Genotoxicity in *Oreochromis niloticus* (Cichlidae) induced by *Microcystis* spp bloom extract containing microcystins

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ABSTRACT

Studies of genotoxicity in fish caused by cyanobacterial extracts containing microcystins (MCs) can be useful in determining their carcinogenic risk due to a genotoxic mechanism. An extract of cyanobacterial *Microcystis* ssp, containing MC-LR and -LA from a bloom collected in a eutrophic lake, showed genotoxicity to *Oreochromis niloticus*. DNA damage (comet assay) was significantly induced in peripheral erythrocytes with both tested concentrations of $6.90 \ \mu g \ kg^{-1}$ bw and $13.80 \ \mu g \ kg^{-1}$ bw through intraperitoneal injection (ip). There was no micronucleus induction after ip injection at concentrations of $6.90 \ \mu g \ kg^{-1}$ bw and $13.80 \ \mu g \ kg^{-1}$ bw. Body exposure resulted in micronucleus induction and DNA damage only at the highest tested concentrations of $103.72 \ \mu g \ L^{-1}$. Thus, comet assay and ip injection revealed the highest levels of the genotoxicity of MCs. Apoptosis-necrosis test carried out at concentrations of $6.90 \ \mu g \ kg^{-1}$ bw and $13.80 \ \mu g \ kg^{-1}$ bw revealed that at low concentrations more apoptosis than necrosis occurred. At higher concentrations more necrosis than apoptosis occurred.

1. Introduction

Microcystins (MCs) are a group of natural toxins produced by cyanobacteria which can be found in lakes, ponds and rivers. These cyanotoxins are hepatotoxic, causing serious human health problems by inhibition of some phosphatase proteins (Terao et al., 1994). MCs cause morphologic damage in the liver, starting with cytoskeletal disruption and loss of sinusoidal structure. Liver weight is increased due to intrahepatic hemorrhage followed by hemodynamic shock, heart failure and death by hemorrhagic shock (Eriksson et al., 1990; Chorus and Bartram, 1999). Zhang et al. (2008), demonstrated the role of reactive oxygen species induced by MC-RR on apoptosis sensitivity of *Carassius auratus* lymphocytes.

In Brazil, tilapia species such as *Tilapia rendalli* and *Oreochromis niloticus* have been introduced for socioeconomic purposes since 1956 (Gurgel and Fernando, 1994). Bioaccumulations in fish were observed in salmon that ate crab larvae containing MC (Williams et al., 1997). Accumulation in liver and muscle of *T. rendalli* was demonstrated by Soares et al. (2004). This latter study showed that toxins could still be found in fish muscle several days after contamination.

Nowadays, billions of humans drink water from freshwater reservoirs, and the quality of this tap water is dependent on characteristics of reservoir water, mainly the eutrophic level. Toxic cyanobacterial blooms in South American water bodies, with occurrence of MCs, reveal the extent of this problem as an emerging concern to public health authorities (Dörr et al., 2010). However not only water, but also food contaminated with cyanotoxins and other pollutants can pose a serious treat for humans, Mohamed and Hussein (2006) found MCs in liver, kidney,

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gut and muscle of *O. niloticus* in an Egyptian fish farm. Cazenave et al. (2005) detected MC in liver, gills, muscle and brain of *Odontesthes bonariensis* collected from a reservoir in Argentina on two sampling dates. Xie et al. (2005) measured MC in gut, liver, kidney, muscle, blood and bile of eight species of fish in Lake Chaohu of China. Jang et al. (2003) measured MC content in body tissue of two native fishes in Hoedong Reservoir.

Lake Paranoá is a Brazilian tropical reservoir that is typically eutrophic due to inadequate sewage treatment associated with high population growth (Altafin et al., 1995). *T. rendalli* and *O. niloticus* are the fish species from Lake Paranoá that are most sold in local markets. Our previous study showed that both species are sensitive to different clastogens such as cyclophosphamide, mitomycin C, 5-fluorouracil and bleomycin (Grisolia and Cordeiro, 2000). The aim in the present study was to evaluate the genotoxicity to tilapia fish *O. niloticus*, as induced by an extract of cyanobacteria containing MCs, using two administration routes and different endpoints, such as micronucleus, comet and apoptosis-necrosis testing.

