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RESEARCH ARTICLE

Lethal and sublethal exposure of *Hemichromis bimaculatus* (Gill, 1862) to malachite green and possible implications for ornamental fish

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Abstract

Malachite green (MG) is a triphenylmethane dye that is widely used in aquaculture as a fungicide, bactericide, ectoparasiticide, and antiprotozoal. There is great debate regarding the potential for this compound to trigger adverse effects. Here, we review the previous findings and then evaluate the lethal and sublethal effects of MG in the species *Hemichromis bimaculatus* (jewelfish). The lethal concentration for 50% of the fish in 96 h was 1 mg/L. We observed a dose-dependent increase in the percentage of fish mortality as well as physical and behavioral changes. We further found that the highest tested sublethal dose significantly increased the DNA damage index identified using the comet assay (74.97 ± 13.8 at a significant level of $P < 0.05$ for the 0.75 mg/L concentration), but did not significantly alter the results of the micronucleus test. Although our results suggest that MG confers risks on exposed fish, the findings were significant only at the highest exposure concentration (0.75 mg/L). At lower concentrations (0.25 mg/L and 0.5 mg/L), no adverse effect was observed. The maximum MG concentration recommended for use in ornamental fish farming is 0.2 mg/L. Therefore, our results suggest that, specifically for the parameters analyzed in this work, MG does not have any adverse effect when users strictly adhere to the recommended concentration criteria for ornamental fish.

Keywords Micronucleus · Comet assay · Lethal concentration · Mutagenicity · Genotoxicity · Jewelfish

Introduction

Malachite green (MG) is a chemical compound that has been used as a dye and in aquaculture since the 1930s. At present, this substance is used mainly for the maintenance of ornamental fish as a fungicide, bactericide, ectoparasiticide, and antiprotozoal. Many aquaculture producers consider it to be irreplaceable due to its low cost, ready availability, and high efficacy (Schmahl et al. 1992; Ruider et al. 1997; Wan et al. 2011).

There is a wide-ranging debate regarding the toxicological effects caused by MG, with some authors considering it to be a safe compound, while others report a number of adverse effects following exposure to MG and its main metabolite, leucomalachite (LM) (Table 1). Different fish species appear to vary widely in their sensitivity to MG (Table 2), with the LC_{50} (lethal concentration to 50% of a population) ranging from 0.154 mg/L for *Micropterus dolomieu* to 5.6 mg/L for *Heteropneustes fossilis*. These results also call attention to the caution that must exist when handling the compost. Given this debate and the widespread use of MG, further studies are needed to fully evaluate its safety and optimum dosage in fish.

The fish species, *Hemichromis bimaculatus* (commonly known as jewelfish), is popular in the aquarist trade because

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Table 1 Evaluation of the effects caused by malachite green and its main metabolite leucomalachite green

Author	Organism	Used concentrations	Concentrations	Damage caused Malachite green (MG)	Damage caused Leucomalachite green (LG)	Time period
(Bergwerff et al. 2004)	*Anguilla anguilla*	0.1 mg/L MG	After 100 days no residues of MG or LG were observed.	Not observed	Not observed	100 days
(Bose et al. 2005)	Culture of embryonic hamster cells	0.025, 0.05, 0.075, and 0.1 mg/mL MG	0.025, 0.05, 0.075, and 0.1 mg/mL MG	Not tested	Apoptosis and breaks in the DNA molecule, cell cycle arrest	24 and 48 h
(Culp et al. 1999)	Rats	0, 25, 100, 300, 600, 1200 ppm MG 0, 290, 580, 1160 ppm MG	1200 ppm MG 1160 ppm LG	Vacuolization of hepatocytes and DNA adducts	Apoptosis in the urinary bladder transition epithelium, vacuolization of hepatocytes and DNA adducts	28 days
(Culp et al. 2002)	Big Blue rats	0, 9, 27, 91, 272, and 543 ppm LG	91, 272, and 543 ppm LG	Not tested	Dose-related DNA adduct. Little mutagenic or carcinogenic consequence	224 days
(Fessard et al. 1999)	Bacteria and mammalian cells	10 to 2000 μg per plate MG	≥ 500 μg per plate ≥ 0,1 μg/mL	Cytotoxic for bacteria and mammalian cells. No present mutagenic activity in bacterial strain. Highly toxic to mammalian cells	No damage was observed	
(Jang et al. 2009)	Embryo of *Danio rerio* zebrafish. Human cell primary culture	125, 150, and 175 ppb MG Concentrations below 125 ppb MG did not cause damage and greater than 175 ppb MG caused death and severe damage	125, 150, and 175 ppb MG	Abnormalities in the cardiovascular system and growth retardation. Significantly inhibited the survival of human umbilical vein endothelial cells	No damage was observed	14 h
(Manjanatha et al. 2004)	Big blue female rats	543 ppm LG	No changes	Not tested	No damage was observed	30 days 90 days 240 days
(Mittelstaedt et al. 2004)	Transgenic mice big blue B6C3F1	450 ppm MG 204 and 408 ppm LG	408 ppm LG	Neither malachite green nor leucomalachite increased the peripheral blood micronucleus frequency or the frequency of the Hprt lymphocyte mutant at any time. The 16-week treatment with 408 ppm of leucomalachite green increased the frequency of the cII mutant in the liver		30 and 90 days
			25, 50, and 100 μM MG		No damage was observed	

Table 1 (continued)

Author	Organism	Used concentrations	Concentrations	Damage caused Malachite green (MG)	Damage caused Leucomalachite green (LG)	Time period
(Nebbia et al. 2017)	Hepatic enzymes of rainbow trout (*Oncorhynchus mykiss*)	25, 50, and 100 μM MG and LG tested in vitro		MG acts as a potent in vitro inhibitor of hepatic trout metabolism		10 min incubation
(Niska et al. 2009)	*Oncorhynchus mykiss* eggs	1, 3, and 6 mg/L MG	Not observed	No damage was observed 96 days after birth. No residues of MG and LG were found in the trout fingerlings.		30 min
(Rao et al. 1998)	Culture of embryonic hamster cells	0.00625 to 0.1 μg/mL MG	0.1 μg/mL MG	Not observed	Induced the dose-dependent G2/M suspension in normal cells	48 h
(Stammati et al. 2005)	Human tumor cells (Caco-2 and HEp-2)	0.26 to 4.0 μM VM or 30–610 μM is equivalent to (0.2 mg/mL) LG to Hep-2 0.1 to 100 μM MG or 25 to 100 μM LG to Caco-2		MG is cytotoxic to two cell lines and impairs mitochondrial activity	LG was not cytotoxic for both cell lines	24 h
(Sundarrajan et al. 2000)	Rats	100 ppm MG	100 ppm MG associated to N-nitrosodimethylamine	Associated to N-nitrosodimethylamine caused liver enlargement, and tumor promotion Note: 100 ppm MG isolated did not cause damage	Not tested	
(Wan et al. 2011)	Sprague-Dawley rats	0, 10, 80, and 160 mg/kg/day LG	80 and 160 mg/kg/day LG	Not tested	Severe reduction in maternal weight and feed intake, skeletal fetal abnormalities	15 days
(Yonar and Yonar 2010)	*Onchorhyncus mykiss*	1/15.000 (66.7 mg/L) 1/150.000 (6.67 mg/L)	1/15.000 (66.7 mg/L) 1/150.000 (6.67 mg/L)	Immunosuppressive effect in fish and oxidative stress	Not tested	30 min 60 min

