Comparative Biochemistry and Physiology, Part C

Textile dyes Maxilon blue 5G and Reactive blue 203 induce acute toxicity and DNA damage during embryonic development of Danio rerio

--Manuscript Draft--

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Corresponding Author:	Mehmet Harbi Calimli Igdir University Igdir, Igdir TURKEY
First Author:	Mine KÖKTÜRK
Order of Authors:	Mine KÖKTÜRK
	Fikret ALTINDAĞ
	Gunes Ozhan
	Mehmet Harbi Calimli
	Mehmet Salih Nas
Abstract:	Common textile dyes used in various industrial sectors are organic compounds and considered for the aquatic environment as pollutants. The textile dye industry is one of the main sectors that have serious impacts on the environment due to a large amount of wastewater released into the ecosystem. Maxilon blue 5G (MB-5G) and Reactive Blue 203 (RB-203) are widely used textile dyes. However, their potential toxicity on living organisms remains to be elucidated. Here, we investigate the acute toxicity and genotoxicity of MB-5G and RB-203 dyes using the zebrafish embryos/larvae. Embryos treated with each dye for 96 h revealed LC 50 values of acute toxicity as 166.04 mg L -1 and 278.32 mg L -1 for MB-5G and RB 203, respectively. When exposed to MB-5G and RB-203 at different concentrations (1, 10, and 100 mg L -1) for 96h, the expression of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, significantly increased in brain tissues as compared to control. MB-5G and RB-203 resulted in common developmental abnormalities including tail malformation, microphthalmia, pericardial edema, curved body axis, and yolk sac/pericardial edemas. Moreover, at its highest dose (100 mg L -1), RB-203 caused premature hatching after 48 hours, while MG-5G did not. Our results collectively reveal that the textile dyes MB-5G and RB-203 cause genotoxicity and teratogenicity during embryonic and larval development of zebrafish. Thus, it is necessary to eliminate these compounds from wastewater or reduce their concentrations to safe levels before discharging the textile industry wastewater into the environment.
Suggested Reviewers:	Muhammed ATAMANALP Ataturk University: Ataturk Universitesi mataman@atauni.edu.tr Prof. ATAMANALP is an expert of toxicology.
	Fikret TÜRKAN Igdir University: Igdir Universitesi fikret.turkan@gmail.com Dr. TÜRKAN has many studies related to toxicology.
	Ercan BURSAL Muş Alparslan Üniversitesi: Mus Alparslan Universitesi e.bursal@alparslan.edu.tr Prof. BURSAL is an expert in Biochemistry.
	Jianchao Liu College of Environment, Hohai University jianchao-liu@hhu.edu.cn

	Dr. Liu has many papers related to aquatic toxicology.
	Jae-Seong Lee Sungkyunkwan University jslee2@skku.edu Dr. Lee is an expert in ecotoxicology.
	Xueping Zhao Zhejiang Academy of Agricultural Sciences zhaoxueping@tom.com Prof. Zhao is an expert in toxicological studies.
Opposed Reviewers:	
Response to Reviewers:	Reviewer 2: The authors have well-revised according to reviewer's suggestion in the revised manuscript. However, there are still some issues that need to be considered before publication of this manuscript. Many thanks for your favor to our work. With your comments, I think everything is clear in the new version and I appreciate this and also for your valuable comments. Hope to be convincing. 1. It is true that there are no references to assessed toxicity of the two dyes, however, as the authors have shown toxicity (LC50) which were 166.04 and 278.32 mg/L, respectively, the experiment should have been performed under these two concentrations to understand molecular toxicity. 1000 mg/L is too high. We understand the concerns of the reviewer. However, since 100 mg/L is a sublethal dose for both dyes we believe that it is an appropriate concentration for our experiments. We also agree with the reviewer that 1000 mg/L is a very high dose; thus we excluded it from the revised version of the manuscript. 2. There is still lack of discussion on mechanistic point of view on toxicity of the two dyes. According to your comment, we have revised the discussion section and the following explanations were added to the manuscript to explain the mechanistic point of on the toxicity of the dyes. The neurotoxic effects of dyes depend on the sensitivity of the brain to oxidative stress. High oxygen consumption rate, high polyunsaturated fatty acids content, regional high iron levels, and proportionately low antioxidant capacity are among the key factors that determine the reaction of the brain to oxidative stress (Noseworthy and Bray, 1998; Meireles et al., 2018). The mechanism of the effect of RB-203 and MB-5G dyes remains largely unknown. However, it is possible that the high 8-OHdG activity in the brain is not only caused by high doses of the dyes but also due to the cationic state of MB-5G that may cause toxicity by binding to the negatively charged DNA with high affinity (Alkan et al., 2008; Dezhampanah et al., 2019). It is also known that binding molecules with a cationic structure can interact with DNA by forming hydrogen bonds with base pairs in small grooves in the DNA structure (Rehman et al., 2014). There is evidence that textile dyes and products of electrolysis can generate DNA damage by interfering with the double helix structure (Uliana et al., 2013). Future studies will not only clarify the molecular mechanisms underlying the toxic effect of these dyes but will also pave the way for mitigating these undesirable impacts in the marine environment. Also, please check English mistakes. Thank you very much for your precision. According to your comment, we have revised the whole manuscript carefully and tried to avoid any grammar or syntax errors (please find the corrected part in green). In addition, we have asked several colleagues who are skilled authors of English language papers to check the English. We hope that the language to be acceptable.

Dear Prof. Martin Grosell

We are excited to submit our recent work entitled, "Textile dyes Maxilon blue 5G and Reactive blue 203 induce acute toxicity and DNA damage during embryonic development of Danio rerio" authored by Mine KÖKTÜRK, Fikret ALTINDAĞ, Gunes OZHAN, Mehmet Salih NAS and me, for publication consideration in *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*. The authors have complied with Elsevier's ethical requirements: the work described has not been published, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out. The submission also implies that, if accepted, it will not be published elsewhere in the same form, in English or any other language, without the written consent of the Publisher. We believe that our recent work is fundamentally important and has all the qualities to provide a great step in advancement. As such, we hope that the popularity of the *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology* provides the opportunity for the dissemination of this work to a broad research community working on the effects of dyestuffs onto the acute toxicity and genotoxicity of dyes. In the meantime, please do not hesitate to contact us, if you have any questions regarding our work and this paper.

Kind regards

Dr. Mehmet Harbi ÇALIMLI

Health Services Vocational School, Igdır University,76000-Iğdır, Turkey

Phone : +90 476 223 00 10/3130

Fax: +90 476 223 00 54

E-mail: calimli.6500@gmail.com

mharbi.calimli@igdir.edu.tr

We do respectfully request that the following scientists can review our manuscript as suggested reviewers;

Dear Prof. Dr. Martin Grosell,

CBPC-D-20-00550

First of all, I would like to thank you and all reviewers of our manuscript (CBPC-D-20-00550). Their comments have helped us to improve readability and the general level of the work. We have modified the sections according to suggestions made by reviewers and editors. In addition, We have carefully double-checked the manuscript taking account of their suggestions.

We have revised the manuscript accordingly, and detailed corrections are listed below point by point. *Please note that the responses or explanations are written in green colors.*

We are looking forward to hearing your positive response. If you have further questions please feel free to ask us.

Best Regards
Dr. Mehmet Harbi ÇALIMLI
Health Services Vocational School, Igdır University,76000-Iğdır, Turkey
Phone : +90 476 223 00 10/3130
Fax: +90 476 223 00 54
E-mail: calimli.6500@gmail.com
mharbi.calimli@igdir.edu.tr

Reviewer 2:

The authors have well-revised according to reviewer's suggestion in the revised manuscript. However, there are still some issues that need to be considered before publication of this manuscript.

Many thanks for your favor to our work. With your comments, I think everything is clear in the new version and I appreciate this and also for your valuable comments. Hope to be convincing.

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Textile dyes Maxilon blue 5G and Reactive blue 203 induce acute toxicity and DNA damage during embryonic development of *Danio rerio*

Mine KÖKTÜRK[1], Fikret ALTINDAĞ[2], Gunes OZHAN[3,4], Mehmet Harbi ÇALIMLI[5*], Mehmet Salih NAS[6]

[1]Department of Organic Farming, College of Applied Sciences, Igdir University, Igdir, Turkey

[2]Department of Histology and Embryology, Medical School, Van Yüzüncü Yıl University, Van, Turkey

[3]Izmir Biomedicine and Genome Center, Dokuz Eylul University Health Campus, Izmir, Turkey

[4]Izmir International Biomedicine and Genome Institute, Dokuz Eylul University, Izmir, Turkey

[5] Department of Medical Services and Techniques, Tuzluca Vocational School, Igdır University, Igdir, Turkey

[6]Department of Environmental Engineering, Faculty of Engineering, Igdır University, Igdir, Turkey

Highlights

- Maxilon Blue 5G (MB-5G) and Reactive Blue 203 (RB-203) exert acute toxicity on zebrafish embryos/larvae
- MB-5G exhibits no changes in the hatching rate, but RB-203 may lead to premature hatching
- MB-5G and RB-203 induce dose-related oxidative DNA damage in the larval zebrafish brain.

ABSTRACT: Common textile dyes used in various industrial sectors are organic compounds and considered for the aquatic environment as pollutants. The textile dye industry is one of the main sectors that have serious impacts on the environment due to a large amount of wastewater released into the ecosystem. Maxilon blue 5G (MB-5G) and Reactive Blue 203 (RB-203) are widely used textile dyes. However, their potential toxicity on living organisms remains to be elucidated. Here, we investigate the acute toxicity and genotoxicity of MB-5G and RB-203 dyes using the zebrafish embryos/larvae. Embryos treated with each dye for 96 h revealed LC_{50} values of acute toxicity as 166.04 mg L^{-1} and 278.32 mg L^{-1} for MB-5G and RB 203, respectively. When exposed to MB-5G and RB-203 at different concentrations (1, 10, and 100 mg L^{-1}) for 96h, the expression of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, significantly increased in brain tissues as compared to control. MB-5G and RB-203 resulted in common developmental abnormalities including tail malformation, microphthalmia, pericardial edema, curved body axis, and yolk sac/pericardial edemas. Moreover, at its highest dose (100 mg L^{-1}), RB-203 caused premature hatching after 48 hours, while MG-5G did not. Our results collectively reveal

that the textile dyes MB-5G and RB-203 cause genotoxicity and teratogenicity during embryonic and larval development of zebrafish. Thus, it is necessary to eliminate these compounds from wastewater or reduce their concentrations to safe levels before discharging the textile industry wastewater into the environment.

Keywords: Maxilon blue 5G; Reactive blue 203**,** 8-OHdG; DNA damage; Zebrafish

Introduction

Today, water pollution based on dyes increased with the growth of colorant activities for textile, leather, food, and agrochemical industries (Hernández-Zamora and Martínez-Jerónimo, 2019a). The textile industries have a significant role in the economy of many countries (Panda et al., 2006). However, due to industrial activities, large amounts of wastewater containing dyes are released into the environment, creating serious problems for aquatic and terrestrial organisms in terms of environmental management (Oliveira et al., 2009; Leme et al., 2015; Nas et al., 2019; Barathi et al., 2020). Water pollution due to textile dyeing causes adverse conditions such as light penetration, decreased photosynthetic activity, lack of oxygen, and change of life cycle in natural organisms (Hernández-Zamora et al., 2014). Generally, the color of a dye can be noticed when its concentration is above 1 mg L^{-1}. However, most dyes have been detected at concentrations of 300 mg L^{-1} in the wastewaters originated from textile activities (Jonstrup et al., 2011; Meng et al., 2012). Approximately 15-50 % of the dyes used in textile activities are released into the aquatic environment (Nojavan et al., 2013). This amount has been reported to be above 50 mg L^{-1} in wastewater, depending on the type of dye and the treatment performed (O'Neill et al., 1999; Nojavan et al., 2013). Synthetic dyes containing the azo-aromatic chromophore group is the largest class of chemical dyes used worldwide (Guaratini and Zanoni, 2000). Due to their high stability, azo-aromatic dyes create permanent pollution and prevent the removal or reduction of toxicity in industrial wastewater containing dye via conventional treatment (O'Neill et al., 1999; Chung, 2000).

Textile dyes can cause mass deaths of species, especially in fish, deteriorate the ecological balance and reduce the self-cleaning capacity of the water bodies that receive these dyes, thus leading to the depletion of water bodies. Several studies have reported the toxic effects of textile dyes on zebrafish embryos (Abe *et al.* 2017, 2018; Meireles *et al.* 2018; Hernández-Zamora and Martínez-Jerónimo 2019a). Some dyes cause genetic damages including mutagenicity and carcinogenicity (Balakrishnan *et al.* 2016, Abe *et al.* 2018, Carvalho da Cruz Brambilla *et al.* 2019). Although the dyes are produced in powder form, they are dissolved in water to be used in the dyeing processes of fabrics. Therefore, textile production facilities cause water pollution that causes serious environmental problems to aquatic biota (Kim, 2001; Kaur et al., n.d.; Puvaneswari et al., 2006; Talaiekhozani et al., 2020). The discovery of textile dyes, especially reactive dyes, and their use in cellulosic fabrics is a breakthrough for the textile industry (Zollinger, 2003). These reactive dyes are known to cause skin irritation, asthma, and allergic reactions (Hunger, 2007; Khattab et al., 2020). Especially Reactive Blue 203 (RB-203) is one

of the most commonly used dyes in the textile industry and a hazardous compound that is found in large amounts of textile wastewater (Ashtekar et al., 1970; Talaiekhozani et al., 2019; Bagheri et al., 2020). Maxilon series consists of mono azo dyes and various textile dyes. As a cationic dye, Maxilon Blue 5G (MB-5G) has been commonly used in histochemical studies and contains various aromatic radicals and aliphatic chemical compounds (de Almeida, 1960). To reveal the toxic mechanism of textile dyes, it is also necessary to determine the toxicity of their degradation products. However, no studies related to the toxicological effects of MB-5G and RB-203 or their degradation products on organisms have been reported to date. Some textile dyes can be carcinogenic without breaking down into aromatic amines (Miller and Miller, 1948). Nevertheless, the carcinogenicity of many dyes is due to their breakdown products such as benzidine and p-phenylenediamine that have been reported to cause various allergies and tumors in humans and animals (K.T. Chung, 2016a; K. T. Chung, 2016b). Exposure to benzidine induces widespread apoptosis in the zebrafish brain and dorsal neurons, resulting in the development of an abnormal telencephalon (Chen et al., 2014). Similarly, some researchers reported that benzidine caused cell proliferation and apoptosis in liver tissues in fish of Gambusia affinis (Lentz et al., 2010).