2. Material and methods

2.1. Animals

O. niloticus used in this study were obtained from a local fish farm, where breeding and sanitary conditions were controlled and monitored constantly. The criterion for fish selection was body length of 7–10 cm. Fish of both sex were acclimatized in the Genetics Laboratory of the University of Brasilia for a week in tanks of 250 L volume, with continuously aerated filtered and dechlorinated tap water. Fish were maintained at a constant temperature of 25 ± 2 °C, conductivity (550 ± 50 μS), pH = 7.0 ± 0.5, photoperiod (14:10 light:dark) and fed twice a day with granular fish chow. The ammonium level in the water was constantly monitored and the water was periodically renewed.

2.2. Microcystis bloom extract characterization – HPLC and mass analyses

The extract was obtained from a bloom taken from Lake Paranoá (Brazil) on June 25, 2006. The lyophilizated sample was resuspended in distilled water and ultrasonicated. Soon afterwards, a small sample aliquot was filtrated in a Microcon unit (Ultracel Ym-10, Millipore) and submitted to HPLC-PDA analyses. The chromatography was carried out under isocratic conditions using a reverse phase C18 column (Synergi 4ì Fusion-RP 80 (250 × 4, 60 mm; Phenomenex)), mobile phase of 20 mM ammonium formate, pH 5.0 and acetonitrile (7:3, v:v) for 30 min. The identification of the MCs produced by the bloom was performed by comparison of the spectrogram similarity index of standard microcystin-LR (SIGMA, CO) in the absorbance range of 200–300 nm. A curve was constructed using different concentrations of Microcystin-LR (SIGMA, CO).

The MC molecular masses were determined by Ultraflex II™ TOF/TOF (Bruker, Bremen, Germany). Aliquots of lyophilized MC fractions were dissolved in Milli-Q water (TFA 0.1%) and mixed with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid (1:3, v/v) and directly applied onto a target (AnchorChip™, Bruker Daltonics). Mass spectrometry was operated in reflector mode for MALDI-TOF or LIFT mode for fully automated MALDI-TOF/ TOF using FlexControl™ software. Calibration of the instrument was performed externally with $[M + H]^+$ ions of angiotensin I, angiotensin II, substance P, bombesin, insulin b-chain and adrenocorticotropic hormones (clip 1–17 and clip 18–39). Each spectrum was produced by accumulating data from 200 consecutive laser shots. Those samples which were analyzed by MALDI-TOF were additionally analyzed using LIFT TOF/TOF MS/MS from the same target.

2.3. Assay

Fish were randomly placed in groups of 8 in glass aquaria of 30 L, and treatments were carried out through intraperitoneal (ip) injection and body exposure. To determine the toxicity (LC50 – 72 h and LD50 – 72 h) the Trimed Spearman-Karber method was used (Hamilton et al., 1977). Treatments with the *Microcystis* extract were performed with the following concentrations: 6.90 μg kg^{-1} bw and 13.80 μg kg^{-1} bw for 72 h in the single ip injection assay, and 5.00 μg L^{-1} and 103.72 μg L^{-1} for 72 h in the exposure assay, plus a respective control. Micronucleus test, comet assay and necrosis versus apoptosis test were carried out on erythrocytes of peripheral blood. Study design was based on the OECD guidelines for testing chemicals – Fish, Acute Toxicity Test 203 (1992), and the Project was approved by the Animal Ethics Committee of the University of Brasilia.

2.4. Micronucleus test

Peripheral blood (50 μL) was obtained by cardiac puncture with a heparinized syringe and immediately smeared. After fixation in ethanol for 15 min, slides were left to air-dry and the concentration of AO in the MN assay was 0.03 mg mL^{-1}. The stained slides were viewed under an epi-fluorescent microscope at a magnification of 1000× and evaluated for the presence of micronuclei exhibiting yellow-green fluorescence in the peripheral blood erythrocytes. For each treatment, all eight fish were sampled and three thousand erythrocyte cells with complete cytoplasm were scored per fish (total of 24,000 cells per treatment). The criteria for the identification of fish micronucleated erythrocytes were as follows: (a) MN should be smaller than one-third of the main nuclei; (b) MN must not touch the main nuclei; (c) MN must be of the same color and intensity as the main nuclei. These data were statistically analyzed by nonparametric Mann–Whitney *U*-test, considering α = 5%.