Table 2 Malachite green toxicity found in various fish groups

Species	LC_{50} (mg/L)	pH	Temp. (C°)	Exposure time (h)	Reference
Lepomis macrochirus (adults)	2.0	8.0	12	3	(Bills et al. 1977)
	7.43	6.5	12	3	
	2.19	7.5	12	3	
Ictarulus punctatus (fingerlings)	0.238	7.5	22	6	(Bills et al. 1977)
	0.960	7.5	12	6	
	0.4	7.5	22	6	
	0.519	9.5	12	6	
	1.72	8.0	12	6	
	1.3	8.0	12	6	
	0.286	8.0	12	24	
Oncorhynchus mykiss (fingerlings)	1.4	7.5	12	3	(Bills et al. 1977)
	2.35	8.0	12	3	
	6.8	8.0	12	6	
Micropterus dolomieu (fingerlings)	0.154	7.5	12	24	(Bills et al. 1977)
Micropterus salmoides (fingerlings)	0.282	7.5	12	24	(Bills et al. 1977)
Oncorhynchus kisutch (fingerlings)	3.0	7.5	12	6	(Bills et al. 1977)
	0.569	7.5	12	24	
	0.383	7.5	12	96	
Salmo salar (fingerlings)	3.560	7.5	12	3	(Bills et al. 1977)
	1.090	7.5	12	6	
	0.497	7.5	12	24	
	0.283	7.5	12	96	
Salmo trutta (fingerlings)	1.730	7.5	12	3	(Bills et al. 1977)
	1.270	7.5	12	6	
	0.352	7.5	12	24	
	0.237	7.5	12	96	
Heteropneustes fossilis (fingerlings)	5.60	7.7	22	24	(Srivastava et al. 1995)
	1.40	7.7	22	48	
	1.25	7.7	22	72	
	1.0	7.7	22	96	
Hemichromis bimaculatus (adults)	1.0	6.8	25	96	Present study

of its rusticity and colors (Rowland 1975; Bhosale et al. 2017). We chose this species as the test organism because it is an ornamental fish (representing the main target group for the use of MG); it is easy to maintain and reproduce in the laboratory; and it has been used in previous physiological and ethological studies (Rowland 1975; Bell 1982; Bhosale et al. 2017) and is thus relevant for studies aimed at determining acute toxicity and concentration of use of MG without causing damage to ornamental fish.

Currently, most studies related to MG have been carried out with species from temperate regions, and although this substance is widely used in aquaculture ornamental and consumer fish, little research has investigated its impact on species present in tropical ecosystems. Although the use is prohibited in fish farming for human consumption, the MG is released for ornamental fish farming, and its improper handling can cause damage to the environment and the people who handle it. Therefore, to increase knowledge regarding the possible adverse effects of MG, we thoroughly examined the effects of lethal and sublethal doses of MG on *Hemichromis bimaculatus*.

Material and methods

Sample

Fishes were adults obtained from and maintained by the Amazonian Ichthyology Laboratory IFPA (Campus Abaetetuba), where the sanitary conditions are constantly monitored. The animals were acclimatized for 15 days with a photoperiod of 12 h, in constantly aerated aquaria with

filtered and dechlorinated water. They received commercial fish feed twice a day. During experiments, we constantly monitored the physical and chemical parameters of the water and kept them optimized for the maintenance of *Hemichromis bimaculatus*: 26 °C ± 2, pH between 6.8 and 7.2, ammonia 0.002 mg/L, dissolved oxygen 6 mg/L, and conductivity 500 ± 50 μS/cm. The study was approved by the Ethics Committee of the Federal University of Pará, permit No. 68/2015. All the tests were done in duplicate to confirm the results obtained.

Acute toxicity test

The fish were separated into six groups (5 experimental and 1 control) of seven fish each. The aquariums with a volume of 35 L of filtered and dechlorinated water each functioned in a static system; the five experimental tanks were treated with five different concentrations of water-diluted MG (0.167 mg/L, 0.5 mg/L, 1.5 mg/L, 4.5 mg/L, and 13.5 mg/L). Concentrations were based on the recommended treatment for fish (approximately 0.2 mg/L); from this concentration, we tested values above and values below in order to find LC_{50} (concentration lethal to 50% of fish). The control groups were left untreated. Each test was applied according to the recommendations established by the OECD - (Organisation for Economic Co-operation and Development) (1992). Fish were exposed for 96 h, and mortality was observed at 24, 48, 72, and 96 h to enable us to calculate the LC_{50} values at these times. The calculation of the LC_{50} was done using the software PROBIT STATGRAPHICS PUS, version 1.8. Results were expressed as mean and standard deviation.

Studies of physical and behavioral changes

The presence or absence of behavioral changes, such as irritability, leakage reflex, contortion, tremors, convulsion, stimulation, hypnosis, anesthesia, loss of direction, and shock against the wall, was observed throughout the experiment. Physical changes, such as mydriasis, depigmentation of the skin, defecation, and swelling of the body and eyes, were also observed. All parameters were analyzed as described by Melo et al. (2015).

