USA). One gram samples were extracted with 5% acetic acid in the water, centrifuged (4000 x g/30 min), filtered (GF/C), clean-upped (ODS cartridge), and stored as a dried residue at 4^{o}C for further analysis (Sivonen and Jones 1999). The LC/IT-MS^2 analysis was performed with 5 µL of sample in a TSK-gel Super-ODS column (2 µm, 2x100 mm, TOSOH, Tokyo, Japan) at 40^{o}C. The mobile phase consisted of water with 0.1 % formic acid (A) and methanol with 0.1 % formic acid (B) under a gradient elution from 10 % to 90 % B for 30 min at a flow rate of 200 µL/min. MS analysis was performed using a Finnigan LCQ Deca XP plus IT mass spectrometer equipped with an ESI interface (Thermo Fisher Scientific, San Jose, CA, USA). Scan ranges were selected according to the molecular *m/z* of analytes (Harada et al. 1996; Mayumi et al. 2006).

Exposure protocol

Juvenile Nile Tilapia (*O. niloticus*) weighing 40 to 60 g (N = 96) were cultivated in the Fish Hatchery Station at the State University of Londrina, Brazil. The fishes were acclimatized in well-aerated water at 25 ± 1^{o}C in 600 L tanks for 2 weeks. Daily feeding was carried out using an AFB_1 negative pellet feed (<2µg/kg by TLC, Soares and Rodrigues-Amaya, 1989), which was withdrawn 24 h before the experiment.

The fishes were exposed to a *M. aeruginosa* BCCUSP 262 cell extract (MC) *via* two routes: intraperitoneal (ip) injection and immersion in a crude cell-extract containing tank. The aflatoxin (AFB_1) was ip-injected with a single dose at a concentration of 10 µg/kg. The ip exposures was carried out in the groups divided into eight tanks, with six fishes each, and designed (i) ipMC: three groups injected with the single doses of ipMC at $2.0x10^5$ $^{(MC1)}$, $4.0x10^5$ $^{(MC2)}$ and $1.0x10^6$ cells/kg $^{(MC3)}$. (ii) ipAFB1+ipMC: three groups injected with ipAFB_1, and after 2 h with ipMC, i.e., AFB_1 + $2.0x10^5$ cells/kg $^{(AFB1+MC1)}$; AFB_1 + $4.0x10^5$ cells/kg $^{(AFB1+MC2)}$ and AFB_1 + $1.0x10^6$ cells/kg $^{(AFB1+MC3)}$. The AFB_1 control comprised six fishes with ipAFB_1, while negative control comprised six fishes ip injected with sterile mineral oil. (iii) i-MC: immersion groups were exposed to MC, resulting in four experimental groups. Fishes were immersed in the tanks A and B with increasing concentrations of MC for 72 h for each exposure, replacing the water immediately after sampling the fishes. Fishes in the tank A (n = 18) were exposed at $1x10^4$ cells/mL, and six fishes were sampled at 72 h $^{(i-MC1)}$; after two months, 12 fishes were exposed at $2x10^4$ cells/mL for 72h and six fishes were sampled $^{(i-MC3)}$. After two months, the last six fishes were exposed at $5x10^4$ cells/mL for 72h $^{(i-MC4)}$. Fishes in the tank B (n = 6) were exposed once at $1x10^5$ cells/mL for 72h $^{(i-MC2)}$. Negative control was carried out in the tank C (18 fish).

Genotoxicity tests were carried outusing a micronucleus and comet assays in ipAFB_1, ipMC and ipAFB_1+MC interaction assay, collecting the blood after 8, 24 and 48 h of exposure. The immunohistochemistry was carried out after 72 h in the liver and muscle of the fishes submitted to ip and immersion in *M. aeruginosa* cell extract $^{(MCs)}$ assays.