2.5. Comet assay

This assay was performed as described by Singh et al. (1988), with some modifications. The cell suspension sampled in the microtubule was mixed with 120 μL low melting agarose (37 °C). Next, 50 μL of the erythrocyte-agarose suspension was placed on a fully frosted slide pre-coated with standard agarose (1.5%) and covered with

a coverslip. The slides were then placed on ice for 15 min to allow complete agarose polymerization and afterwards in a chilled lysing solution (NaCl 2.5 M; EDTA 100 mM; Tris 10 mM; N-laurolyl-sarcosine 1%; Triton-X 1%; DMSO 10%; pH = 10). Then the slides were placed on a horizontal gel electrophoresis platform and covered with a chilled alkaline solution consisting of 300 mM NaOH and 1 mM Na2EDTA (pH = 13); they were left in the dark at 4 °C for 30 min, and then the DNA was electrophoresed at 4 °C in the dark for 30 min at 25 V and approximately 350 mA. The slides were gently rinsed twice with 400 mM Tris (pH = 7.5) to neutralize the alkali. Each slide was stained with 30 μL of 20 μg/mL ethidium bromide and covered with a coverslip. One hundred cells from each replicate were randomly chosen (50 from each duplicate slide), and analyzed under an optical fluorescence microscope (Axioskop-2, Carl Zeiss), with a 510–560 nm filter and a 590 nm barrier filter, with a magnification of 400×. For damage index calculation, cells were sorted into four classes, according to tail size. The index of damage (ID) is the sum of classes of the 100 cells analyzed per fish, and may vary from 0 (all cells undamaged – 0 × 100) to 400 (all cells highly damaged – 4 × 100). The damage index is based on the length of migration and on the amount of DNA in the tail, and it is considered a sensitive measurement of detectable DNA damage. Statistical analysis was carried out with the MINITAB program, using the ANOVA parametric test and Tukey's parametric linear correlation, with a significance

level of 95%. To quantify the damage to the DNA, the following formula was used:

$$ID_{(au)} = \frac{N1 + 2N2 + 3N3 + 4N4}{S/100}$$

where ID = index of DNA damage, au = arbitrary unit, $N1$–$N4$ = nucleoids in levels 1, 2, 3 and 4, S = number of nucleoids analyzed, including level 0.