Genotoxicity assays

Genotoxicity was assessed at sublethal concentrations, which were calculated from the LC_{50} determined in the acute toxicity test. The fish were separated into 4 groups (3 experimental groups and 1 negative control) with six animals in each group. They were exposed to 0.25 mg/L, 0.50 mg/L, and 0.75 mg/L, which corresponded to 25%, 50%, and 75% of the LC_{50}, respectively. After 96 h of exposure, they were euthanized by marrow section, and myelotomy was performed. From the cut made in the marrow, blood was collected. The fish length was determined, and the weights of the liver and total animal were quantified. For this experiment, aquariums with a volume of 35 L of filtered and dechlorinated water were used, in the proportion of 1 g of animal weight for each liter of water as recommended by OECD - (Organisation for Economic Co-operation and Development) (1992).

Comet assay

The comet assay was performed as described by Hartmann and Speit (1997). Blood (5 μL) was collected with a micropipette and transferred to an Eppendorf tube containing 500 μL of fetal bovine serum (FBS), and 40 μL of the mixture was combined with 120 μL of low-melting-point agarose (0.5%) kept in a water bath at 37 °C. The material was placed on microscope slides that had been previously lined with normal agarose (1.5%) and covered with coverslips. The slides were refrigerated, the coverslips were removed, and the slides were submerged in freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris; pH 10.0–10.5) containing 1% Triton X-100 and 10% DMSO and held at 4 °C for 1 h. The slides were then alkaline treated for 30 min in pH 13 buffer solution (300 mM NaOH and 1 mM EDTA) previously cooled in an ice bath. Electrophoresis was performed using a horizontal system at 25 V and 300 mA for 30 min. The slides were neutralized (0.4 M Tris-HCl, pH 7.5) three times for 5 min each time, stained with 30 μL of ethidium bromide aqueous solution (Gibco Industries Inc., USA) and observed under a × 400 fluorescence microscope with a 515- to 560-nm excitation filter and a 590-nm barrier. One hundred nucleoids were analyzed per slide, as recommended by Collins et al. (2001). The cells were classified into categories from tail size in classes 0 to 4 (0 = no tail, 1 = little damage, 2 = moderate damage, 3 = severe damage, 4 = maximum damage level, 80% DNA or more in the tail), according to Collin et al. (1995). One hundred cells were analyzed per treatment. The damage index (ID) was calculated using the following formula:$ID = (0 \times n0) + (1 \times n1) + (2 \times n2) + (3 \times n3) + (4 \times n4)$, where n is the number of cells in each class analyzed (Collins et al. 2001; Silva-Pereira et al. 2005). Finally, the total score per specimen, which represented the frequency of breaks in DNA, was calculated as the mean of the scores of obtained from the two slides corresponding to that specimen. For each fish, slices were made in duplicate.

Micronucleus test

Blood (5 μL) was collected by a micropipette and transferred to an Eppendorf tube containing 500 μL of FBS, and 50 μL per slide was used to prepare two blood smears per animal. The slides were dried for 24 h at room temperature, fixed in absolute methanol for 10 min, and stained with Giemsa (10%)

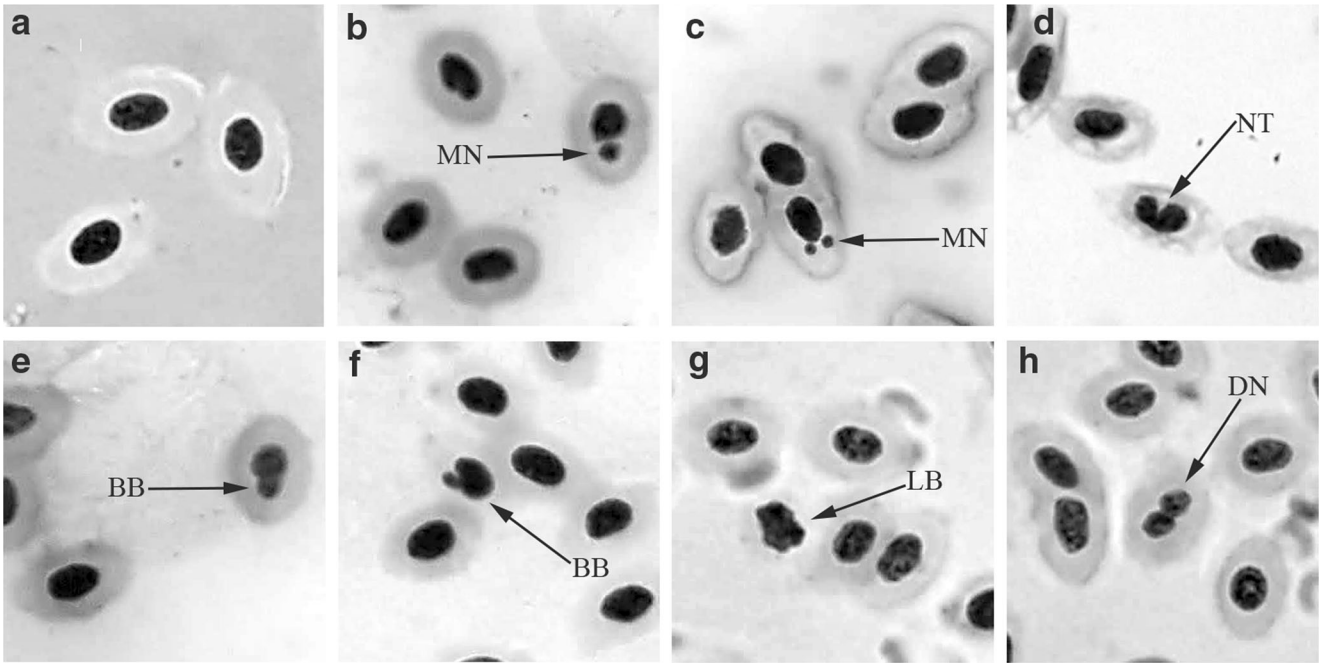

Fig. 1 Red blood cells of *H. bimaculatus*, exposed to MG. Normal cells (**a**). Micronucleus, MN (**b** and **c**). Notched, NT (**d**). Blebbed, BB (**e** and **f**). Lobed, LB (**g**). Double nucleus, DN (**h**). Images from the authors

diluted in phosphate buffer (pH 6.8) for 10 min. The slides were coded to blind the analyst and avoid bias and analyzed under a light microscope using a × 100x objective. For each fish, 3000 erythrocytes were analyzed for the frequency of micronuclei and nuclear erythrocyte abnormalities (NEAs). For the micronucleus analysis, we used the criteria of Cavalcante et al. (2008), in that a micronucleus must be morphologically similar to the main nucleus; have a diameter between 1/16 and 1/3 of the main core; not be refringent; do not have a physical connection with the nucleus; and have the same staining intensity as the main nucleus. Cells were considered to have NEAs if they were binucleate or had blebbed, lobed, or notched nuclei, as described by Carrasco et al. (1990) (Fig. 1).