DNA damage is one of the most important consequences of oxidative stress. 8-Hydroxy-2′-deoxyguanosine (8-OHdG), a major product of oxidative DNA damage, is induced by reactive oxygen species (ROS) that have adverse effects such as mutation and cell death (Anjana Vaman et al., 2013; Meng et al., 2014; Valavanidis et al., 2009; Zhang et al., 2014; Alak et al., 2017). Besides, 8-OHdG is widely used as an important biomarker for the neurotoxic effects of xenobiotics in aquatic organisms (Topal et al., 2017; Gyimah et al., n.d.; Sökmen et al., 2020). Zebrafish embryos and larvae are commonly used as a platform to assess the toxic effects of textile dyes (Shen et al., 2015; de Oliveira et al., 2016; Hernández-Zamora and Martínez-Jerónimo, 2019a; Meireles et al., 2018). Owing to its distinctive features including small size, embryo transparency, rapid development, low-cost maintenance, and high genetic, anatomical, and physiological homology to mammals zebrafish provides a convenient model of toxicology that allows for easy physiological evaluation *in vivo* (Schaeck et al., 2013; Gutiérrez-Lovera et al., 2019), (Schlegel, 2016; Ijaz and Hoffman, 2016; Sant and Timme-Laragy, 2018; James et al., 2019; Köktürk et al., 2020). In this study, we aim at unraveling the potentially toxic and genotoxic effects of MB-5G and RB-203 dyes using the zebrafish embryos and larvae.

2. MATERIAL AND METHODS

2.1. Textile dyes

Maxilon Blue 5G (MB-5G) ($C_{16}H_{26}N_3O$, 266 g mol^{-1}) and Reactive Blue 203 (RB-203) ($C_{28}H_{29}N_5Na_4O_{21}S_6$, 617.54g mol^{-1}) were purchased from Setaş and Eksoy Textile Co. (Bursa, Turkey). MB-5G and RB-203 were of analytical grade with a purity > 99.5 % and used without any purification. MB-5G is a cationic dye (Alkan et al., 2008) and cationic dyes can react with anionic substances due to the cationic region on their surface. RB-203 is an anionic dye (Bagheri et al., 2020) and contains anionic regions on its surface. RB-203 reacts with cationic substances in these anionic regions.

2.2. Zebrafish maintenance and ethics statement

Adult wild-type zebrafish were maintained in the Zebrafish Facility of Izmir Biomedicine and Genome Center at about 28°C with a 14-h light/10-h dark cycle, and bred according to standard procedures (Westerfield, 1995). Fish were fed twice a day (9 a.m - 3 p.m) with flake food: TetraMin Tropical Flakes (48% protein, 8% fat, and 2% fiber) in the morning and artemia in the afternoon. The zebrafish larvae used in the study were younger than 5-days-old. Thus, the study does not require any license (Directive 86/609/EEC and EU Directive, 2010/63/EU).

2.3. Experimental design

The study was planned according to a semi-static test using 4 repeats (Ensibi et al., 2014). The experiment consisted of 5 groups with 30 embryos each: one control and four repeats of treatment. Embryos were kept in the E3 medium (0.17 mM KCI, 0.33 mM $MgSO_4$, 5 mM NaCI, 0.33 mM $CaCI_2$). The dye solutions were prepared in E3 medium without the addition of other solvents. Different concentrations of RB-203 and MB-5G dyes (1, 10, 100, and 1000 mg L^{-1}) were applied to the embryos and the limiting pretest was performed for LC_{50} values. The embryos were transferred to six-well plates and RB203 and MB5G dyes were applied at concentrations of 1, 10, and 100 mg L^{-1} in 5 mL of E3 for 4 to 96 h (Westerfield, 1995). In the evening, males and females were placed in mating tanks, separated by a transparent plastic divider. At 9:00 in the morning, the divider was removed to allow zebrafish to mate and spawn. The eggs were collected in Petri dishes containing E3 medium. On the first day of the experiment, randomly selected eggs were transferred to an E3 medium containing the dyes at 4 hours-post-fertilization (hpf) (Köktürk et al. 2020). The culture medium was completely refreshed every 24 h. All experimental groups were incubated at 28.5°C.

2.4. Assessment of acute toxicity and mortality

The embryos and larvae of all groups were visualized under a stereomicroscope (SZX10 Olympus microscope with SC50 Olympus camera) at 24, 48, 72, and 96 h after the addition of the dyes. Larvae were recorded as dead if they did not survive until 24 hpf or no heartbeat was observed at 24 hpf.

2.5. Hatching rate

The zebrafish larvae normally hatch around 48 hours-post-fertilization (hpf). The effect of RB-203 and MB-5G dyes on larval hatching was observed and quantified in the 48- 96 hour-interval.

2.6. Histopathological examination

Larvae at 96 hpf were used for brain histological examination with minor changes on the protocols described previously (Hallare et al., n.d.; Hill et al., 2002; Li et al., n.d.; Sabaliauskas et al., n.d.). In this regard, 10 larvae that were contacted with MB-5G and RB-203 dyes and showed various malformations were selected. For brain histopathological evaluation, zebrafish larvae were fixed in a 10% neutral buffered formalin. After passing through routine histological tissue follow-up steps, zebrafish larvae were embedded in paraffin (Copper et al., 2018). Whole larval sections of 4 µm were cut in the microtome (Leica, RM2125, China). The sections were stained with Hematoxylin-Eosin (HE) and the brain was examined under a light microscope

(Nikon Y-IM 7551012, Japan). A random sampling of histopathological sections was performed and an average of 15-17 areas for each experimental group was evaluated semiquantitatively. The evaluation was performed based on the average number of lesions observed: Normal: - (no lesion), mild: + (1-4 lesions), moderate: ++ (5-8 lesions) and severe: +++ (9 or more lesions) (Topal et al., 2017).

2.7. Immunohistochemical examination

For immunohistochemical measurements, six groups were selected randomly from each group. After sections were deparaffinized and dehydrated, they were kept in 3% H_2O_2 for 10 minutes to block the endogenous peroxidase. The sections were next heated up in antigen retrieval (citrate buffer, pH 6.1) solution in a microwave oven twice for 5 min each to prevent antigen masking in the nucleus. To prevent nonspecific binding, sections were incubated in protein blocking buffer for 10 min. Sections were next incubated with the monoclonal antibody anti-8-OHdG (1/50; sc-66036, Santa Cruz) at +4 ^{o}C o/n. The next day, sections were washed with PBS and incubated with biotinylated goat anti-polyvalent and streptavidin-peroxidase conjugate (Thermo Fisher Scientific, s21024-5, USA) for 10 and 30 min, respectively. Sections were then stained using the chromogen diaminobenzidine, counterstained with Mayer's hematoxylin, and examined under a light microscope (Nikon Y-IM 7551012, Japan). Cells were counted as negative, slightly dense, moderately dense, or very dense based on the immunopositivity grades and evaluated statistically by applying H-score (Topal et al., 2017).

2.8. Statistical analysis

In probit analyses for LC_{50} values and the whole statistic calculations, the Microsoft Excel and SPSS 21.0 software programs were used, respectively (Finney and Stevens, 1948). Mukhi et al., 2005; Reddy et al., 2016) Mean value and standard error were calculated for hatching rate, malformation, and survival rate. Statistical analyses were performed using one-way ANOVA and Tukey's post hoc test ($p<0.05$). Kruskal-Wallis test was used to analyze the semi-quantitative data obtained in histopathological examinations and the Mann-Whitney U test was used for the comparison of paired groups.

3. Results

3.1. LC_{50} values for MB-5G and RB-203 textile dyes

To determine the LC_{50} values for MB-5G and RB-203 dyes, we treated the zebrafish larvae with different doses of the dyes for 96 h and scored the dead and alive embryos. There was no death in the control group. We determined the LC_{50} values as 166.04 mg L^{-1} and 278.32 mg L^{-1} for MB-5G and RB-203, respectively.

3.1. MB-5G and RB-203 reduce the survival rate of zebrafish larvae

To examine the survival rates of zebrafish embryos and larvae upon exposure to MB-5G and RB-203, we treated the embryos with different concentrations of dyes and monitored them at 24, 48, 72, and 96 h. The survival rate of embryos/larvae treated with RB-203 dye at the end of 96 h was significantly decreased 100 mg L^{-1} treatment group but not in 1 mg L^{-1}, 10 mg L^{-1}

groups (Fig.1A). The survival rate reduced significantly only in the 100 mg L^{-1} MB-5G group (Fig.2A).

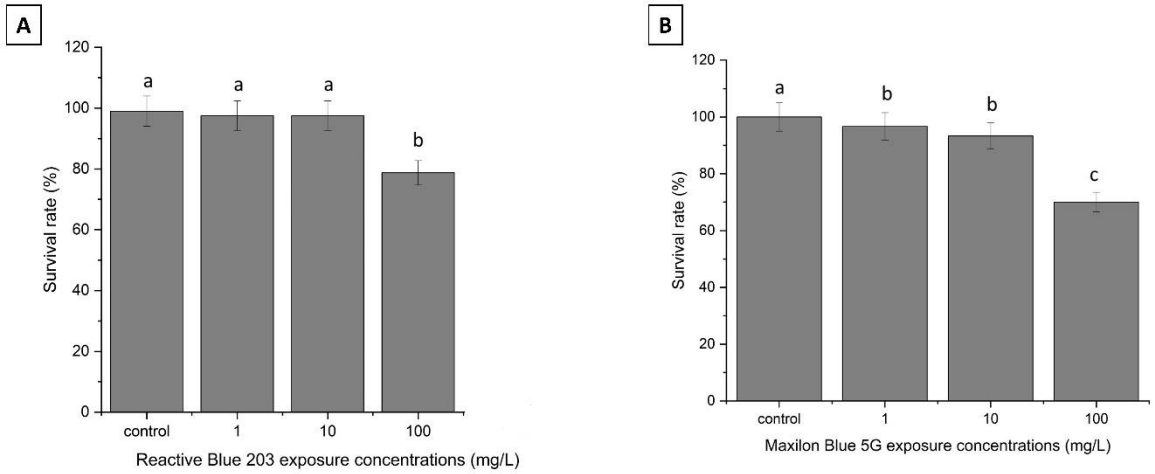

Figure 1. The dose-dependent survival effects of RB-203 (A) and MB-5G (B) on zebrafish embryo/larvae at 96 hpf. Different letters indicate significant differences between the groups ($p < 0.05$).

3.2. RB-203, but not MB-5G, causes premature larval hatching

To determine the developmental toxicity effects of RB-203 and MB-5G on zebrafish, we calculated the hatching rates of zebrafish embryos/larvae at 24, 48, 72, and 96 h. RB-203 treatment caused premature hatching at 48 h at its highest dose (100 mg L^{-1}) compared to the control group (Fig. 2A). However, there was no significant difference in the hatching rate in any of the MB-5G-treated groups as compared to the control group (Fig. 2B).

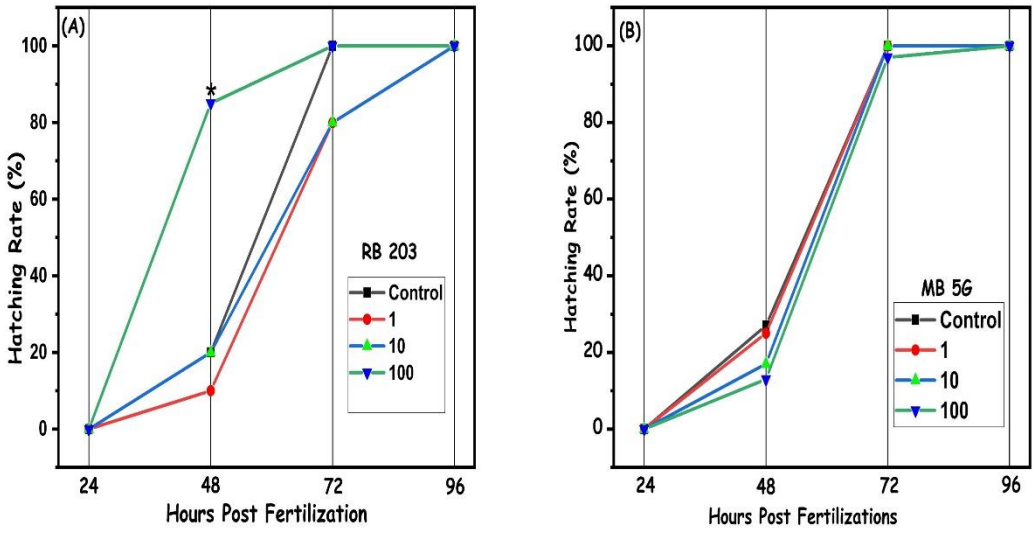

Figure 2. The hatching rate of zebrafish embryos exposed textile dyes (A) RB-203 and (B) MB-5G from 24 to 96 hpf.* $p < 0.05$

3.3. RB-203 and MB-5G induce embryonic malformations

To examine any morphological abnormalities caused by RB-203 and MB-5G, we closely monitored the dye-treated embryos and evaluated them morphologically at 96 hpf. The most severe malformation was the formation of a curved body axis in 28% of larvae, which was significantly different than the control at the two high concentrations (100 mg L^{-1}) of RB-203 (Fig. 3). On the other hand, MB-5G treatment caused severe malformations including yolk sac edema, pericardial edema, curved body axis, tail malformation, and microphthalmia in all concentrations of MB-5G (Fig. 4). Nevertheless, pericardial edema was the most notable malformation observed in 23 % of embryos treated with 100 mg L^{-1} of MB-5G (Fig. 4). These results suggest that RB-203 and MB 5G cause severe malformations during embryonic development.