2.6. Apoptosis-necrosis test

Treatments were carried out in groups of eight fish through intraperitoneal injection of extract of *Microcystis* spp at 6.90 μg kg^{-1} bw and 13.80 μg kg^{-1} bw for 72 h. 0.1 mL of peripheral blood was obtained from cardiac puncture and diluted in 2.0 ml of fetal bovine serum at room temperature of 23 °C. A smear of 15 μL of cell suspension was made immediately, 1 μL of Acridine Orange (3.0 μg L^{-1})/Ethidium Bromide (3.0 μg L^{-1}), (1:1 v/v) stain was added and the slides were covered with a coverslip. Slides were analyzed with a fluorescence Axioskop-2 Zeiss microscope with 1000× magnification using a wavelength of 510–560 nm. Viable peripheral fish erythrocyte cells were identified by greenish nuclei with dark cytoplasm. Apoptotic erythrocyte cells were identified as fragmented greenish nuclei. Necrotic cells were identified as round cells with reddish nuclei. Five hundred peripheral erythrocytes were analyzed and
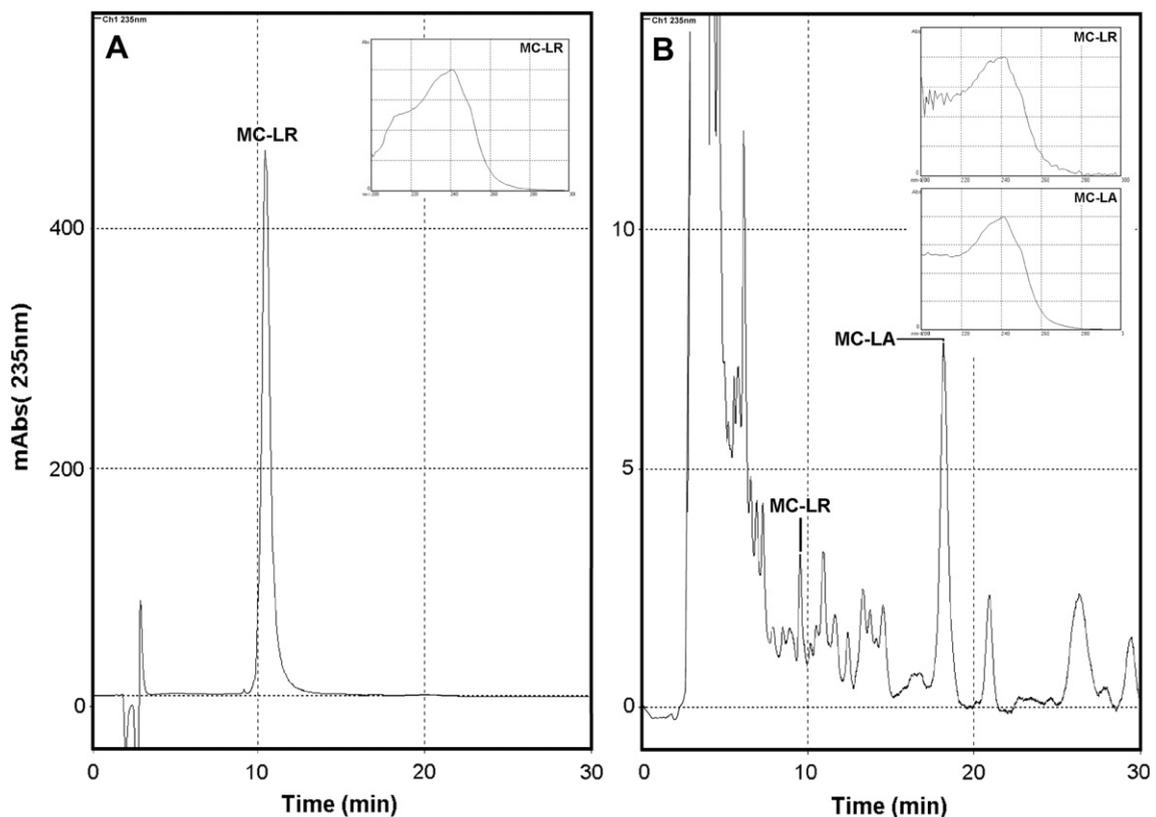

Fig. 1. Chromatogram profile of (A) Microcystin-LR Standard, and (B) ultrafiltered bloom extract, suggesting the presence of 2 MCs. Inset (A) UV Spectrogram profile range 200–300 of Microcystin-LR (SIGMA), and (B) the chromatographic fractions denominated Microcystin-LR and Microcystin-LA.
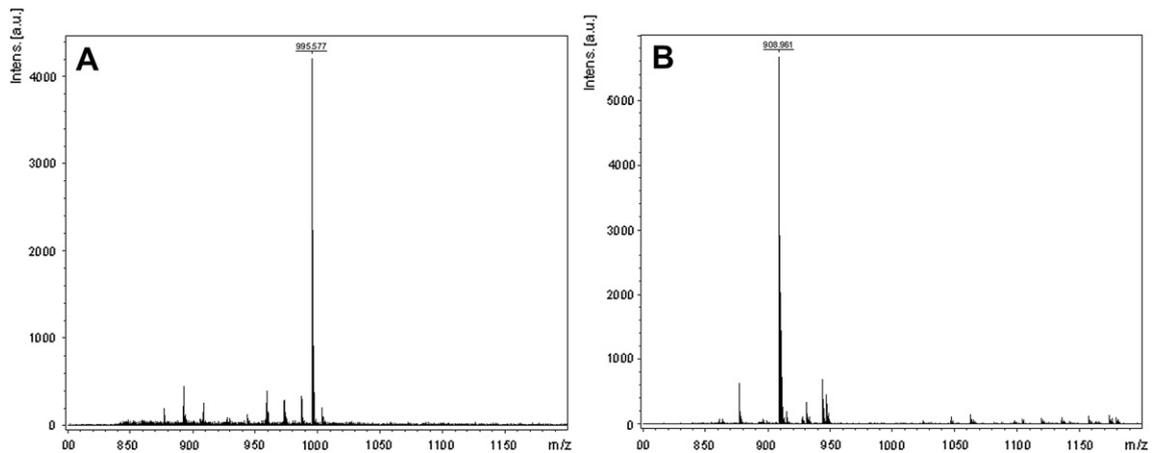
Fig. 2. Mass Spectrogram profile of the chromatographic fractions (A) Microcystin-LR and (B) Microcystin-LA.

classified as viable, necrotic or apoptotic. Results were statistically analyzed t-test, with significance of 0.05.