Statistical analysis

Statistical analysis was performed with the Biostat 5.0 program (Ayres et al. 2007). The utilized test was chosen according to the sample normality, which was assessed using the Kolmogorov-Smirnov test. ANOVA (multiple Tukey comparisons) was used for normally distributed data, while the Kruskal-Wallis test (multiple Dunn comparisons) was used for non-normally distributed data.

Results

Acute toxicity and determination of the LC_{50}

The values found for LC_{50} at 24, 48, 72, and 96 h are presented in Table 3. The highest concentration tested (13.5 mg/L) caused mortality in fish beginning at 24 h after MG exposure. The LC_{50} value increased significantly from 24 to 48 h and then remained stable to 96 h. As shown in Fig. 2, the mortality

Table 3 Values of LC_{50} (mg/L) in *H. bimaculatus* after exposure to MG at different times

Exposure time (h)	LC_{50} (mg/L)
24	4.3 (± 0.14)*
48	1.87 (± 0.0)
72	1.34 (± 0.47)
96	1.0 (± 0.0)

*Differs statistically in relation to the other exposure times ($P < 0.05$)

ANOVA (multiple Tukey comparisons)

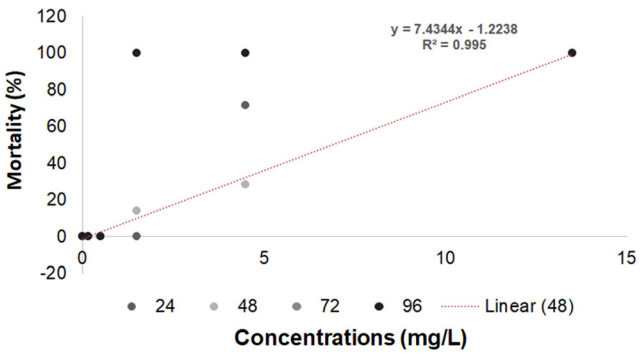

Fig. 2 Graphical dispersion demonstrates a strong linear correlation of *H. bimaculatus* mortality as a function of MG concentration in the acute toxicity test. Pearson linear correlation test

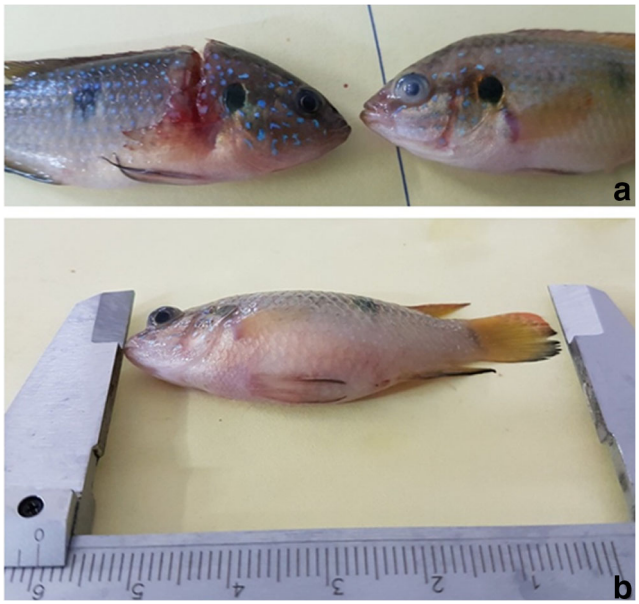

Fig. 3 Changes observed in fish exposed to MG. **a** Left fish: negative control with normal characteristics of pigmentation and absence of dilation in the eyes. Right fish treated with MG (0.75 mg/L) where depigmentation and swelling in the eyes is observed. **b** Abdominal swelling, depigmentation, and eye swelling in fish treated with 0.75 mg/L of MG

rate increased with the MG concentration. The value of the linear regression coefficient calculated for the obtained data ($R^2 = 0.995$) confirmed that there was a concentration-dependent relationship.

Physical and behavioral changes

Among the sublethally exposed and control fish, those exposed to the control condition and lower concentrations of MG (0.25 and 0.5 mg/L) were active and fed normally. In contrast, those exposed to the highest concentration (0.75 mg/L) did not feed well from the first day of the experiment. They also exhibited the following behavioral manifestations: loss of leakage reflex, slow lateral flaps, increased irritability, writhing, respiration on the surface of the aquarium, swiveling, and decreased flow rates. Physically, fish exposed to the highest dose of MG exhibited abdominal swelling due to fluid retention, depigmentation of the scales, and swelling of the eyes (Fig. 3).

Measurements of length, fish weight and liver weight

For sublethally exposed fish, Fig. 4 presents the total weight and length of them and the weight of the fish liver. The results indicate that the treated fish did not exhibit any between-group difference in weight or length, relative to the control (Fig. 4a and b), but there was a statistically significant increase in fish liver weight at the highest concentration of MG exposure (0.75 mg/L) (Fig. 4c).

Comet assay

The results of the comet assay indicated that there was a significant increase in the total and high damage index in the erythrocyte DNA of *H. bimaculatus* exposed to the highest sublethal concentration of MG (0.75 mg/L), compared with the other treatment groups (Fig. 5).

Micronucleus test

The MN test was evaluated by comparing the frequency of MNs found in the different tested concentrations of MG (0.25 mg/L, 0.50 mg/L, and 0.74 mg/L) in relation to the negative control. Table 4 shows the means and standard deviations of the frequency of MNs and NEAs for the different concentrations. The observed results did not show significant difference for any of the concentrations, being able to be visualized in the average of the frequency of MNs and NEAs, and confirmed by the ANOVA test (multiple Tukey comparisons) ($P < 0.05$).