Immunohistochemical assay

The M8H5 monoclonal antibody (mAb) anti-microcystin was used as the primary antibody. A microcystin immunohistochemical assay was performed with paraffin-embedded tissues (5 µm, liver and muscle) adhered onto 5% polyvinyl acetate-coated glass slides, deparaffinized in xylene, and hydrated in an ethanol solution (80^{o}GL). Endogenous peroxidase was denatured with 3 % H_2O_2 in methanol for 5 min, and washed with PBS solution (pH 7.2). M8H5 mAb diluted in PBS (1:10, 1:100 and 1:1000) was added, and after incubation for 30 min, unbounded antibodies were removed by washing sections in PBS. The slides were then incubated at 4^{o}C with a secondary antibody, comparing two peroxidase systems: avidin-biotin peroxidase (ABPx) overnight and polymeric peroxidase (polymer-Px) system after

30 min (Dako Envision System, Cambridgeshire, UK). After washing with PBS, the slides were incubated with dihydrate 3,3'-diaminobenzidine tetrahydrochloride (DAB-substrate system, Sigma, St. Louis, USA) and an H2O2 solution for 3 min. The slides were washed with distilled water, counterstained with Mayer hematoxylin, dehydrated in ethanol and were applied with cover slips in an aqueous mounting medium for microscopy (EWS 2100 capture software - Leica Microscopy, Wetzlar, German).

Genotoxicity tests

Two slides were prepared with fine blood smears from each fish exposed to AFB1, *M. aeruginosa* extract(MCs) or AFB1+MC interaction assay and after 24 h, were fixed with absolute methanol prior to Giemsa staining (1:20 in phosphate buffer pH 6.8, 4.0827 g KH_2PO_4 and 7.099 g Na_2HPO_4 in 1L of H_2O). Micronucleus frequency (‰) was examined in 2,000 erythrocytes per fish, following the scoring criteria based essentially on micronuclei diameter which was in accordance with criteria described by Matsumoto and Cólus (2000). Comet assay was performed as described by Singh et al. (1988) and modified by Speit and Hartmann (1999). A 10 µL blood sample (1:100 in 7.4 g NaCl, 0.36 g KCl, 0.17 g $CaCl_2$, 0.31 g $NaHCO_3$, 1.6 g Na_2HPO_4, 0.4 g NaH_2PO_4 in 1L of H_2O) was mixed with 120 µL of 0.5 % LMP-agarose in PBS, spread onto a slide previously coated with normal agarose, and conditioned at 4^oC for 10 min. After cell lyses (60 min at 4^oC in lyses solution), the slides were immersed in 10 M NaOH with 200 mM EDTA, pH>13. The DNA was denatured for 20 min, and nuclei electrophoresis was carried out at 300 mA and 25 V (~1.0 V/cm). The slides were then neutralized in a Tris buffer, fixed in ethanol for 10 min, and stained with ethidium bromide (200 µg/mL). The fluorescence microscopy analysis was carried out in 100 cells per fish (Nikon EX 420-490, BA 520, Melville, NY, USA with excitation filter of 515 to 560 nm and a barrier filter of 590 nm). On each slide, the cells were scored visually and the nucleus was classified in four classes, 0 for undamaged to 3 for extensively-damaged according the DNA migration, and the score was calculated by multiplying the number of cells in each class by the damage class (Speit, Hartmann 1999).

Statistical Analysis

The frequency of the MN and comet data from *M. aeruginosa* cell extract (MCs), AFB1+MC interaction assays, and the negative control group were compared by one way analysis of variance (ANOVA), followed by the Tukey test for multiple comparison of averages (p=0.05) using System for Statistical Analysis (Statistica 9.0, Statsoft, Tulsa, Oklahoma, USA)