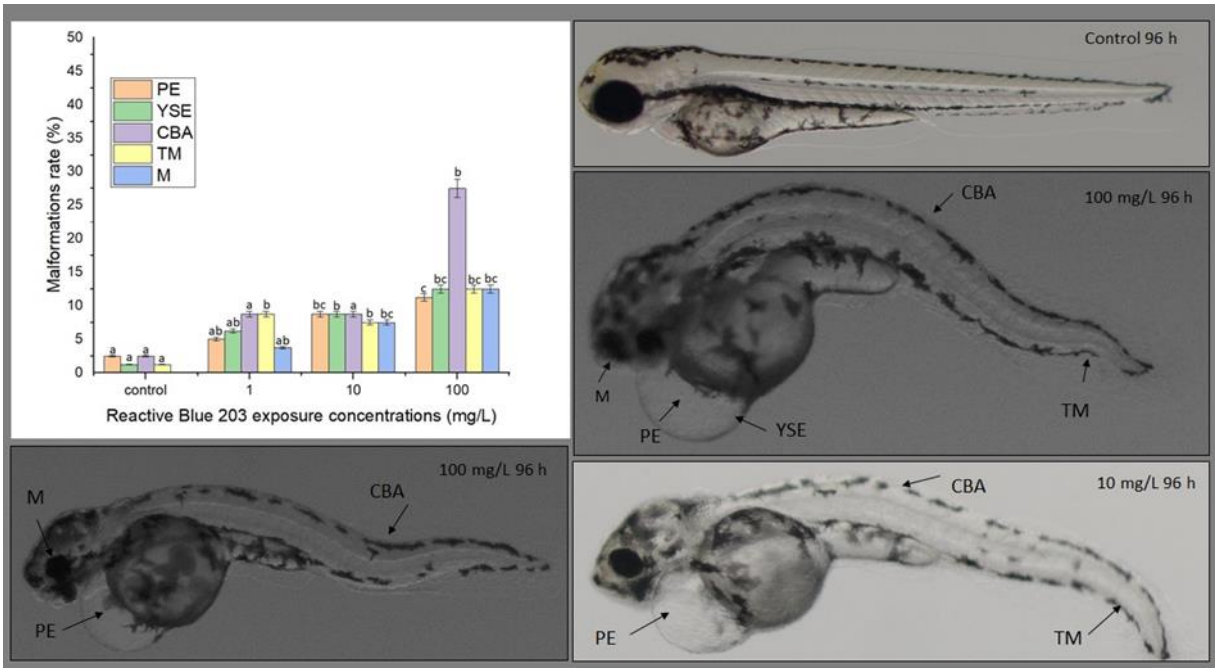

Figure 3. Microscopic images of embryos and percentage of observed malformations after reactive blue 203 exposure during 96 h. YSE: yolk sac edema; PE: pericardial edema; CBA: curved body axis; TM: tail malformation, M: microphthalmia). Different letters indicate significant differences between the groups ($p < 0.05$) and each value is the average ± SEM.

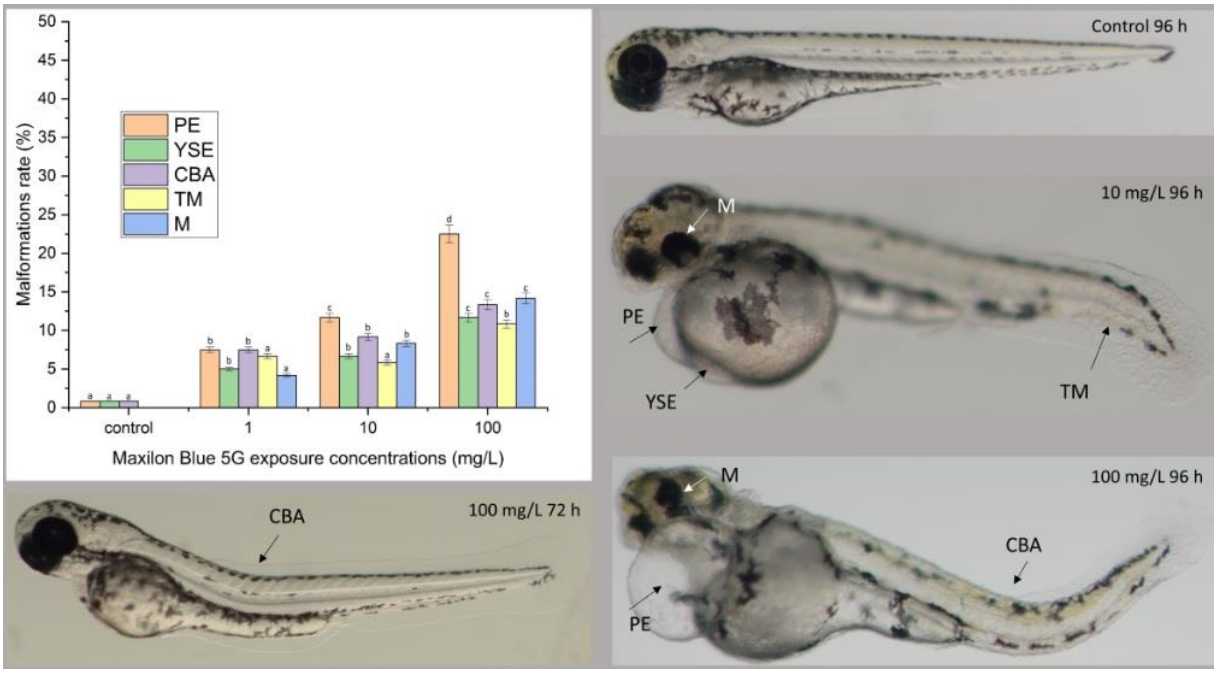

Figure 4. Microscopic images of embryos and percentage of observed malformations after Maxilon blue 5G exposure during 96 h. YSE: yolk sac edema; PE: pericardial edema; CBA: curved body axis; TM: tail malformation, M: microphthalmia). Different letters indicate significant differences between the groups ($p < 0.05$) and each value is the average ± SEM.

3.4. RB-203 and MB-5G cause degeneration and necrosis

To evaluate the influence of the textile dyes on embryonic development from a histopathological perspective, we collected sections of zebrafish larvae exposed to different concentrations of the dyes at 96 hpf. We did not detect any difference in the brain architecture of the larvae treated with MB-5G at 1 mg L^{-1} in comparison to the control (Fig. 5_1 and 5_2). MB-5G treatment caused mild tissue degeneration at 10 mg L^{-1} (Fig. 5_3) or severe tissue degeneration and necrosis at 100 mg L^{-1} (Fig. 5_4).

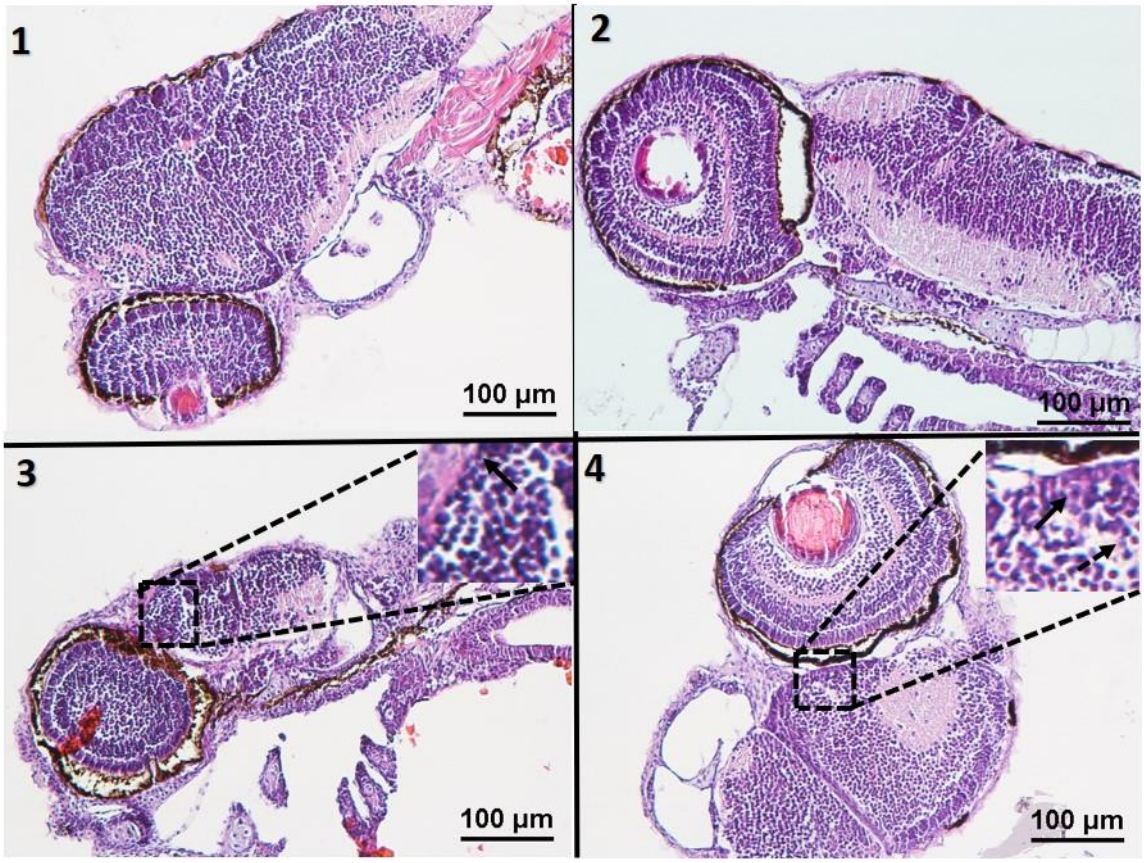

Figure 5. Histological images of larvae treated with MB-5G. 1: Control, 2: 1 mg L^{-1}, 3: 10 mg L^{-1}, 4: 100 mg L^{-1}. 1st and 2nd groups in the normal histological structure architecture. Mild degeneration (arrow) in group 3, severe degeneration (arrow), and necrosis (dashed arrow) in group 4. H-E. 200x.

On the other hand, RB-203 treatment led to mild tissue degeneration at 1 mg L^{-1} (Fig. 6_2), moderate degeneration at 10 mg L^{-1} (Fig. 6_3), or severe degeneration and necrosis at 100 mg L^{-1} (Fig. 6_4).

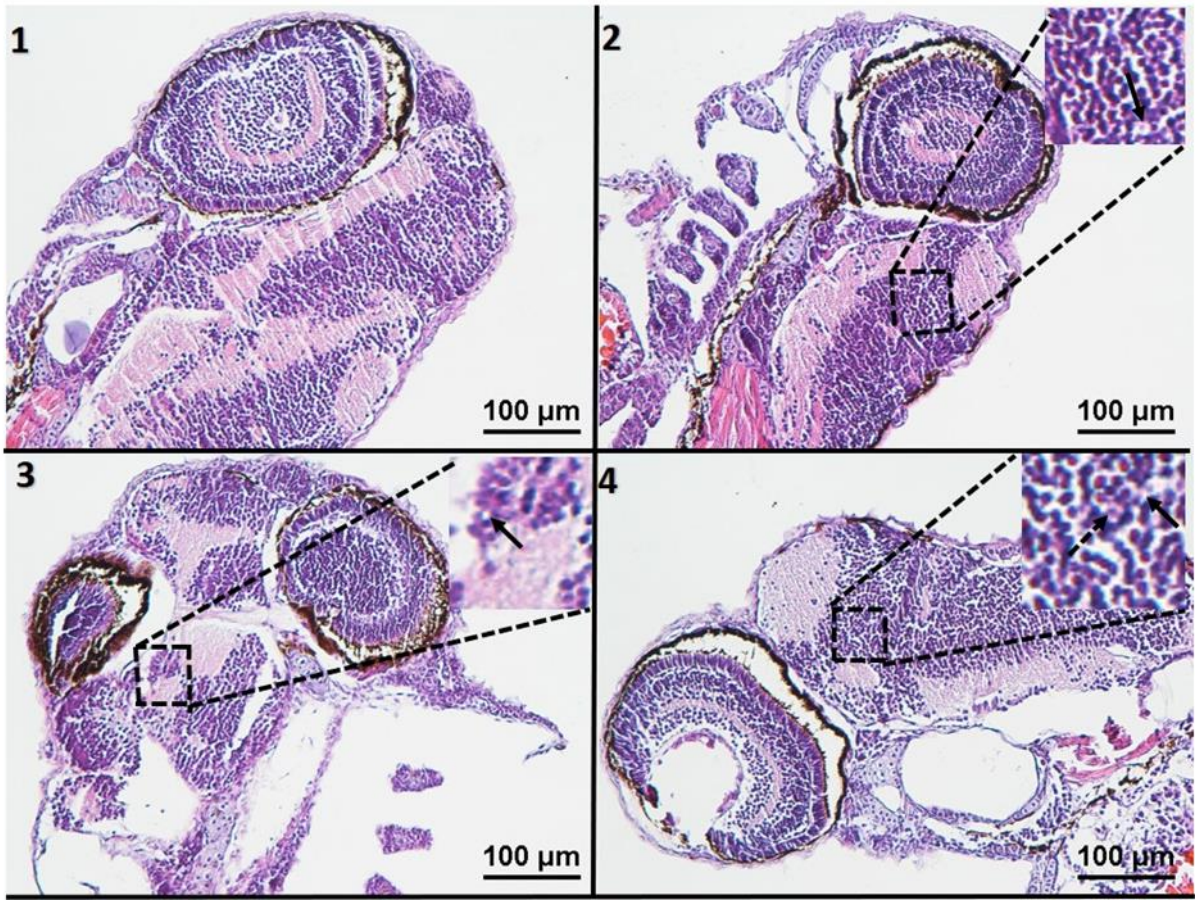

Figure 6. Histopathological images of the larvae were treated with RB-203. 1: Control, 2: 1 mg L^{-1}, 3: 10 mg L^{-1}, 4: 100 mg L^{-1}. The Control group (1st group) displays normal histological architecture. Mild degeneration (arrow) in the 2nd group, moderate degeneration (arrow) in the 3rd group, severe degeneration (arrow), and necrosis (broken arrow) in 4th. H-E. 200x.