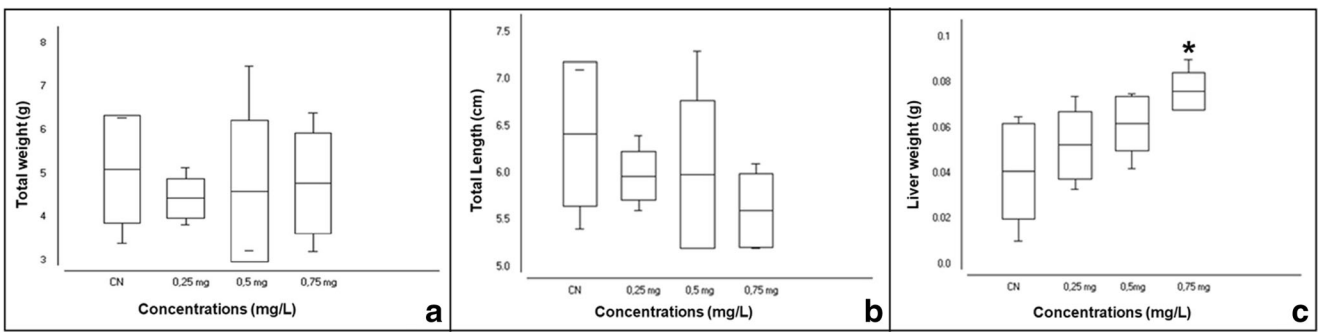

Fig. 4 Mean and standard deviation of weight and total length of animals and liver weight exposed to sublethal concentrations of MG. **a** Total weight. **b** Total length. **c** Liver weight. Parametric test one way ANOVA; multiple Tukey comparisons. *Differs statistically from the other treatments ($P < 0.05$)

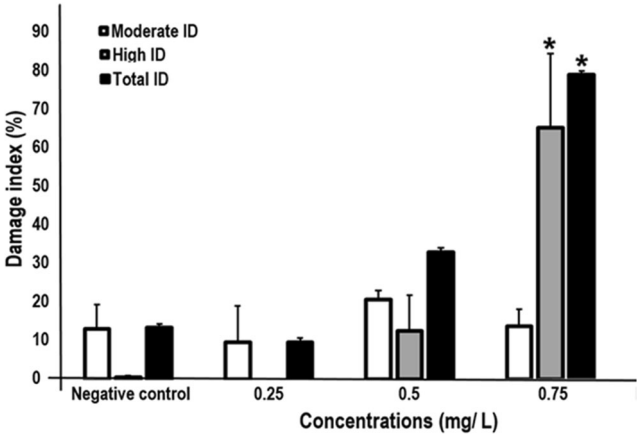

Fig. 5 Mean and standard deviation of the indices of moderate, elevated, and total damage in erythrocytes of *H. bimaculatus* exposed to MG in the Comet assay. Parametric test one way ANOVA; multiple Tukey comparisons. *Differs statistically from other treatments ($P < 0.05$)

Discussion

The realization of bioassays in fish gives important answers on the effects of MG on a main target organism. Here, we report that the LC_{50}-96 h value for *H. bimaculatus* is 1 mg/L of MG. The comparison with the corresponding values for other fish species shows that there is a large variation in the toxic effects of MG on fish (Table 2). This may reflect differences in the utilized experimental conditions, such as temperature and pH. The ionization constant (pK) of MG is 6.9. It is 100% ionized at pH 4.0, 50% ionized at pH 6.9, 25% at 7.4, and 0% at pH 10.1 (Goldacre and Phillips 1949). According to Kubitza and Kubtiza (2004), the tolerance of fish to MG is lower under high temperatures and high pH, although there are species- and fish size–related differences in this pattern. Thus, we might expect that the toxicity of MG would increase with the acidity of the water. Regarding temperature, fish are poikilothermic animals and their metabolic activities increase with temperature, suggesting that the toxic effects of MG could be more severe at higher temperatures.

The strong correlation observed herein ($R^2 = 0.995$) (Fig. 2) indicates that the MG concentration and exposure time can strongly influence the toxicity of this substance. Srivastava et al. (1995) also observed different LC_{50} values for MG in *Heteropneustes fossilis* at different exposure times. Thus, we propose that the between-study variations in MG toxicity (Table 2) are likely to reflect differences in the administration conditions. It is difficult to compare the toxicity of MG in different species given that the information was collected under various combinations of water temperature, pH, hardness, and dissolved oxygen (Schoettger 1970; Smith and Heath 1979; Gluth and Hanke 1983; Doerge et al. 1998).

Under the conditions tested in this work, we were able to determine the LC_{50} and examine the effects of sublethal doses of MG. We saw adverse effects only at the highest sublethal exposure concentration tested (0.75 mg/L). The observed behavioral changes, such as surface clogging and an unusual operculum beat, suggest that MG is likely to interfere with the respiratory system of fish, making it difficult for them to exchange gas with the environment. Other authors also detected respiratory discomfort in MG-treated fish of additional species, such as rainbow trout (Ross et al. 1985) and Nile tilapia (Omoregie et al. 1998). According to Werth and Boiteux (1968), the compound blocks respiratory enzymes.

The liver weight was increased in animals treated with 0.75 mg/mL of MG, suggesting that the treatment may affect this organ. Since the liver comes into direct contact with toxic substances that have entered the body, liver weight is considered to be an excellent marker of toxicity. An increase in the liver weight of rats treated with MG (100 ppm) was reported by Sundarrajan et al. (2000), who associated this change with N-nitrosodimethylamine that potentializes the induction of tumors in the liver of rats. In studies on rats fed with MG and LM, the authors observed histological injuries, such as necrosis and vacuolization of liver cells (Culp et al. 1999, 2002). The possible toxic effect of MG on the liver is highly relevant, since changes in this organ can lead to general health problems.

Another issue is related to nuclear abnormalities, described in the literature as an important indicator of genotoxicity (Ferraro et al. 2004; Özkan et al. 2011). It is known that the damage observed depends mainly on the type of pollutant involved and the species of fish exposed (Kousar and Javed 2015). In the present study, the species analyzed exposed to MG do not have a significant increase in nuclear abnormalities, although they are in higher number when compared with micronucleus. This is due to the presence of NEAs being brought together in a single counting point. Although the mechanisms responsible for the formation of nuclear abnormalities are not yet fully understood (Bolognesi et al. 2006; Çavaş 2008; Strunjak-Perovic et al. 2009), some authors have already described that several substances can interfere in the DNA synthesis of the exposed organism and thus result in this type of observation (Da Silva and Fontanetti 2006). An example is the study by de Campos Venturaet al. (2008) reporting

Table 4 Mean and standard deviation of micronuclei (MN) and nuclear erythrocytic alterations (NEAs) observed in *H. bimaculatus*, exposed to malachite green

Treatments	MN	NEAs
Negative control	0 (± 0)	0.58 (± 0.52)
Malachite green (0.25 mg/L)	0.05 (± 0.04)	0.24 (± 0.31)
Malachite green (0.50 mg/L)	0.09 (± 0.07)	0.53 (± 0.52)
Malachite green (0.75 mg/L)	0.02 (± 0.03)	0.61 (± 0.30)

ANOVA (multiple Tukey comparisons)

that invaginations, which were also observed in our work, may be the result of aneuploidies that occur due to tubulin failures. This failure makes the action of the mitotic spindle difficult, generating aneuploid cells with this type of abnormality after cell division (Fernandes et al. 2007).