RESULTS AND DISCUSSION

The *M. aeruginosa* strain BCCUSP 262 produced 1,054.75 and 8.65 µg/g of 7D-MCLR and MCLW per lyophilized biomass, respectively. The main microcystin detected in the cell extracts was 7D-MCLR ([M+H]+, m/z 981) by LC-ITMS. The ip doses at $2x10^5$, $4x10^5$, and $1x10^6$ cells/kg corresponded to 0.602, 1.204, and 3.011 µg/kg of 7D-MCLR, respectively. Immersion doses at $1x10^4$, $2x10^4$, $5x10^4$ and $1x10^5$ cells/mL corresponded to 30.1, 60.2, 150.5, and 301.0 µg/L of 7D-MCLR, respectively. The higher immersion exposure ($1x10^5$ cells/mL) simulated the alert level 2 according to Alert Framework of Guidance in Drinking Water (WHO 1998). However, the desmethylation of Mdha residues in MCLR (7D-MCLR) decreased the toxicity to intermediate levels in mice (Harada et al. 1991).

The liver and kidneys are the primary targets of microcystin in fish (Fischer and Dietrich 2000). Considering that fish erythropoiesis takes place in the kidney, the peripheral blood has been proved to be adequate in biomonitoring purpose (Palhares and Grisolia 2002). Therefore genotoxicity was evaluated in the tilapia's blood (Fig. 1). Immunohistochemistry was developed (Table 1) and carried out in the liver and the edible portion of fish muscle (Table 2). The effectiveness of the avidine-biotine-peroxidase versus the polymeric-peroxidase system in immunohistochemistry was compared using an anti-microcystin M8H5 mAb at 1:10 to 1:1000 dilutions (Table 1). The Polymeric-peroxidase system showed higher performance for the demarcation of microcystin in the fish liver using M8H5 mAb diluted to 1:10 and 1:100. This demarcation system also reduced the 24 h analysis time to 12 h, when compared with the avidine-biotine-peroxidade system. Thus, the polymeric-peroxidase system with mAb diluted to 1:100 was used in the immunohistochemical assay (Table 2).

The enhanced signal response in this technology was reached using an enzyme-labeled inert spine molecule of dextran attached with approximately 70 enzymes and 10 mAbs (Dako Envision Systems, Cambridgeshire, UK).

Table 1 - Immunohistochemistry: comparison of microcystin detection using avidin-biotin peroxidase and polymer peroxidase systems in liver tissue of ip exposed Nile Tilapia to crude cell extract of *M. aeruginosa* BCCUSP 262

Immunohistochemical System	mAb* (dilution)	Microcystin**
Avidin-Biotin peroxidase	1:1000	(-)
	1:100	(-)
	1:10	(-)
Polymer peroxidase	1:1000	(-)
	1:100	(+)
	1:10	(+)

*: M8H5 monoclonal antibody was used; **: Positive (+) and negative (-) reactions to microcystin immune-staining.

In the experiment, a *M. aeruginosa* crude cell extract was used, permitting other cell components such as lipopolysaccharide (LPS) to be involved in cell toxicity (Table 2, Fig. 1). LPS indirectly activates the renin angiotensin system, increasing the microcystin uptake in immersion assay, as well as reducing the glutathione S transferase activity involved in detoxification (Best et al. 2003). No fish death was observed in the ip or immersion group; however erratic swimming was observed in the group treated with a higher ipMC dose.

Circulation is the major portal of entry to the body, which also exposes the blood cells to high concentration of chemicals. Figure 1B displays the genotoxic effects in erythrocytes of all the treatments ($p<0.05$). However, class 0, representing the undamaged nuclei, predominated in all the groups, while the frequency of class 3 was higher in ipMC group ($1x10^6$ cells/kg, 3.011 μg/kg of 7D-MCLR), increasing the comet score independently for AFB_1 (10 μg/kg) introduction (data not shown). The AFB_1 dose fixed to half the concentration of the Brazilian guideline (ANVISA, 2002) was based on a previous aflatoxin survey of 42 feed aquaculture samples collected from the Northern Parana state, Brazil (Hashimoto et al. 2003).

Concerning the comet assay, all the treatments showed comet score higher than the negative control group. In the micronucleus test, AFB1 treatment (8h), i-MC (8, 24 and 48h), MC1 (24h) and MC2 (8h) had not different frequency of negative control micronuclei ($p <0.05$).