RB-203 and MB-5G lead to oxidative DNA damage during the development

Next, to determine the effect of the dyes on oxidative DNA damage, we stained the larval sections immunohistochemically for 8-OHdG and evaluated using H-score. In MB-5G or RB-203 treatments, there was no statistically significant difference in the 1 mg L^{-1} groups as compared to the control administration group ($p > 0.05$) (Figs.7_1 and 7_2; Figs. 8_1 and 8_2). However, 8-OHdG immunoreactivity displayed a significant increase in MB-5G groups of 10 and 100 mg L^{-1} (Fig. 7_3 and 7_4).

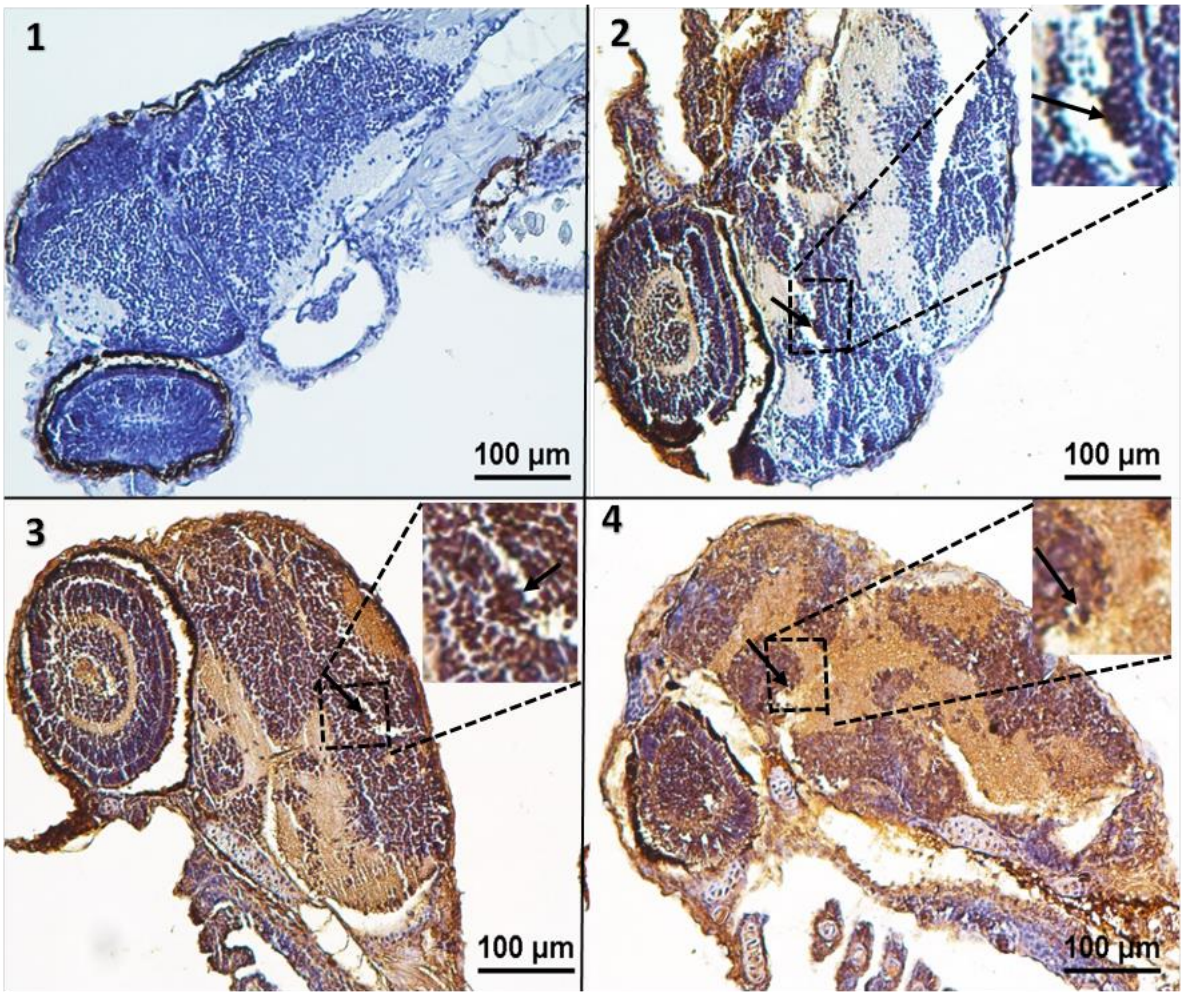

Figure 7. 8-OHdG immunohistochemical sections of larval brains at 96 hpf after MG-5G treatment. No immunoreactivity in the 1st group, low level in the 2nd group, high level of 8-OHdG immunoreactivity (arrows) in the 3rd and 4th groups. Control, 2: 1 mg L^{-1}, 3: 10 mg L^{-1}, 4: 100 mg L^{-1}. H-E. 200x.

RB-203 likewise induced a significant increase in 8-OHdG immunoreactivity when applied at concentrations of 10 and 100 mg L^{-1} ($p<0.05$) (Figs. 8_3 and 8_4). Thus we conclude that the textile dyes RB-203 and MB-5G induce oxidative DNA damage during embryogenesis.

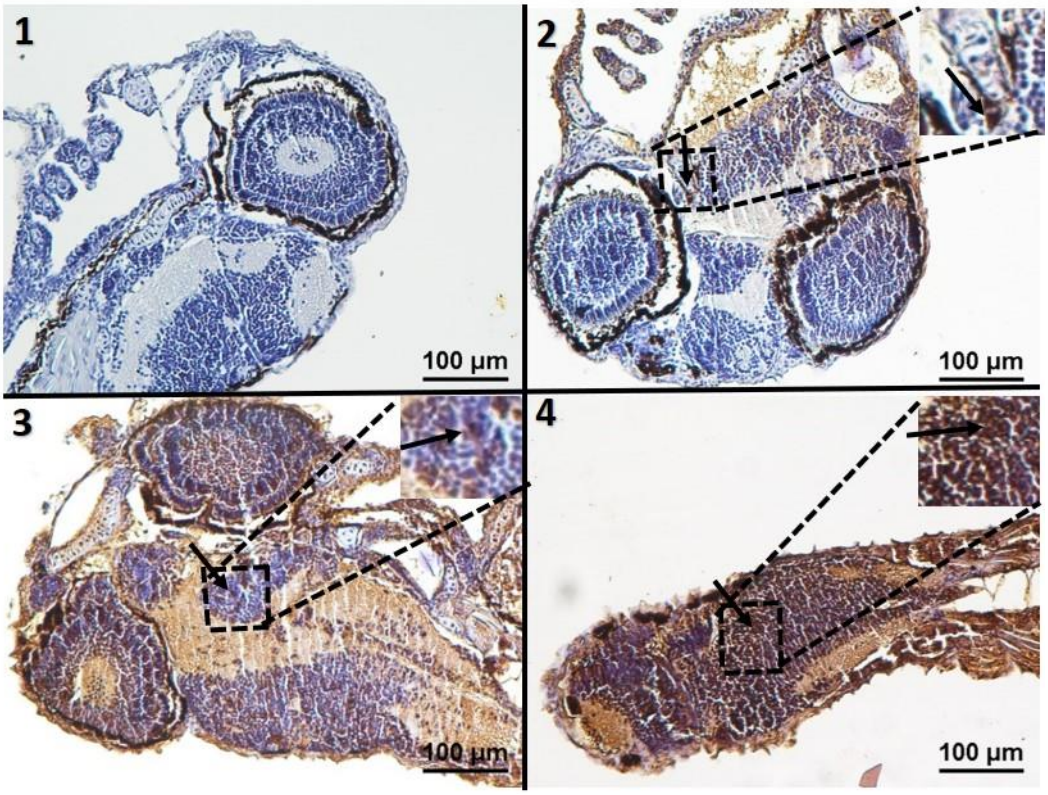

Figure 8. 8-OHdG immunohistochemical sections of larval brains at 96 hpf after RB-203 treatment. No immunoreactivity in the 1st group. Low level of 8-OHdG immunoreactivity (arrows) in the 2nd group, high level in the 3rd and 4th groups. 1: Control, 2: 1 mg L^{-1}, 3: 10 mg L^{-1}, 4: 100 mg L^{-1} . 200x.

Discussion

Global consumption of synthetic dyes is approximately 7×10^5 tons per year and the synthetic dye consumption generates large amounts of wastewater discharged into aquatic ecosystems(Hernández-Zamora and Martínez-Jerónimo, 2019b). Despite the widespread use in the world, there are no data available on the potential toxicity mechanisms of RG-203 and MB-5G textile dyes. In this paper, we determine the LC_{50} values of RG-203 and MB-5G at 96 as 278.32 mg L^{-1} and 166.04 mg L^{-1}, respectively. The absorption rate of chemicals with a large molecular weight (MW) is slower than those with a small molecular weight (Balogh et al., 2008; Kuna et al., 2018; Zhu et al., 2019). This is an important feature that affects the toxicity of dyes on organisms (Chakraborty, 2015). Of the two textile dyes we used, the MW of RB-203 is greater than that of MB-5G, explaining the lower LC_{50} value of MB-5G. Shen et. al., have likewise identified the LC_{50} values for Direct Red 28 and Basic Violet 14 dyes as 476.84 µg ml^{-1} and 60.63 µg ml^{-1}, respectively, with the former dye having a MW of 696.66 g mol^{-1} and the latter one of 337.8 g mol^{-1} (Shen et al., 2015). These findings are in line with our LC_{50} data for MB-5G and RB-203 dyes.

Hatching in fish is the primary and most important developmental phenomenon caused by a series of morphogenetic events (Karthik et al., 2019). Hatching success and time are important ecotoxicological criteria (Kataoka et al., 2018). Although both RB-203 and MG-5G dyes appear to affect the hatching of zebrafish embryos in a concentration-dependent manner, we observed the most striking effect with RB-203 at its highest dose (100 mg L^{-1}) as premature hatching at

48 hpf. This can be explained by the blockage of the chorion pores, which causes a hypoxic state and prevents the excretion of metabolites. These conditions can increase the release of enzymes that facilitate the rupture of the chorion (Carvalho da Cruz Brambilla *et al.* 2019). Larval hatching time can be affected by the activity of chorionic hatching enzymes and embryonic motility (Cheng et al., 2007; Yamagami, 1981). Chemicals such as xenobiotics that prevent the oxygen exchange of the embryo and result in an increase in respiratory rate or increased stimulation of hatching enzyme activity may cause premature hatching (Du et al., 2012; Manjunatha et al., 2014; Samaee et al., 2015).

Premature hatching may cause developmental deformities such as reduced growth, the curvature of the body and tail, and yolk sac edema (Karthik et al., 2019; Liang et al., 2017; Samaee et al., 2015). We have observed tail malformations, pericardial edema, yolk sac edema, microphthalmia, and curved body axis in all groups of embryos/larvae exposed to RB-203 and MG-5G dyes. Most of these abnormalities have also been observed for the azo dye Direct blue 15, the artificial food dye tartrazine, and the hair dye Basic Red 51 in zebrafish embryos (Hernández-Zamora and Martínez-Jerónimo, 2019a) (Abe *et al.* 2017, Joshi and Katti 2018). In our study, we determined the highest malformation rate as the curved body axis and pericardial edema in embryos treated with RB-203 and MG-5G, respectively. Acid Red 26 dye appears to exert cardiovascular toxicity at concentrations above 2500 µg/ml (Shen et al., 2015). Azo dyes likewise cause cardiac edema and a decrease in heart rate in zebrafish embryos (Jiang et al., 2020). Some textile dyes have also been reported to cause cardiac edema in zebrafish embryos and larvae (Hernández-Zamora and Martínez-Jerónimo, 2019a; Shen et al., 2015).