At the DNA level, our comet assay showed that MG has genotoxic potential in the tested fish species. Unexpectedly, however, our analysis of micronuclei and erythrocytic nuclear alterations failed to find any significant treatment-related change. Two hypotheses may be suggested to explain this: (1) the DNA is repaired before a micronucleus is formed, or (2) the DNA-damaged cells enter apoptosis and do not complete another cell cycle, so no micronucleus is formed. We suggest that the latter possibility is more likely, as eukaryotic cells with high levels of DNA damage and errors tend to be earmarked for apoptosis (Bose et al. 2005; Alberts et al. 2008). According to Fessard et al. (1999), the bactericidal and cytotoxic activity of MG can hide mutagenic effects at higher concentrations. Even though cell death may be a beneficial event when programmed, excessive apoptosis may be indicative of toxicity caused by external agents (Grisolia et al. 2009). Therefore, we speculate that the breaks detected by the comet assay may reflect DNA fragmentation and that this induces the cells to undergo apoptosis.

Few previous reports have addressed the ability of MG to cause DNA fragmentation (Bose et al. 2005). To our knowledge, this is the first study to use the comet assay to examine the effect of MG on DNA in fish. Our review of previous reports on the cytotoxic, genotoxic, and carcinogenic effects of MG and LM in fish and mammals (Table 1) found that there are a number of contradictions in dosage weighing to the actual effect caused by the compound. It is possible that these variations reflect the lack of standardization in the exposure dosages. For example, Bergwerff et al. (2004) treated *Anguilla anguilla* with 0.1 mg/L of MG and observed no side effect or persistence of residues after 100 days. Niska et al. (2009) treated *Oncorhynchus mykiss* eggs with 1 mg/L, 3 mg/L, and 6 mg/L and failed to observe any teratogenic effect after hatching. However, Yonar and Yonar (2010) observed immunosuppression and oxidative stress in *Oncorhynchus mykiss* treated with 1/15,000 (66.7 mg/L) and 1/150,000 (6.67 mg/L) of MG. Nebbia et al. (2017) treated *Oncorhynchus mykiss* with 25 μM (9.12 mg/L), 50 μM (18.25 mg/L), and 100 μM (36.50 mg/L) of MG and found that it had potent in vitro inhibitory effects on hepatic metabolism. Thus, the concentrations applied in these studies varied widely, from 0.1 mg/L to 66.7 mg/L, likely explaining the differences in the obtained results. When studying sublethal effects, it is important to determine an appropriate concentration range. Here, we first determined the LC_{50} in our model organism and then used this information to examine sublethal effects. It was possible to observe that in many studies, the investigators did not to establish the LC_{50} for the organism, being therefore a failure to determine the effect in sublethal dosages.

Although GM is not approved by Food and Drug Administration (FDA) for or use in aquaculture, its low cost and high efficacy in the control and prevention of zoonoses mean that it is often used (and potentially abused) in the production of fish for both the aquarium trade and food consumption (Yonar and Yonar 2010). Therefore, it is important to promote actions that define the safe use of MG, which can have cytotoxic and mutagenic effects and cause serious problems when used at inappropriate concentrations. In addition, environmental pollution and contamination of exposed fish and people may be recurring problems associated with its indiscriminate use. The use of activated charcoal filters has been shown to reduce the risk of adverse effects from MG, in addition to having antiparasitic effects (Bills et al. 1977; Niska et al. 2009).

We note that all of the significant adverse effects observed herein were seen only at the highest tested sublethal concentration of MG (0.75 mg/L). No adverse effect was seen in fish treated with the lower doses of 0.25 mg/L and 0.5 mg/L. At the higher dose, however, the damage was severe, indicating that we must take care in using this compound. Previous studies found that MG and ML had dose-dependent effects on DNA (Culp et al. 1999) and that MG dose dependently induced severe cell death in the intersomitic region of the *Danio rerio* embryo (Jang et al. 2009). Biosafety tests regarding the carcinogenicity of MG and LM indicate that these compounds can dose dependently trigger tumor formation (Mittelstaedt et al. 2004) and LM was reported to dose dependently increase skeletal abnormalities in rats (Wan et al. 2011). The meticulous work of Bassleer (2009) in several species of ornamental fish suggested that the maximum safe dose of MG is 0.2 mg/L. Therefore, adherence to appropriate sanitary criteria for ornamental fish would seem likely to guard against possible MG toxicity.

Conclusion

Above all, our findings emphasize that more research is needed to clarify the effects of MG on various relevant parameters beyond those examined herein. This will help ensure that the use of MG, when allowed, can be properly managed. We must seek to maximize the effectiveness of MG without damaging treated organisms and the environment.

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References

Alberts B, Wilson J, Hunt T (2008) Molecular biology of the cell. Garland Science

Ayres M, Ayres Jr M, Ayres DL, Santos AS (2007) BioEstat 5.0 – Aplicações estatísticas nas áreas das Ciências Biológicas e Médicas. Belém: Sociedade Civil Mamirauá, 5th edn. p 290

Bassleer G (2009) The new illustrated guide to fish diseases in ornental tropical and pond fish, 3rd Edition. Bassleer Biofish, Westmeerbeek

Bell DM (1982) Physiological and psychophysical spectral sensitivities of the cichlid fish, Hemichromis bimaculatus. J Exp Zool 223:29–32. https://doi.org/10.1002/jez.1402230105

Bergwerff AA, Kuiper RV, Scherpenisse P (2004) Persistence of residues of malachite green in juvenile eels (Anguilla anguilla). Aquaculture 233:55–63. https://doi.org/10.1016/j.aquaculture.2003.11.013

Bhosale MM, Felix S, Aruna S, et al (2017) Induced breeding of African jewelfish Hemichromis bimaculatus (Gill, 1862) using gonadotropic signaling molecular analogue WOVA-FH. ~ 2547 ~ J Entomol Zool Stud 5

Bills TD, Marking LL, Chandler JH Jr (1977) Malachite green: its toxicity to aquatic organisms, persistence, and removal with activated carbon. US Fish Wildl Serv