Table 2 - Immunohistochemistry of microcystin in liver and muscle tissue of Nile Tilapia exposed to crude cell extract of *M. aeruginosa* BCCUSP 262 (^{ip}injection and immersion assay).

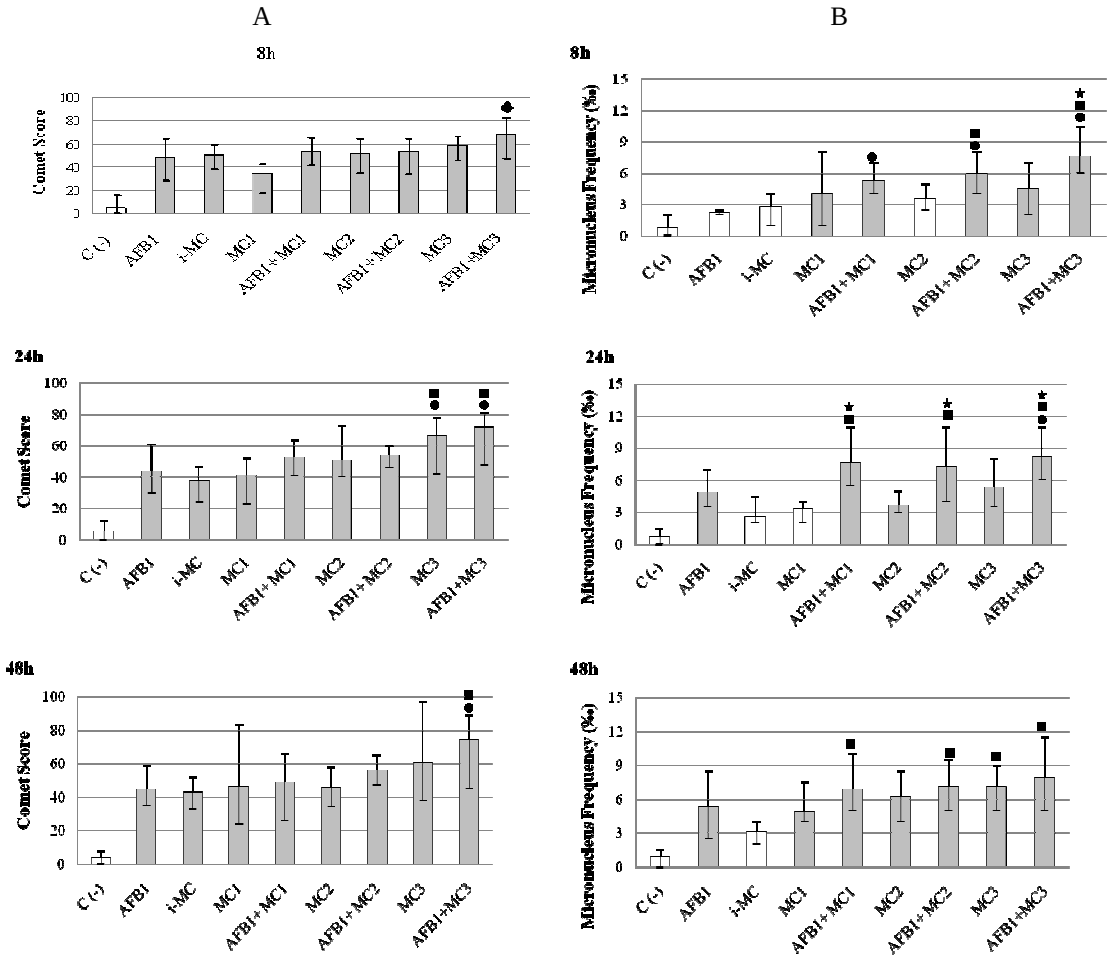
M. aeruginosa (exposure)		Microcystin		
		7desmethyl-MCLR* (cell extract of *M. aeruginosa*)	Immunohistochemistry**	
			liver	muscle
^{ip}injection	Dose (cells/kg)	(μg/kg)		
MC1	$2.0x10^5$	0.602	(+)	(-)
MC2	$4.0x10^5$	1.204	(+)	(-)
MC3	$1.0x10^6$	3.011	(+)	(-)
Immersion	Conc. in water (cells/mL)	(μg/L)		
i-MC1	$1.0x10^4$	30	(-)	(-)
i-MC2	$1.0x10^5$	300	(+)	(-)
i-MC3	$1.0x10^4 + 2.0x10^4$	30 + 60	(-)	(-)
i-MC4	$1.0x10^4 + 2.0x10^4 + 5.0x10^4$	30 + 60 + 150	(-)	(-)