Interestingly, we have observed that the larval cardiac edema was accompanied by degeneration and necrosis in the brain tissues at high doses of MB-5G and RB-203 dyes. Several clinical studies in patients have suggested a similar relationship between acute cardiac dysfunction and brain hemorrhage (Yoshimura et al., 2008, Lee et al., 2016, Chen et al., 2017). Thus the combined effect of MB-5G and RB-203 dyes on heart and brain tissues deserves further examination.

Oxidative stress is a complex biological process that results from an imbalance between the production of reactive oxygen species (ROS) and antioxidant defense systems (van Velzen et al., 2017). ROS can attack deoxyribose phosphate backbones and nucleobases of DNA nucleotides, generating a wide variety of base- and sugar-modified products (Dalle-Donne et al., 2006).Oxidative damage induced by reactive oxygen species is a major factor in the progression of many neurodegenerative and oncological diseases. DNA lesions and 8-OHdG are known as biomarkers of oxidative damage (Cooke et al., 2003; Hussein et al., 2017). There is a limited number of studies on mechanisms of the DNA damage of textile dyes (Oliveira *et al.* 2018, Carvalho da Cruz Brambilla *et al.* 2019). We detected a dose-dependent increase in 8-OHdG levels in the brain tissues of zebrafish larvae treated with MB-5G or RB-203. This increase may activate the sympathetic nervous system by stimulating the hypothalamus and cardiac activities (Jia et al., 2015). The neurotoxic effects of dyes depend on the sensitivity of the brain to oxidative stress. High oxygen consumption rate, high polyunsaturated fatty acids content, regional high iron levels, and proportionately low antioxidant capacity are among the key factors that determine the reaction of the brain to oxidative stress (Noseworthy and Bray,

1998; Meireles et al., 2018). The mechanism of the effect of RB-203 and MB-5G dyes remains largely unknown. However, it is possible that the high 8-OHdG activity in the brain is not only caused by high doses of the dyes but also due to the cationic state of MB-5G that may cause toxicity by binding to the negatively charged DNA with high affinity (Alkan et al., 2008; Dezhampanah et al., 2019). It is also known that binding molecules with a cationic structure can interact with DNA by forming hydrogen bonds with base pairs in small grooves in the DNA structure (Rehman et al., 2014). There is evidence that textile dyes and products of electrolysis can generate DNA damage by interfering with the double helix structure (Uliana et al., 2013). Future studies will not only clarify the molecular mechanisms underlying the toxic effect of these dyes but will also pave the way for mitigating these undesirable impacts in the marine environment.

Conclusion

The findings of this paper are summarized as follows:

- MB-5G and RB-203 dyes exert toxicity and genotoxicity during zebrafish embryonic development.
- Based on the LC_{50} values, MB-5G appears to be more toxic than RB-203.
- RB-203 and MB-5G dyes can induce DNA damage due to oxidative stress, causing irreversible damage to embryos/larvae.
- RB-203 and MB-5G result in developmental abnormalities such as yolk sac edema, pericardial edema, curved body axis, tail malformation, and microphthalmia, strongly suggesting that they have detrimental effects on reproductive success.
- The embryotoxic effect of the dyes indicates a potential teratogenic effect.
- The synthetic textile dyes that are discharged into the water pose a threat to aquatic organisms and their habitats. Thus they must be considered as major pollutants and subject to strict environmental protection regulations.

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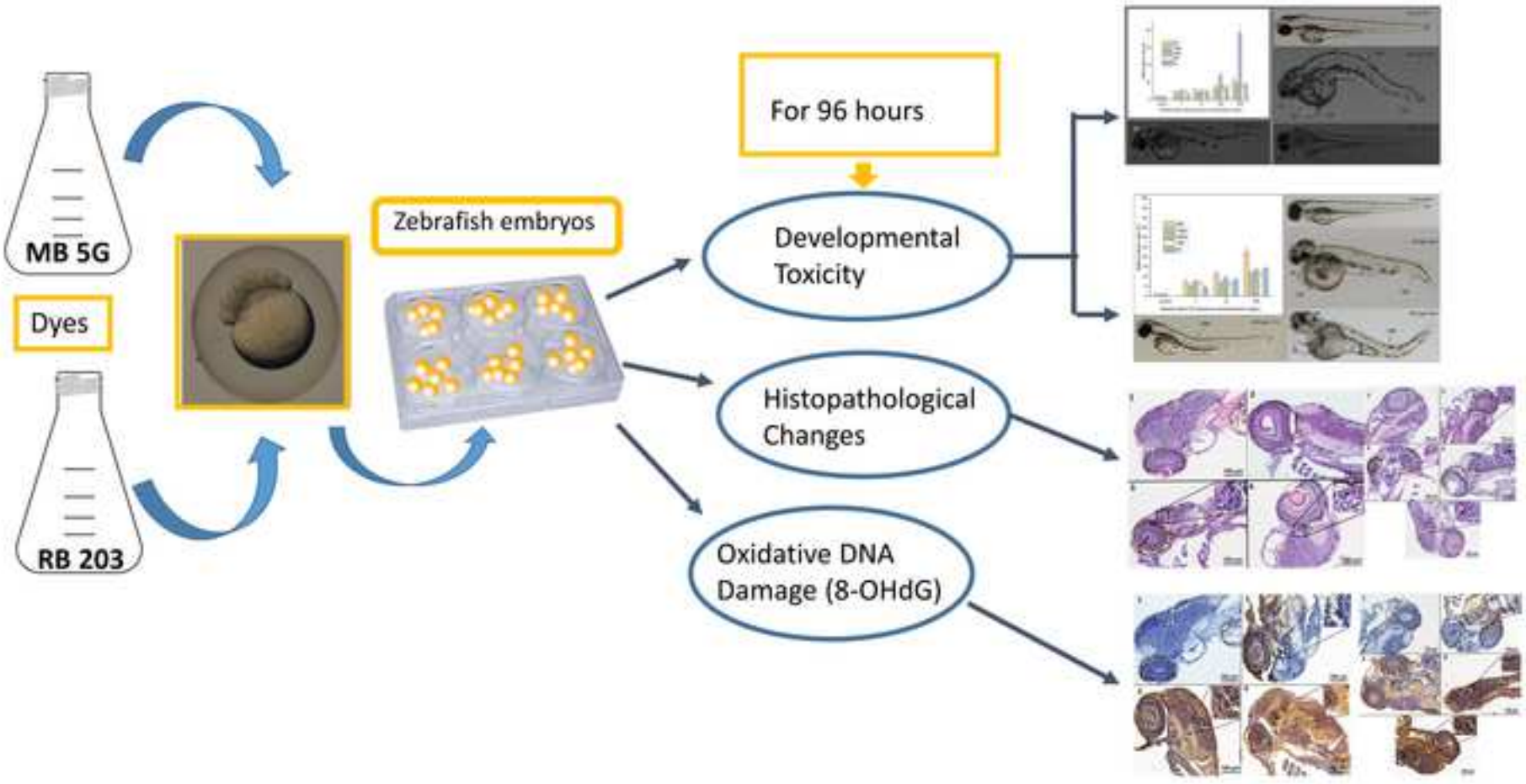
Highlights

- Maxilon Blue 5G (MB-5G) and Reactive Blue 203 (RB-203) exert acute toxicity on zebrafish embryos/larvae
- MB-5G exhibits no changes in the hatching rate, but RB-203 may lead to premature hatching
- MB-5G and RB-203 induce dose-related oxidative DNA damage in the larval zebrafish brain.

Graphical Abstract



Textile dyes Maxilon blue 5G and Reactive blue 203 induce acute toxicity and DNA damage during embryonic development of *Danio rerio*

Mine KÖKTÜRK[1], Fikret ALTINDAĞ[2], Gunes OZHAN[3,4], Mehmet Harbi ÇALIMLI[5*], Mehmet Salih NAS[6]

[1]Department of Organic Farming, College of Applied Sciences, Igdir University, Igdir, Turkey

[2]Department of Histology and Embryology, Medical School, Van Yüzüncü Yıl University, Van, Turkey

[3]Izmir Biomedicine and Genome Center, Dokuz Eylul University Health Campus, Izmir, Turkey

[4]Izmir International Biomedicine and Genome Institute, Dokuz Eylul University, Izmir, Turkey

[5] Department of Medical Services and Techniques, Tuzluca Vocational School, Igdır University, Igdir, Turkey

[6]Department of Environmental Engineering, Faculty of Engineering, Igdır University, Igdir, Turkey

Highlights

- Maxilon Blue 5G (MB-5G) and Reactive Blue 203 (RB-203) exert acute toxicity on zebrafish embryos/larvae
- MB-5G exhibits no changes in the hatching rate, but RB-203 may lead to premature hatching
- MB-5G and RB-203 induce dose-related oxidative DNA damage in the larval zebrafish brain.

ABSTRACT: Common textile dyes used in various industrial sectors are organic compounds and considered for the aquatic environment as pollutants. The textile dye industry is one of the main sectors that have serious impacts on the environment due to a large amount of wastewater released into the ecosystem. Maxilon blue 5G (MB-5G) and Reactive Blue 203 (RB-203) are widely used textile dyes. However, their potential toxicity on living organisms remains to be elucidated. Here, we investigate the acute toxicity and genotoxicity of MB-5G and RB-203 dyes using the zebrafish embryos/larvae. Embryos treated with each dye for 96 h revealed LC_{50} values of acute toxicity as 166.04 mg L^{-1} and 278.32 mg L^{-1} for MB-5G and RB 203, respectively. When exposed to MB-5G and RB-203 at different concentrations (1, 10, and 100 mg L^{-1}) for 96h, the expression of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, significantly increased in brain tissues as compared to control. MB-5G and RB-203 resulted in common developmental abnormalities including tail malformation, microphthalmia, pericardial edema, curved body axis, and yolk sac/pericardial edemas. Moreover, at its highest dose (100 mg L^{-1}), RB-203 caused premature hatching after 48 hours, while MG-5G did not. Our results collectively reveal

that the textile dyes MB-5G and RB-203 cause genotoxicity and teratogenicity during embryonic and larval development of zebrafish. Thus, it is necessary to eliminate these compounds from wastewater or reduce their concentrations to safe levels before discharging the textile industry wastewater into the environment.

Keywords: Maxilon blue 5G; Reactive blue 203**,** 8-OHdG; DNA damage; Zebrafish

Introduction

Today, water pollution based on dyes increased with the growth of colorant activities for textile, leather, food, and agrochemical industries (Hernández-Zamora and Martínez-Jerónimo, 2019a). The textile industries have a significant role in the economy of many countries (Panda et al., 2006). However, due to industrial activities, large amounts of wastewater containing dyes are released into the environment, creating serious problems for aquatic and terrestrial organisms in terms of environmental management (Oliveira et al., 2009; Leme et al., 2015; Nas et al., 2019; Barathi et al., 2020). Water pollution due to textile dyeing causes adverse conditions such as light penetration, decreased photosynthetic activity, lack of oxygen, and change of life cycle in natural organisms (Hernández-Zamora et al., 2014). Generally, the color of a dye can be noticed when its concentration is above 1 mg L^{-1}. However, most dyes have been detected at concentrations of 300 mg L^{-1} in the wastewaters originated from textile activities (Jonstrup et al., 2011; Meng et al., 2012). Approximately 15-50 % of the dyes used in textile activities are released into the aquatic environment (Nojavan et al., 2013). This amount has been reported to be above 50 mg L^{-1} in wastewater, depending on the type of dye and the treatment performed (O'Neill et al., 1999; Nojavan et al., 2013). Synthetic dyes containing the azo-aromatic chromophore group is the largest class of chemical dyes used worldwide (Guaratini and Zanoni, 2000). Due to their high stability, azo-aromatic dyes create permanent pollution and prevent the removal or reduction of toxicity in industrial wastewater containing dye via conventional treatment (O'Neill et al., 1999; Chung, 2000).

Textile dyes can cause mass deaths of species, especially in fish, deteriorate the ecological balance and reduce the self-cleaning capacity of the water bodies that receive these dyes, thus leading to the depletion of water bodies. Several studies have reported the toxic effects of textile dyes on zebrafish embryos (Abe *et al.* 2017, 2018; Meireles *et al.* 2018; Hernández-Zamora and Martínez-Jerónimo 2019a). Some dyes cause genetic damages including mutagenicity and carcinogenicity (Balakrishnan *et al.* 2016, Abe *et al.* 2018, Carvalho da Cruz Brambilla *et al.* 2019). Although the dyes are produced in powder form, they are dissolved in water to be used in the dyeing processes of fabrics. Therefore, textile production facilities cause water pollution that causes serious environmental problems to aquatic biota (Kim, 2001; Kaur et al., n.d.; Puvaneswari et al., 2006; Talaiekhozani et al., 2020). The discovery of textile dyes, especially reactive dyes, and their use in cellulosic fabrics is a breakthrough for the textile industry (Zollinger, 2003). These reactive dyes are known to cause skin irritation, asthma, and allergic reactions (Hunger, 2007; Khattab et al., 2020). Especially Reactive Blue 203 (RB-203) is one

of the most commonly used dyes in the textile industry and a hazardous compound that is found in large amounts of textile wastewater (Ashtekar et al., 1970; Talaiekhozani et al., 2019; Bagheri et al., 2020). Maxilon series consists of mono azo dyes and various textile dyes. As a cationic dye, Maxilon Blue 5G (MB-5G) has been commonly used in histochemical studies and contains various aromatic radicals and aliphatic chemical compounds (de Almeida, 1960). To reveal the toxic mechanism of textile dyes, it is also necessary to determine the toxicity of their degradation products. However, no studies related to the toxicological effects of MB-5G and RB-203 or their degradation products on organisms have been reported to date. Some textile dyes can be carcinogenic without breaking down into aromatic amines (Miller and Miller, 1948). Nevertheless, the carcinogenicity of many dyes is due to their breakdown products such as benzidine and p-phenylenediamine that have been reported to cause various allergies and tumors in humans and animals (K.T. Chung, 2016a; K. T. Chung, 2016b). Exposure to benzidine induces widespread apoptosis in the zebrafish brain and dorsal neurons, resulting in the development of an abnormal telencephalon (Chen et al., 2014). Similarly, some researchers reported that benzidine caused cell proliferation and apoptosis in liver tissues in fish of Gambusia affinis (Lentz et al., 2010).