Bolognesi C, Perrone E, Roggieri P, Pampanin DM, Sciutto A (2006) Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions. Aquat Toxicol 78:S93–S98. https://doi.org/10.1016/j.aquatox.2006.02.015

Bose B, Motiwale L, Rao KVK (2005) DNA damage and G2/M arrest in Syrian hamster embryo cells during malachite green exposure are associated with elevated phosphorylation of ERK1 and JNK1. Cancer Lett 230:260–270. https://doi.org/10.1016/j.canlet.2005.01.001

Carrasco KR, Tilbury KL, Myers MS (1990) Assessment of the piscine micronucleus test as an in situ biological indicator of chemical contaminant effects. Can J Fish Aquat Sci 47:2123–2136. https://doi.org/10.1139/f90-237

Cavalcante DGSM, Martinez CBR, Sofia SH (2008) Genotoxic effects of roundup® on the fish Prochilodus lineatus. Mutat Res Genet Toxicol Environ Mutagen 655:41–46. https://doi.org/10.1016/j.mrgentox.2008.06.010

Çavaş T (2008) In vivo genotoxicity of mercury chloride and lead acetate: micronucleus test on acridine orange stained fish cells. Food Chem Toxicol 46:352–358. https://doi.org/10.1016/j.fct.2007.08.015

Collins AR, Ai-guo A, Duthie SJ (1995) The kinetics of repair of oxidative DNA damage (strand breaks and oxidised pyrimidines) in human cells. Mutat Res 336:69–77

Collins AR, Dusinská M, Horská A (2001) Detection of alkylation damage in human lymphocyte DNA with the comet assay. Acta Biochim Pol 48:611–614

Culp SJ, Blankenship LR, Kusewitt DF, Doerge DR, Mulligan LT, Beland FA (1999) Toxicity and metabolism of malachite green and leucomalachite green during short-term feeding to Fischer 344 rats and B6C3F1 mice. Chem Biol Interact 122:153–170. https://doi.org/10.1016/S0009-2797(99)00119-2

Culp SJ, Beland FA, Heflich RH, Benson RW, Blankenship LR, Webb PJ, Mellick PW, Trotter RW, Shelton SD, Greenlees KJ, Manjanatha MG (2002) Mutagenicity and carcinogenicity in relation to DNA adduct formation in rats fed leucomalachite green. Mutat Res Fundam Mol Mech Mutagen 506–507:55–63. https://doi.org/10.1016/S0027-5107(02)00152-5

Da Silva ST, Fontanetti CS (2006) Micronucleus test and observation of nuclear alterations in erythrocytes of Nile tilapia exposed to waters affected by refinery effluent. Mutat Res Toxicol Environ Mutagen 605:87–93. https://doi.org/10.1016/j.mrgentox.2006.02.010

de Campos Ventura B, de Angelis DF, Marin-Morales AMA (2008) Mutagenic and genotoxic effects of the Atrazine herbicide in *Oreochromis niloticus* (Perciformes, Cichlidae) detected by the micronuclei test and the comet assay. Pestic Biochem Phys 90(1):42–51

Doerge DR, Churchwell MI, Gehring TA, Pu YM, Plakas SM (1998) Analysis of malachite green and metabolites in fish using liquid chromatography atmospheric pressure chemical ionization mass spectrometry. Rapid Commun Mass Spectrom 12:1625–1634. https://doi.org/10.1002/(SICI)1097-0231(19981115)12:21<1625::AID-RCM373>3.0.CO;2-I

Fernandes TCC, Mazzeo DEC, Marin-Morales MA (2007) Mechanism of micronuclei formation in polyploidizated cells of Allium cepa exposed to trifluralin herbicide. Pestic Biochem Physiol 88:252–259. https://doi.org/10.1016/j.pestbp.2006.12.003

Ferraro MVM, Fenocchio AS, Mantovani MS, Ribeiro CO, Cestari MM (2004) Mutagenic effects of tributyltin and inorganic lead (Pb II) on the fish H. malabaricus as evaluated using the comet assay and the piscine micronucleus and chromosome aberration tests. Genet Mol Biol 27:103–107. https://doi.org/10.1590/S1415-47572004000100017

Fessard V, Godard T, Huet S, Mourot A, Poul JM (1999) Mutagenicity of malachite green and leucomalachite green in in vitro tests. J Appl Toxicol 19:421–430. https://doi.org/10.1002/(SICI)1099-1263(199911/12)19:6<421::AID-JAT595>3.0.CO;2-6

Gluth G, Hanke W (1983) The effect of temperature on physiological changes in carp, Cyprinus carpio L., induced by phenol. Ecotoxicol Environ Saf 7:373–389. https://doi.org/10.1016/0147-6513(83)90003-9

Goldacre RJ, Phillips JN (1949) 370. The ionization of basic triphenylmethane dyes. J Chem Soc 0:1724. https://doi.org/10.1039/jr9490001724

Grisolia CK, Oliveira-Filho EC, Ramos FR, Lopes MC, Muniz DHF, Monnerat RG (2009) Acute toxicity and cytotoxicity of Bacillus thuringiensis and Bacillus sphaericus strains on fish and mouse bone marrow. Ecotoxicology 18:22–26. https://doi.org/10.1007/s10646-008-0252-7

Hartmann A, Speit G (1997) The contribution of cytotoxicity to DNA-effects in the single cell gel test (comet assay). Toxicol Lett 90:183–188. https://doi.org/10.1016/S0378-4274(96)03847-7

Jang GH, Park IS, Lee SH, Huh TL, Lee YM (2009) Malachite green induces cardiovascular defects in developing zebrafish (Danio rerio) embryos by blocking VEGFR-2 signaling. Biochem Biophys Res Commun 382:486–491. https://doi.org/10.1016/j.bbrc.2009.01.118

Kousar S, Javed M (2015) Studies on induction of nuclear abnormalities in peripheral blood erythrocytes of fish exposed to copper. 886:879–886. https://doi.org/10.4194/1303-2712-v15

Kubitza F, Kubtiza L (2004) Principais parasitoses e doenças dos peixes cultivados, 4ª Edição

Manjanatha MG, Shelton SD, Bishop M, Shaddock JG, Dobrovolsky VN, Heflich RH, Webb PJ, Blankenship LR, Beland FA, Greenlees KJ, Culp SJ (2004) Analysis of mutations and bone marrow micronuclei in big blue® rats fed leucomalachite green. Mutat Res Fundam Mol Mech Mutagen 547:5–18. https://doi.org/10.1016/j.mrfmmm.2003.11.009