* 7desmethyl-MCLR in crude cell extract of *M. aeruginosa* BCCUSP 262 was analyzed by LC/IT-MS^2.

** Positive (+) and negative (-) reactions to microcystin immune-staining. A polymer peroxidase system with M8H5 monoclonal antibody was used for microcystin detection in tilapia tissues.

^{ip} injection: crude cell extract of *M. aeruginosa* injected at MC1, MC2 and MC3 doses.

Immersion: tilapia immersed in water with increasing concentration / re-exposure of crude cell extract of *M. aeruginosa*.



Treatments: C (-) :
Negative Control; AFB1;
AFB_1 10µg/Kg (i.p.);
i-MC: immersed in $1x10^5$ cells/mL of *M. aeruginosa*;
MC1: 2.0×10^5 cells/Kg (i.p.) of *M. aeruginosa*
MC2: 4.0×10^5 cells/Kg (i.p.) of *M. aeruginosa*;
MC3: 1.0×10^6 cells/Kg (i.p.) of *M. aeruginosa*.

Response: Tukey Test ($p = 0.05$)
grey color column > than negative control (white)
◆ > than MC1 group
● > than AFB1 10µg/Kg (i.p.)
■ > than immersed group in 1x105 cells/mL of *M. aeruginosa*
★ > than the correspondent MC dose, due to AFB1 additional effect

Figure 1 - (A) Comet score and (B) Micronucleus frequency (‰) in Nile Tilapia erythrocytes exposed to AFB_1, crude cell extract of *M. aeruginosa* BCCUSP262 and AFB_1+MC combination.

For the evaluation of the treatments, followings were compared: exposure form (■) by immersion exposure with i.p. (i-MC x MC1, MC2 and MC3), increasing doses i.p. in MC1 x MC2 x MC3 (◆); the additional effect of MC (●) on AFB1 (AFB_1 x AFB_1 + MC) and the additive effect of AFB_1 (★) in MC (MC1x AFB_1 + MC1, MC2 x AFB_1 + MC2 and MC3 x AFB_1 + MC3). Comparing ip AFB_1 with non-ipAFB_1 data (MC versus AFB_1+MC), the AFB_1 increased the mutagenicity in the highest MC dose at 8h and in the all MC doses at 24 h ($2x10^5$ to $1x10^6$ cells/kg) (Fig. 1B, data ★ $p<0.05$).

Concerning the AFB_1, no difference was detected in the MN frequency at the 8 h treatment, but a high MN frequency occurred in all the treatments after 24 h, when compared with the negative control ($p<0.05$, Fig. 1B). Such a delay could be related with the variable behavior of *O. niloticus* against genotoxic agents (Palhares and Grisolia 2002), as well as the maximum MN induction that normally occurred at 1 to 5 days post-exposure (Al-Sabti and Metcalfe 1995). The MN showed chromosome fragmentation or whole chromosomes loss during the anaphase, hence the

MN frequency depended on the kinetic of cell cycle in a population (Al-Sabti and Metcalfe, 1995). The addition of ipMC ($1x10^6$ cells/kg) dose increased the AFB_1 comet score at 24 and 48h, as well AFB_1 MN frequency was increased at 8h with all ipMC doses ($2x10^5$ to $1x10^6$ cells/kg) and the highest dose at 24h (Fig. 1, .data ● $p<0.05$).