DNA damage is one of the most important consequences of oxidative stress. 8-Hydroxy-2′-deoxyguanosine (8-OHdG), a major product of oxidative DNA damage, is induced by reactive oxygen species (ROS) that have adverse effects such as mutation and cell death (Anjana Vaman et al., 2013; Meng et al., 2014; Valavanidis et al., 2009; Zhang et al., 2014; Alak et al., 2017). Besides, 8-OHdG is widely used as an important biomarker for the neurotoxic effects of xenobiotics in aquatic organisms (Topal et al., 2017; Gyimah et al., n.d.; Sökmen et al., 2020). Zebrafish embryos and larvae are commonly used as a platform to assess the toxic effects of textile dyes (Shen et al., 2015; de Oliveira et al., 2016; Hernández-Zamora and Martínez-Jerónimo, 2019a; Meireles et al., 2018). Owing to its distinctive features including small size, embryo transparency, rapid development, low-cost maintenance, and high genetic, anatomical, and physiological homology to mammals zebrafish provides a convenient model of toxicology that allows for easy physiological evaluation *in vivo* (Schaeck et al., 2013; Gutiérrez-Lovera et al., 2019), (Schlegel, 2016; Ijaz and Hoffman, 2016; Sant and Timme-Laragy, 2018; James et al., 2019; Köktürk et al., 2020). In this study, we aim at unraveling the potentially toxic and genotoxic effects of MB-5G and RB-203 dyes using the zebrafish embryos and larvae.

2. MATERIAL AND METHODS

2.1. Textile dyes

Maxilon Blue 5G (MB-5G) ($C_{16}H_{26}N_3O$, 266 g mol^{-1}) and Reactive Blue 203 (RB-203) ($C_{28}H_{29}N_5Na_4O_{21}S_6$, 617.54g mol^{-1}) were purchased from Setaş and Eksoy Textile Co. (Bursa, Turkey). MB-5G and RB-203 were of analytical grade with a purity > 99.5 % and used without any purification. MB-5G is a cationic dye (Alkan et al., 2008) and cationic dyes can react with anionic substances due to the cationic region on their surface. RB-203 is an anionic dye (Bagheri et al., 2020) and contains anionic regions on its surface. RB-203 reacts with cationic substances in these anionic regions.

2.2. Zebrafish maintenance and ethics statement

Adult wild-type zebrafish were maintained in the Zebrafish Facility of Izmir Biomedicine and Genome Center at about 28°C with a 14-h light/10-h dark cycle, and bred according to standard procedures (Westerfield, 1995). Fish were fed twice a day (9 a.m - 3 p.m) with flake food: TetraMin Tropical Flakes (48% protein, 8% fat, and 2% fiber) in the morning and artemia in the afternoon. The zebrafish larvae used in the study were younger than 5-days-old. Thus, the study does not require any license (Directive 86/609/EEC and EU Directive, 2010/63/EU).

2.3. Experimental design

The study was planned according to a semi-static test using 4 repeats (Ensibi et al., 2014). The experiment consisted of 5 groups with 30 embryos each: one control and four repeats of treatment. Embryos were kept in the E3 medium (0.17 mM KCI, 0.33 mM $MgSO_4$, 5 mM NaCI, 0.33 mM $CaCI_2$). The dye solutions were prepared in E3 medium without the addition of other solvents. Different concentrations of RB-203 and MB-5G dyes (1, 10, 100, and 1000 mg L^{-1}) were applied to the embryos and the limiting pretest was performed for LC_{50} values. The embryos were transferred to six-well plates and RB203 and MB5G dyes were applied at concentrations of 1, 10, and 100 mg L^{-1} in 5 mL of E3 for 4 to 96 h (Westerfield, 1995). In the evening, males and females were placed in mating tanks, separated by a transparent plastic divider. At 9:00 in the morning, the divider was removed to allow zebrafish to mate and spawn. The eggs were collected in Petri dishes containing E3 medium. On the first day of the experiment, randomly selected eggs were transferred to an E3 medium containing the dyes at 4 hours-post-fertilization (hpf) (Köktürk et al. 2020). The culture medium was completely refreshed every 24 h. All experimental groups were incubated at 28.5°C.

2.4. Assessment of acute toxicity and mortality

The embryos and larvae of all groups were visualized under a stereomicroscope (SZX10 Olympus microscope with SC50 Olympus camera) at 24, 48, 72, and 96 h after the addition of the dyes. Larvae were recorded as dead if they did not survive until 24 hpf or no heartbeat was observed at 24 hpf.

2.5. Hatching rate

The zebrafish larvae normally hatch around 48 hours-post-fertilization (hpf). The effect of RB-203 and MB-5G dyes on larval hatching was observed and quantified in the 48- 96 hour-interval.

2.6. Histopathological examination

Larvae at 96 hpf were used for brain histological examination with minor changes on the protocols described previously (Hallare et al., n.d.; Hill et al., 2002; Li et al., n.d.; Sabaliauskas et al., n.d.). In this regard, 10 larvae that were contacted with MB-5G and RB-203 dyes and showed various malformations were selected. For brain histopathological evaluation, zebrafish larvae were fixed in a 10% neutral buffered formalin. After passing through routine histological tissue follow-up steps, zebrafish larvae were embedded in paraffin (Copper et al., 2018). Whole larval sections of 4 µm were cut in the microtome (Leica, RM2125, China). The sections were stained with Hematoxylin-Eosin (HE) and the brain was examined under a light microscope

(Nikon Y-IM 7551012, Japan). A random sampling of histopathological sections was performed and an average of 15-17 areas for each experimental group was evaluated semiquantitatively. The evaluation was performed based on the average number of lesions observed: Normal: - (no lesion), mild: + (1-4 lesions), moderate: ++ (5-8 lesions) and severe: +++ (9 or more lesions) (Topal et al., 2017).

2.7. Immunohistochemical examination

For immunohistochemical measurements, six groups were selected randomly from each group. After sections were deparaffinized and dehydrated, they were kept in 3% H_2O_2 for 10 minutes to block the endogenous peroxidase. The sections were next heated up in antigen retrieval (citrate buffer, pH 6.1) solution in a microwave oven twice for 5 min each to prevent antigen masking in the nucleus. To prevent nonspecific binding, sections were incubated in protein blocking buffer for 10 min. Sections were next incubated with the monoclonal antibody anti-8-OHdG (1/50; sc-66036, Santa Cruz) at +4 ^{o}C o/n. The next day, sections were washed with PBS and incubated with biotinylated goat anti-polyvalent and streptavidin-peroxidase conjugate (Thermo Fisher Scientific, s21024-5, USA) for 10 and 30 min, respectively. Sections were then stained using the chromogen diaminobenzidine, counterstained with Mayer's hematoxylin, and examined under a light microscope (Nikon Y-IM 7551012, Japan). Cells were counted as negative, slightly dense, moderately dense, or very dense based on the immunopositivity grades and evaluated statistically by applying H-score (Topal et al., 2017).

2.8. Statistical analysis

In probit analyses for LC_{50} values and the whole statistic calculations, the Microsoft Excel and SPSS 21.0 software programs were used, respectively (Finney and Stevens, 1948). Mukhi et al., 2005; Reddy et al., 2016) Mean value and standard error were calculated for hatching rate, malformation, and survival rate. Statistical analyses were performed using one-way ANOVA and Tukey's post hoc test ($p<0.05$). Kruskal-Wallis test was used to analyze the semi-quantitative data obtained in histopathological examinations and the Mann-Whitney U test was used for the comparison of paired groups.

3. Results

3.1. LC_{50} values for MB-5G and RB-203 textile dyes

To determine the LC_{50} values for MB-5G and RB-203 dyes, we treated the zebrafish larvae with different doses of the dyes for 96 h and scored the dead and alive embryos. There was no death in the control group. We determined the LC_{50} values as 166.04 mg L^{-1} and 278.32 mg L^{-1} for MB-5G and RB-203, respectively.

3.1. MB-5G and RB-203 reduce the survival rate of zebrafish larvae

To examine the survival rates of zebrafish embryos and larvae upon exposure to MB-5G and RB-203, we treated the embryos with different concentrations of dyes and monitored them at 24, 48, 72, and 96 h. The survival rate of embryos/larvae treated with RB-203 dye at the end of 96 h was significantly decreased 100 mg L^{-1} treatment group but not in 1 mg L^{-1}, 10 mg L^{-1}

groups (Fig.1A). The survival rate reduced significantly only in the 100 mg L^{-1} MB-5G group (Fig.2A).

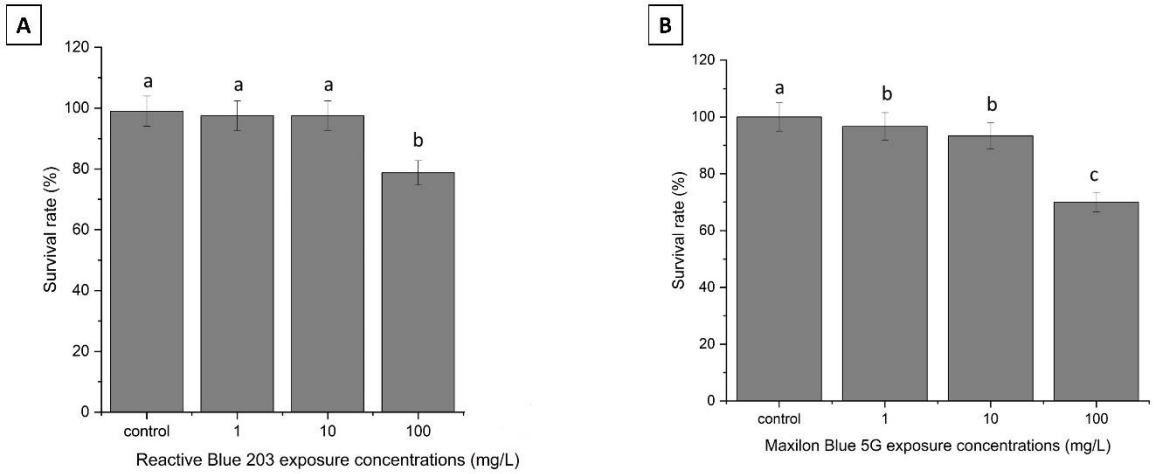


Figure 1. The dose-dependent survival effects of RB-203 (A) and MB-5G (B) on zebrafish embryo/larvae at 96 hpf. Different letters indicate significant differences between the groups ($p < 0.05$).

3.2. RB-203, but not MB-5G, causes premature larval hatching

To determine the developmental toxicity effects of RB-203 and MB-5G on zebrafish, we calculated the hatching rates of zebrafish embryos/larvae at 24, 48, 72, and 96 h. RB-203 treatment caused premature hatching at 48 h at its highest dose (100 mg L^{-1}) compared to the control group (Fig. 2A). However, there was no significant difference in the hatching rate in any of the MB-5G-treated groups as compared to the control group (Fig. 2B).

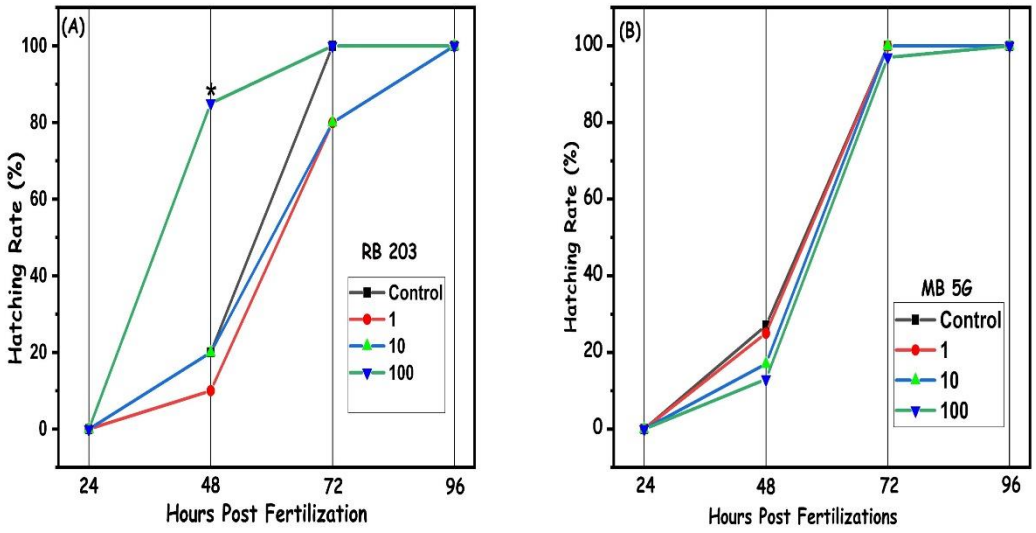


Figure 2. The hatching rate of zebrafish embryos exposed textile dyes (A) RB-203 and (B) MB-5G from 24 to 96 hpf.* $p < 0.05$

3.3. RB-203 and MB-5G induce embryonic malformations

To examine any morphological abnormalities caused by RB-203 and MB-5G, we closely monitored the dye-treated embryos and evaluated them morphologically at 96 hpf. The most severe malformation was the formation of a curved body axis in 28% of larvae, which was significantly different than the control at the two high concentrations (100 mg L^{-1}) of RB-203 (Fig. 3). On the other hand, MB-5G treatment caused severe malformations including yolk sac edema, pericardial edema, curved body axis, tail malformation, and microphthalmia in all concentrations of MB-5G (Fig. 4). Nevertheless, pericardial edema was the most notable malformation observed in 23 % of embryos treated with 100 mg L^{-1} of MB-5G (Fig. 4). These results suggest that RB-203 and MB 5G cause severe malformations during embryonic development.