3. Results

The bloom extract showed the presence of 2 MCs (Fig. 1), while the mass spectrometry analyses on reflective mode showed the mass components at 995.577 Da and 908.961 Da, suggesting the presence of Microcystin-LR and Microcystin-LA respectively (Fig. 2). The mass spectrometry analyses in lift mode confirmed the presence of MCs by showing in the fragment pattern the ion 135.0, an ADDA fragment exclusive to microcystin toxins (Data not shown).

The concentration of total MCs estimated in the extract was 138.30 μg mL^{-1}. Separately, the concentrations were 41.0 μg mL^{-1} and 97.9 μg mL^{-1} for MC-LR and MC-LA respectively.

Neither mortality nor visible adverse effects were observed in the two exposure routes during the experiments. Doses of 13.80 μg kg^{-1} bw for 72 h in the ip assay and 103.72 μg L^{-1} for 72 h in the exposure assay were considered the maximum tolerated doses, because previously tested higher doses caused toxic signals such as refusing food, scale loss, alteration in the behavior and mortality. The LC50 was greater than 103.72 μg L^{-1} of microcystin in the body exposure route and the LD50 was greater than 13.80 μg kg^{-1} bw via ip. The micronucleus test showed that only the highest concentration of 103.72 μg L^{-1}, via body exposure, was statistically significant ($P = 0.040$, Table 1). Treatment by intraperitoneal injection did not induce MN (Table 1). The highest treatment via body exposure and both treatments via intraperitoneal injection significantly increased the index of DNA damage (Table 2). Apoptosis-necrosis test showed that at lower concentrations more apoptosis was found. On the other hand, at higher concentrations more necrosis was found (Table 3).

4. Discussion

Gaudin et al. (2008) reported DNA damage in different tissues through single cell gel electrophoresis (comet assay) in mice after single acute oral administration and intraperitoneal injection of MCs. An extract of cyanobacteria containing MCs from a water source in China was mutagenic to Salmonella typhimurium (Ames test), induced DNA damage in rat hepatocytes (comet assay) and induced micronucleated polychromatic erythrocytes in mouse bone marrow (Ding et al., 1999). Zegura et al. (2004) demonstrated in human hepatoma HepG2 cells that MC-LR increases the levels of reactive oxygen species (ROS), providing evidence that the observed genotoxicity was mediated by ROS. MC-LR and -RR increased the antioxidant enzymatic activities of superoxide dismutase and catalase in tilapia fish Oreochromis sp (Jos et al., 2005; Prieto et al., 2006). In our previous study in Astyanax bimaculatus (Pisces: characidae), this same batch of microcystin induced comets and MN (Silva et al., 2010). Thus, there is evidence that MCs are genotoxic to different aquatic

Table 1
Means (SD) of micronuclei in O. niloticus after treatment with extracts at different concentrations, considering two exposure routes.

Treatment	Exposure route	MN (Mean + SD)	P Mann–Whitney U-test
Control	–	2.5 ± 2.72	
5.00 μg L^{-1}	Body exposure	2.3 ± 1.89	0.788
103.72 μg L^{-1}	Body exposure	7.1 ± 6.19	0.040*
6.90 μg kg^{-1} bw	Intraperitoneal	2.5 ± 1.77	0.652
13.80 μg kg^{-1} bw	Intraperitoneal	3.0 ± 2.93	0.620

bw = body weight.

Table 2
Means (SD) of DNA damage index obtained from comet assay from peripheral erythrocytes of O. niloticus after treatment with extracts at different concentrations, considering two exposure routes.