Melo KM, Oliveira R, Grisolia CK, Domingues I, Pieczarka JC, de Souza Filho J, Nagamachi CY (2015) Short-term exposure to low doses of rotenone induces developmental, biochemical, behavioral, and histological changes in fish. Environ Sci Pollut Res 22:13926–13938. https://doi.org/10.1007/s11356-015-4596-2

Mittelstaedt RA, Mei N, Webb PJ, Shaddock JG, Dobrovolsky VN, McGarrity LJ, Morris SM, Chen T, Beland FA, Greenlees KJ, Heflich RH (2004) Genotoxicity of malachite green and leucomalachite green in female big blue B6C3F1 mice. Mutat Res Genet Toxicol Environ Mutagen 561:127–138. https://doi.org/10.1016/j.mrgentox.2004.04.003

Nebbia C, Girolami F, Carletti M, Gasco L, Zoccarato I, Giuliano Albo A (2017) In vitro interactions of malachite green and leucomalachite green with hepatic drug-metabolizing enzyme systems in the rainbow trout (Onchorhyncus mykiss). Toxicol Lett 280:41–47. https://doi.org/10.1016/j.toxlet.2017.07.900

Niska K, Korkea-aho T, Lindfors E, Kiuru T, Tuomainen M, Taskinen J, Peltonen K (2009) Disappearance of malachite green residues in fry of rainbow trout (Oncorhynchus mykiss) after treatment of eggs at the hatching stage. Aquaculture 297:25–30. https://doi.org/10.1016/j.aquaculture.2009.08.037

OECD - (Organisation for Economic Co-operation and Development) (1992) Guideline for Testing of Chemicals, 203. Fish, acute toxicity test. OECD, Paris, France Available at: <www.oecd.org>

Omoregie E, Ofojekwu PC, Anosike JC, Adeleye AO (1998) Acute toxicity of malachite green to the Nile tilapia, Oreochromis niloticus (L.). J Aquac Tropics 13:233–237

Özkan F, Gündüz SG, Berköz M, Özlüer Hunt A (2011) Induction of micronuclei and other nuclear abnormalities in peripheral erythrocytes of Nile tilapia, Oreochromis niloticus, following exposure to sublethal cadmium doses. Turkish J Zool 35:585–592. https://doi.org/10.3906/zoo-0907-77

Rao KVK, Mahudawala DM, Redkar AA (1998) Malignant transformation of Syrian hamster embryo (SHE) cells in primary culture by malachite green: transformation is associated with abrogation of G2/M checkpoint control. Cell Biol Int 22:581–589. https://doi.org/10.1006/cbir.1998.0300

Ross LG, Ward KMH, Ross B (1985) The effects of formalin, malachite green and suspended solids on the respiratory activity of rainbow trout, Salmo gairdneri Richardson. Aquac Res 16:129–138. https://doi.org/10.1111/j.1365-2109.1985.tb00302.x

Rowland WJ (1975) The effects of dummy size and color on behavioral interaction in the Jewel cichlid, Hemichromis bimaculatus gill. Behaviour 53:109–125

Ruider S, Schmahl G, Mehlhorn H, Schmidt H, Ritter G (1997) Effects of different malachite green derivatives and metabolites on the fish ectoparasite, Ichthyophthirius multifiliis, Fouquet 1876 (Hymenostomatida, Ciliophora). Eur J Protistol 33:375–388. https://doi.org/10.1016/S0932-4739(97)80049-1

Schmahl G, Ruider S, Mehlhorn H et al (1992) Treatment of fish parasites - 9. Effects of a medicated food containing malachite green on Ichthyophthirius multifiliis Fouquet, 1876 (Hymenostomatida, Ciliophora) in ornamental fish. Parasitol Res 78:183–192. https://doi.org/10.1007/BF00931725

Schoettger RA (1970) Toxicology of thiodan in several fish and aquatic invertebrates. Investig Fish Control 35:2–31

Silva-Pereira LC, Cardoso PCS, Leite DS, Bahia MO, Bastos WR, Smith MAC, Burbano RR (2005) Mercury genotoxicity on human lymphocyte cultures. Braz J Med Biol Res 38:901–907

Smith MJ, Heath AG (1979) Acute toxicity of copper, chromate, zinc, and cyanide to freshwater fish: effect of different temperatures. Bull Environ Contam Toxicol 22:113–119. https://doi.org/10.1007/BF02026917

Srivastava SJ, Singh ND, Srivastava AK, Sinha R (1995) Acute toxicity of malachite green and its effects on certain blood parameters of a catfish, Heteropneustes fossilis. Aquat Toxicol 31:241–247. https://doi.org/10.1016/0166-445X(94)00061-T

Stammati A, Nebbia C, De Angelis I et al (2005) Effects of malachite green (MG) and its major metabolite, leucomalachite green (LMG), in two human cell lines. Toxicol in Vitro 19:853–858. https://doi.org/10.1016/j.tiv.2005.06.021

Strunjak-Perovic I, Coz-Rakovac R, Topic Popovic N, Jadan M (2009) Seasonality of nuclear abnormalities in gilthead sea bream Sparus aurata (L.) erythrocytes. Fish Physiol Biochem 35:287–291. https://doi.org/10.1007/s10695-008-9208-3

Sundarrajan M, Fernandis AZ, Subrahmanyam G, Prabhudesai S, Krishnamurthy SC, Rao KVK (2000) Overexpression of G1/S cyclins and PCNA and their relationship to tyrosine phosphorylation and dephosphorylation during tumor promotion by metanil yellow and malachite green. Toxicol Lett 116:119–130. https://doi.org/10.1016/S0378-4274(00)00216-2

Wan H, Weng S, Liang L, Lu Q, He J (2011) Evaluation of the developmental toxicity of leucomalachite green administered orally to rats. Food Chem Toxicol 49:3031–3037. https://doi.org/10.1016/j.fct.2011.10.003

Werth G, Boiteux A (1968) Toxicity of triphenylmethane dyes. Malachite green as an uncoupling agent of oxidative phosphorylation in vivo and in vitro. Arch Toxikol 23:82–103

Yonar ME, Yonar SM (2010) Changes in selected immunological parameters and antioxidant status of rainbow trout exposed to malachite green (Oncorhynchus mykiss, Walbaum, 1792). Pestic Biochem Physiol 97:19–23. https://doi.org/10.1016/j.pestbp.2009.11.009