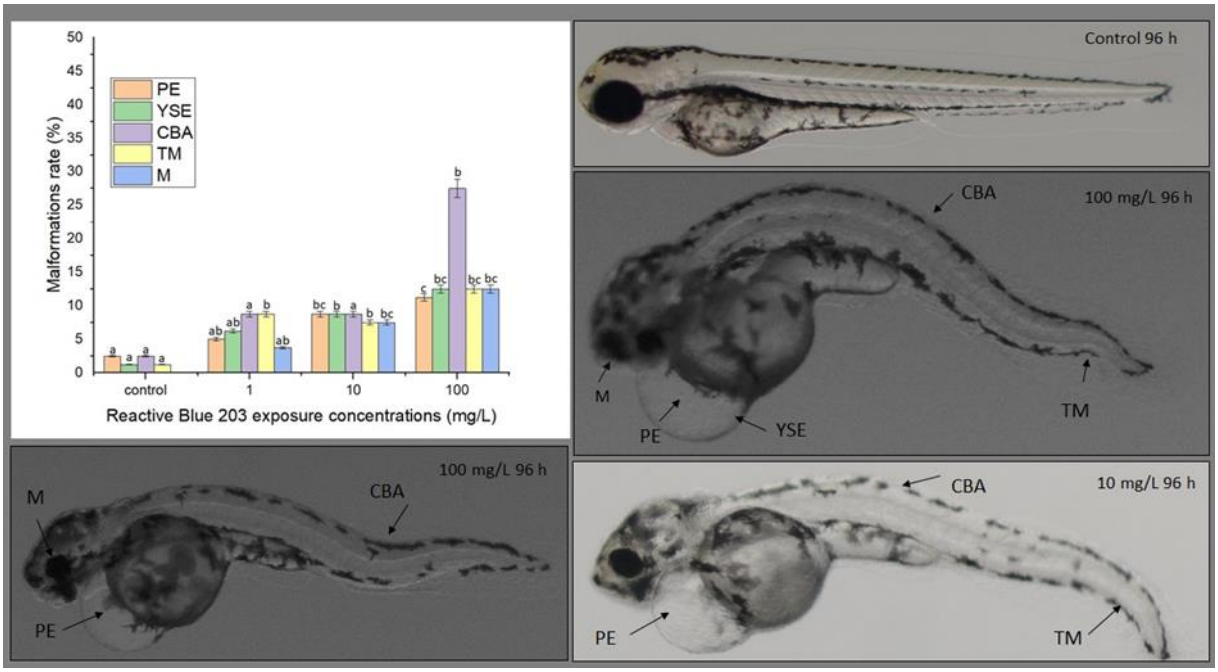


Figure 3. Microscopic images of embryos and percentage of observed malformations after reactive blue 203 exposure during 96 h. YSE: yolk sac edema; PE: pericardial edema; CBA: curved body axis; TM: tail malformation, M: microphthalmia). Different letters indicate significant differences between the groups ($p < 0.05$) and each value is the average ± SEM.

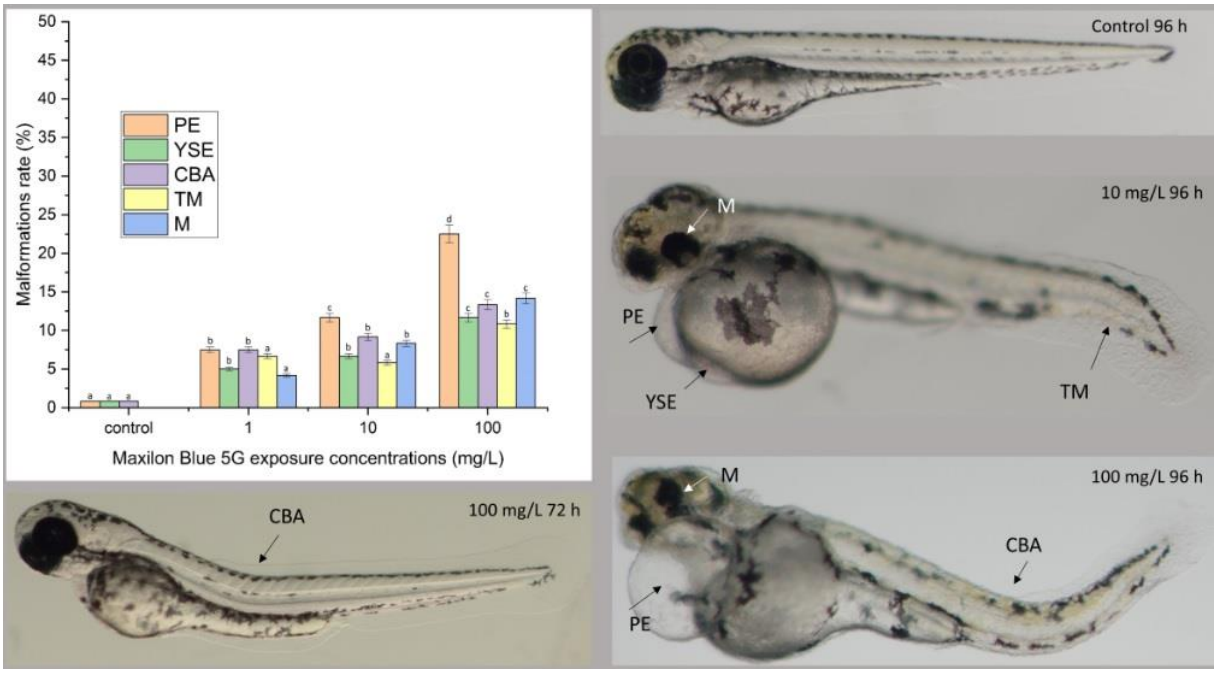


Figure 4. Microscopic images of embryos and percentage of observed malformations after Maxilon blue 5G exposure during 96 h. YSE: yolk sac edema; PE: pericardial edema; CBA: curved body axis; TM: tail malformation, M: microphthalmia). Different letters indicate significant differences between the groups ($p < 0.05$) and each value is the average ± SEM.

3.4. RB-203 and MB-5G cause degeneration and necrosis

To evaluate the influence of the textile dyes on embryonic development from a histopathological perspective, we collected sections of zebrafish larvae exposed to different concentrations of the dyes at 96 hpf. We did not detect any difference in the brain architecture of the larvae treated with MB-5G at 1 mg L^{-1} in comparison to the control (Fig. 5_1 and 5_2). MB-5G treatment caused mild tissue degeneration at 10 mg L^{-1} (Fig. 5_3) or severe tissue degeneration and necrosis at 100 mg L^{-1} (Fig. 5_4).

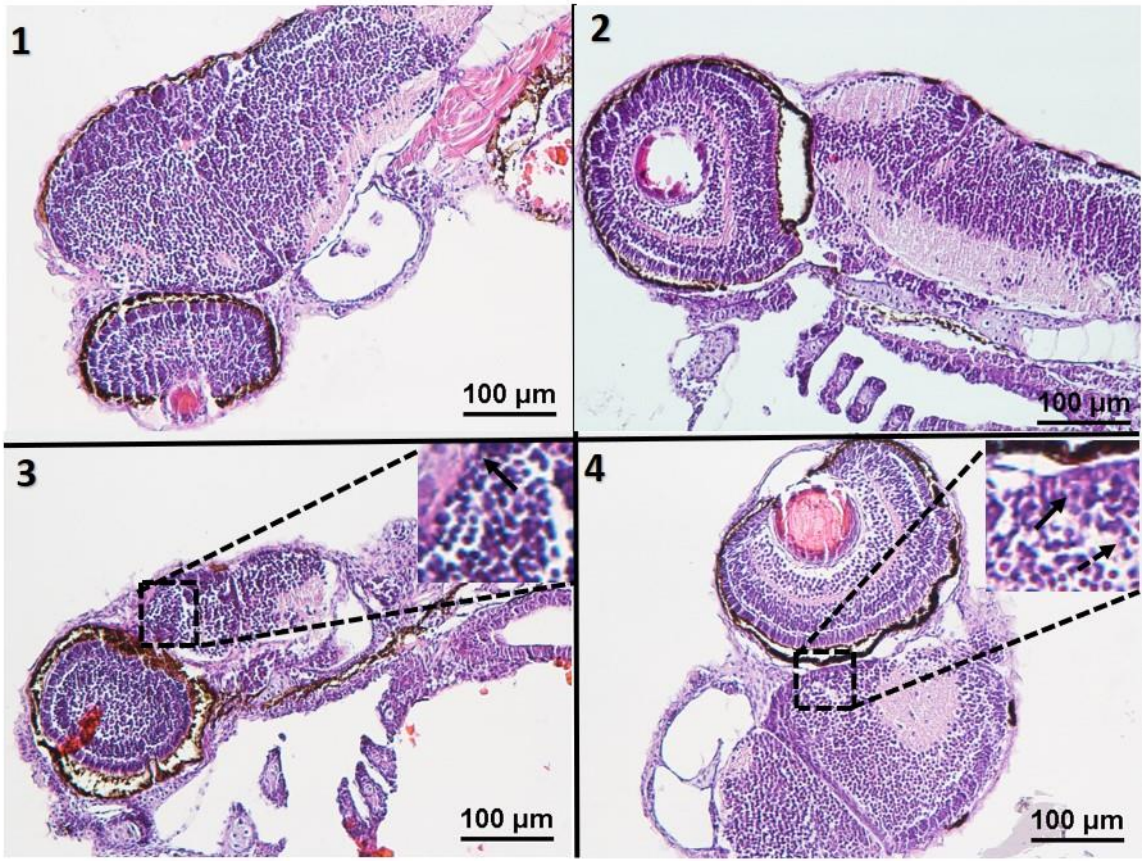


Figure 5. Histological images of larvae treated with MB-5G. 1: Control, 2: 1 mg L^{-1}, 3: 10 mg L^{-1}, 4: 100 mg L^{-1}. 1st and 2nd groups in the normal histological structure architecture. Mild degeneration (arrow) in group 3, severe degeneration (arrow), and necrosis (dashed arrow) in group 4. H-E. 200x.

On the other hand, RB-203 treatment led to mild tissue degeneration at 1 mg L^{-1} (Fig. 6_2), moderate degeneration at 10 mg L^{-1} (Fig. 6_3), or severe degeneration and necrosis at 100 mg L^{-1} (Fig. 6_4).

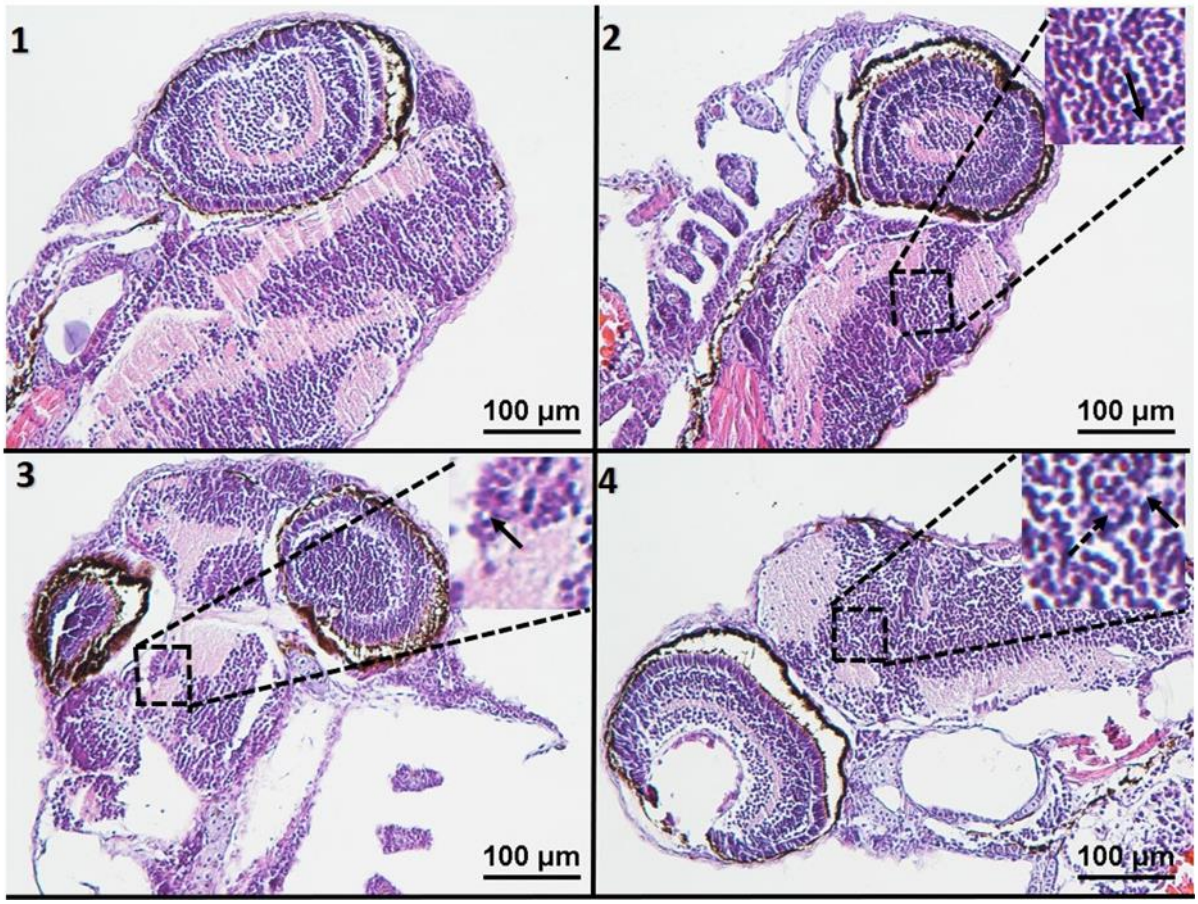


Figure 6. Histopathological images of the larvae were treated with RB-203. 1: Control, 2: 1 mg L^{-1}, 3: 10 mg L^{-1}, 4: 100 mg L^{-1}. The Control group (1st group) displays normal histological architecture. Mild degeneration (arrow) in the 2nd group, moderate degeneration (arrow) in the 3rd group, severe degeneration (arrow), and necrosis (broken arrow) in 4th. H-E. 200x.