Treatment	Exposure route	ID (Mean ± SD)	P Mann–Whitney U-test
Control	–	18.26 ± 6.23	
5.00 μg L^{-1}	Body exposure	32.56 ± 17.68	0.0500
103.72 μg L^{-1}	Body exposure	59.20 ± 22.76	0.0002*
6.90 μg kg^{-1}	Intraperitoneal	190.59 ± 13.41	0.0004*
13.80 μg kg^{-1}	Intraperitoneal	149.23 ± 30.45	0.0004*

*$P < 0.05$, significant.

Table 3
Means (SD) of viable, necrotic and apoptotic peripheral erythrocyte cells of *O. niloticus* and their respective percentages after ip injection of extracts of microcystis spp.

Treatment	Viable	Necrosis	Apoptosis	% Viables	% Necrosis	% Apoptosis
Control	473.0 ± 38.2	20.75 ± 34	4.62 ± 3.7	94.9	4.2	0.9
6.90 μg kg^{-1}	441.4 ± 88.5	58.75 ± 88.8	12.5 ± 6.0	86.1	11.5	2.4*
13.80 μg kg^{-1}	312.5 ± 123.5	175.0 ± 135.7	5.62 ± 2.8	63.4	35.5*	1.1

*$P < 0.05$, significant, SD = standard deviation.

species and mammals, including human cells. In contrast, Abramsson-Zetterberg et al. (2010) reported no *in vivo* micronucleus induction in young mouse erythrocytes and nor *in vitro* micronucleus induction in human lymphocytes after exposure to pure MC-LR and cyanobacterial extracts from different lakes in Sweden. Extracts collected from different blooms as well as different parts of world may contain also other components of cyanotoxins, having different profiles of toxicity.

O. niloticus was susceptible to genotoxicity of an extract of *Microcystis* collected in a water bloom during the dry season. Induction of micronucleated cells was observed only at higher concentration through body exposure. No micronucleus increases were found with treatments via ip. According to Gaudin et al. (2008), genotoxicity caused by MCs could be variable in different organs of mice, such as blood, liver, kidney, colon and intestine, and it also depends on the administration route. Apoptosis-necrosis analysis to study cell viability and mode of cell death induced by toxins using double fluorescent stain is rapid, repeatable and easy to perform. Brockmann et al. (2006) showed that apoptosis starts at much lower concentrations than cytotoxic concentrations when cells are exposed to genotoxic compounds. Intraperitoneal injection is an inappropriate route for fish models in genotoxicity studies, although normally, ip injections give a more precise exposure level to the studied toxins and show a better response than aquatic exposure. Our results showed differences in genotoxicity comparing ip injection with body exposure. Ip injection induced comets, but not MN. Thus, we should be more conservative in the evaluation of MC's genotoxicity due to an uncommon route of exposure. In this case, the ip injection was probably very toxic, causing inhibition of cell divisions, so that micronuclei were not observed. On the other hand, comets followed by ip injection were found because these did not need cell divisions. The comet assay has been successfully applied in laboratory and field conditions as a non-specific, sensitive, rapid and economical biomarker for detection of genetic damage in natural biota (Jha, 2008). This author suggested also that comet assay is capable of detecting oxidized DNA bases in fish exposed to environmental contaminants. Our results are in accordance with this purpose. Otherwise, we should also consider that the tested crude cyanobacterial extract can contain other components, besides MCs.

Induction of comet cells occurred probably due to DNA strand breaks caused by oxidative stress induced by MCs. Exposure of cells to genotoxic compounds induces apoptosis by a mechanism that is initiated by DNA damage. In contrast, necrosis can be started by non-specific external stimuli, such as ischemia, trauma, infection, cell membrane break or any kind of cell disruption. Our data showed that a microcystic extract, when in low concentrations, could activate cellular oxidative stress, causing genotoxicity, as proposed by many authors and cited above. Obviously, cell death by necrosis occurred due to exposure to a high concentration of a well-known toxic compound. Fessard and Bernard (2003) and Bazin et al. (2010) observed that cylindrospermopsin, another cyanotoxin produced by freshwater cyanobacteria, is genotoxic without reacting directly with DNA, indicating that its metabolism is required. So, they suggested that this toxin is a progenotoxin. It seems that there may be different mechanisms of action to explain the genotoxicity of cyanotoxins. Therefore, cyanobacterial blooms in ponds represent a genotoxic risk to fish and consequently to human health.

Conflict of interest

None.

Acknowledgements

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