The MC dose-response was non-significant ($p>0.05$) in MN and only at 8h, comet score of the group i.p. exposed to MC3 ($1x10^6$ cells/kg) was higher than the MC1 ($2x10^5$ cells/kg) group (Fig. 1A, data ◆ $p<0.05$). Microcystin is a potent tumor promoter (Nishiwaki-Matsushima et al. 1992), which can also act as tumor initiator (Ito et al. 1997), with increased evidence of genotoxicity. A high microcystin dose has been demonstrated to be involved in clastogenic chromosome breakage (Repavich et al. 1990).

Comparing the immersion and i.p. MC exposition, a significant difference (Fig. 1A, data ■$p>0.05$) was found between the fishes immersed at $1x10^5$ cells/mL (*alert level 2* of Alert Levels Framework, WHO Guideline, 1993) and the highest dose of the MC ($1x10^6$ cells/kg) in the comet score at 24h and MN at 48h. Immersion concentration was genotoxic with an ip dose at 0.602-1.204 μg/kg. However, the spontaneous MN frequency (negative control) did not differ from the MN frequency in the fishes immersed at $1x10^5$ cells/mL with 300 μg/L 7D-MCLR ($p>0.05$). Comparing the comet score and the MN frequency in all the treatments (Fig. 1A and 1B), the comet assay was more sensitive than the MN test for this dose-concentration range used (Fig. 1 and 2). Lankoff et al. (2004) reported a negative correlation between the apoptotic cell frequency and the level of DNA-damage. That is, MCLR-induced DNA damage in the comet assays may indicate an earlier stage of apoptosis, which triggers cytotoxicity without genotoxicity.

In summary, the reversible capacity of the alkaline version of the comet assay permitted DNA repair kinetics to be measured, while an impaired repair of DNA damage was detected by a micronucleus assay, i.e., mutagenicity (Lankoff et al. 2004). Taking into account the genotoxic effects on blood cells, the bioaccumulation profile was evaluated for the irrigated tissues. Liver tissue, and not the muscle tissue, was microcystin-positive by polymeric-peroxidase immunohistochemistry, suggesting target specificity of this toxin (Table 2). The unique organotropy of microcystin in the liver was reported for both ip and oral administration in the mice and rat, with an immediate massive intra-hepatic hemorrhage and cellular necrosis (Tencalla and Dietrich 1997). Residual microcystin in the muscle tissue was observed in the fishes collected in the highly contaminated Jacarepaguá Lagoon, Rio de Janeiro, Brazil (Magalhães et al. 2003).

All ipMC fishes showed microcystin marking in their liver, contrasting with the findings from the immersion test, where only higher dose was positive ($1.0x10^5$ cells/mL, 300 μg/L of 7D-MCLR). Microcystin was non-detectable in the muscle and liver of immersed animals in $<1.0x10^5$ cells/mL (Table 2). Gill and skin epithelia in the freshwater fishes hinder microcystin entrance, making oral ingestion the primary route of microcystin uptake. Fishes immersed in a dense culture of toxigenic *M. aeruginosa* remained unaffected for several days, but ip injection of same strain with purified microcystin caused lethal liver and kidney damage within hours (Carbis et al. 1996).

Immunohistochemical analyses performed with the M8H5-mAb detected MCLR in the cytoplasm and some nuclei of fishes hepatocytes (Table 2). This suggested that the modulation of nucleolar phosphatase affected the cellular growth and differentiation (Yoshida et al. 1998). If the microcystin-response targets in the nuclear DNA of AFB_1-iniatiated hepatic cells possess a high affinity for microcystins, the combined effect of these toxins can be expected for tumor promotion (Sekijima et al. 1999).

Immunohistochemistry is an attractive, direct method for detecting the toxic residue contamination *in situ*, which, combined with genotoxicity tests, can fulfill the exigent requirement in quality control intended for the profitable biomonitoring in aquaculture.

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