RB-203 and MB-5G lead to oxidative DNA damage during the development

Next, to determine the effect of the dyes on oxidative DNA damage, we stained the larval sections immunohistochemically for 8-OHdG and evaluated using H-score. In MB-5G or RB-203 treatments, there was no statistically significant difference in the 1 mg L^{-1} groups as compared to the control administration group ($p > 0.05$) (Figs.7_1 and 7_2; Figs. 8_1 and 8_2). However, 8-OHdG immunoreactivity displayed a significant increase in MB-5G groups of 10 and 100 mg L^{-1} (Fig. 7_3 and 7_4).

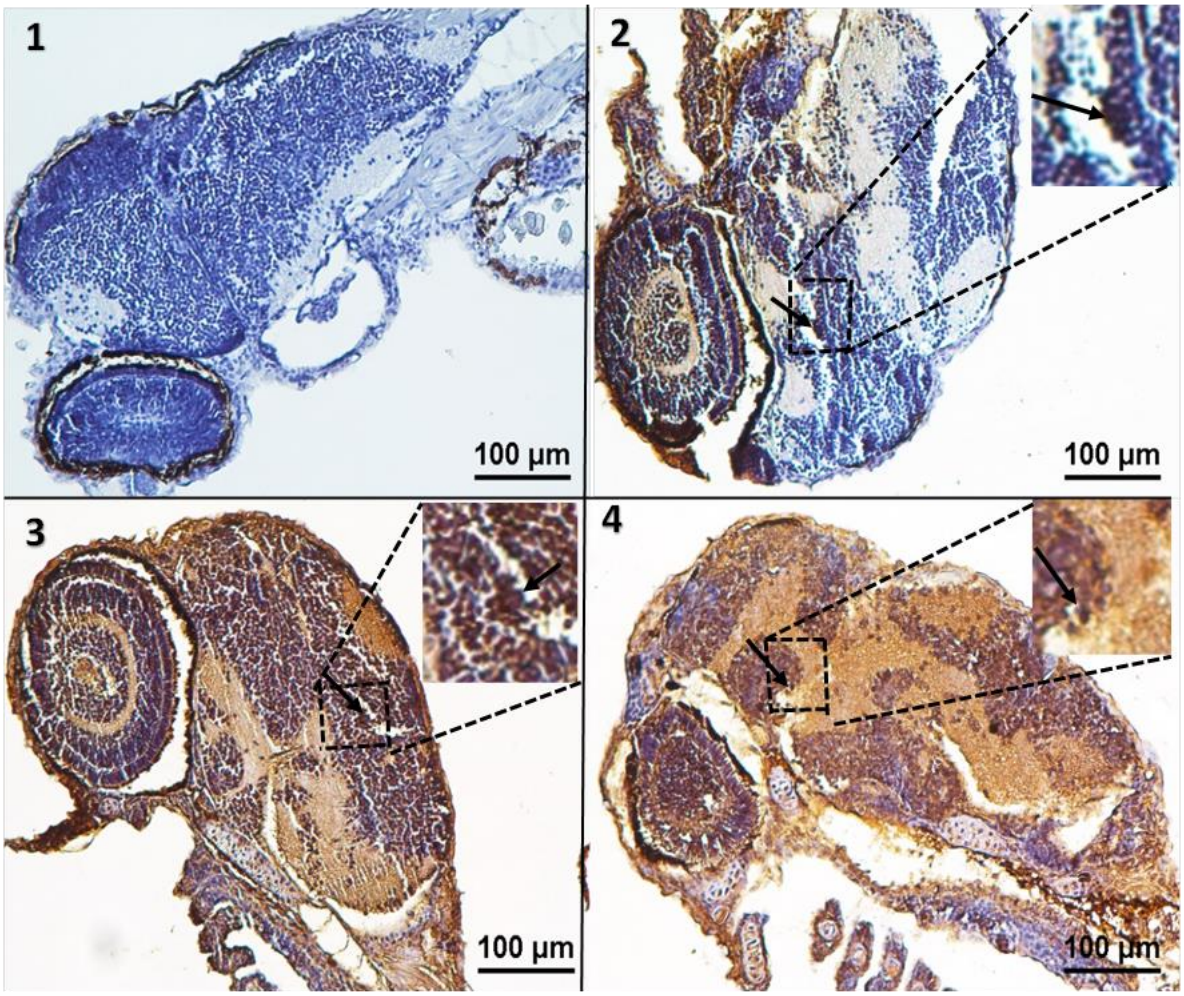


Figure 7. 8-OHdG immunohistochemical sections of larval brains at 96 hpf after MG-5G treatment. No immunoreactivity in the 1st group, low level in the 2nd group, high level of 8-OHdG immunoreactivity (arrows) in the 3rd and 4th groups. Control, 2: 1 mg L^{-1}, 3: 10 mg L^{-1}, 4: 100 mg L^{-1}. H-E. 200x.

RB-203 likewise induced a significant increase in 8-OHdG immunoreactivity when applied at concentrations of 10 and 100 mg L^{-1} ($p<0.05$) (Figs. 8_3 and 8_4). Thus we conclude that the textile dyes RB-203 and MB-5G induce oxidative DNA damage during embryogenesis.

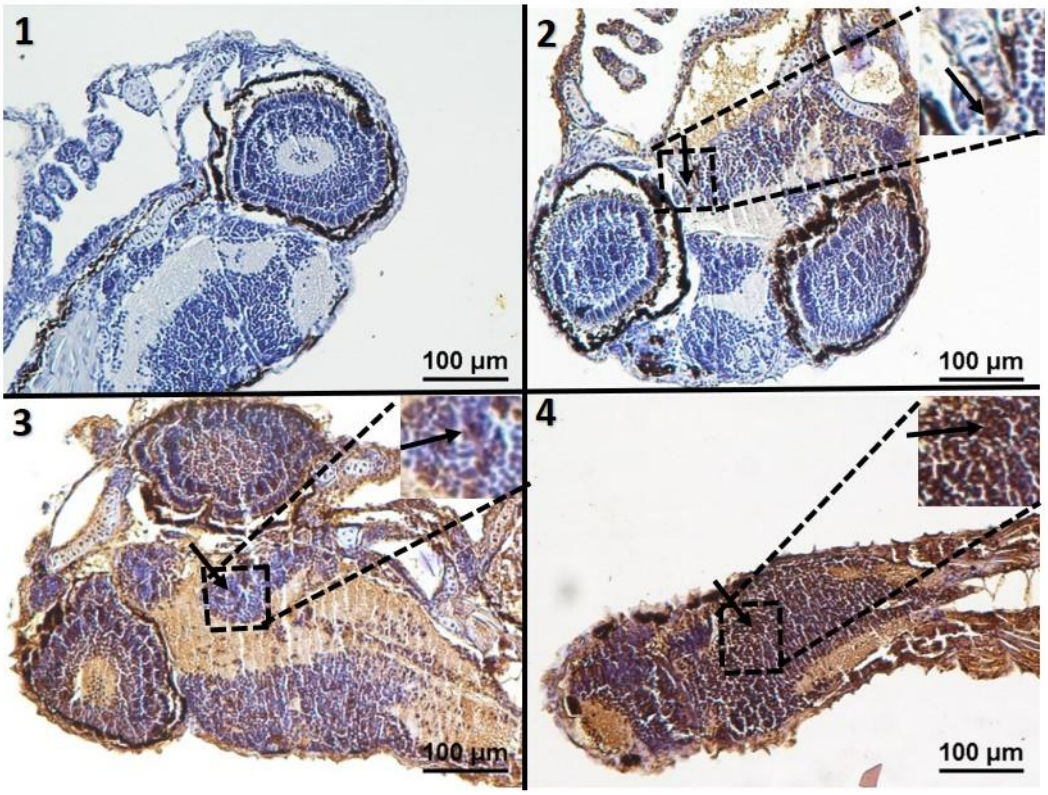


Figure 8. 8-OHdG immunohistochemical sections of larval brains at 96 hpf after RB-203 treatment. No immunoreactivity in the 1st group. Low level of 8-OHdG immunoreactivity (arrows) in the 2nd group, high level in the 3rd and 4th groups. 1: Control, 2: 1 mg L^{-1}, 3: 10 mg L^{-1}, 4: 100 mg L^{-1} . 200x.

Discussion

Global consumption of synthetic dyes is approximately 7×10^5 tons per year and the synthetic dye consumption generates large amounts of wastewater discharged into aquatic ecosystems(Hernández-Zamora and Martínez-Jerónimo, 2019b). Despite the widespread use in the world, there are no data available on the potential toxicity mechanisms of RG-203 and MB-5G textile dyes. In this paper, we determine the LC_{50} values of RG-203 and MB-5G at 96 as 278.32 mg L^{-1} and 166.04 mg L^{-1}, respectively. The absorption rate of chemicals with a large molecular weight (MW) is slower than those with a small molecular weight (Balogh et al., 2008; Kuna et al., 2018; Zhu et al., 2019). This is an important feature that affects the toxicity of dyes on organisms (Chakraborty, 2015). Of the two textile dyes we used, the MW of RB-203 is greater than that of MB-5G, explaining the lower LC_{50} value of MB-5G. Shen et. al., have likewise identified the LC_{50} values for Direct Red 28 and Basic Violet 14 dyes as 476.84 µg ml^{-1} and 60.63 µg ml^{-1}, respectively, with the former dye having a MW of 696.66 g mol^{-1} and the latter one of 337.8 g mol^{-1} (Shen et al., 2015). These findings are in line with our LC_{50} data for MB-5G and RB-203 dyes.

Hatching in fish is the primary and most important developmental phenomenon caused by a series of morphogenetic events (Karthik et al., 2019). Hatching success and time are important ecotoxicological criteria (Kataoka et al., 2018). Although both RB-203 and MG-5G dyes appear to affect the hatching of zebrafish embryos in a concentration-dependent manner, we observed the most striking effect with RB-203 at its highest dose (100 mg L^{-1}) as premature hatching at

48 hpf. This can be explained by the blockage of the chorion pores, which causes a hypoxic state and prevents the excretion of metabolites. These conditions can increase the release of enzymes that facilitate the rupture of the chorion (Carvalho da Cruz Brambilla *et al.* 2019). Larval hatching time can be affected by the activity of chorionic hatching enzymes and embryonic motility (Cheng et al., 2007; Yamagami, 1981). Chemicals such as xenobiotics that prevent the oxygen exchange of the embryo and result in an increase in respiratory rate or increased stimulation of hatching enzyme activity may cause premature hatching (Du et al., 2012; Manjunatha et al., 2014; Samaee et al., 2015).

Premature hatching may cause developmental deformities such as reduced growth, the curvature of the body and tail, and yolk sac edema (Karthik et al., 2019; Liang et al., 2017; Samaee et al., 2015). We have observed tail malformations, pericardial edema, yolk sac edema, microphthalmia, and curved body axis in all groups of embryos/larvae exposed to RB-203 and MG-5G dyes. Most of these abnormalities have also been observed for the azo dye Direct blue 15, the artificial food dye tartrazine, and the hair dye Basic Red 51 in zebrafish embryos (Hernández-Zamora and Martínez-Jerónimo, 2019a) (Abe *et al.* 2017, Joshi and Katti 2018). In our study, we determined the highest malformation rate as the curved body axis and pericardial edema in embryos treated with RB-203 and MG-5G, respectively. Acid Red 26 dye appears to exert cardiovascular toxicity at concentrations above 2500 µg/ml (Shen et al., 2015). Azo dyes likewise cause cardiac edema and a decrease in heart rate in zebrafish embryos (Jiang et al., 2020). Some textile dyes have also been reported to cause cardiac edema in zebrafish embryos and larvae (Hernández-Zamora and Martínez-Jerónimo, 2019a; Shen et al., 2015).

Interestingly, we have observed that the larval cardiac edema was accompanied by degeneration and necrosis in the brain tissues at high doses of MB-5G and RB-203 dyes. Several clinical studies in patients have suggested a similar relationship between acute cardiac dysfunction and brain hemorrhage (Yoshimura et al., 2008, Lee et al., 2016, Chen et al., 2017). Thus the combined effect of MB-5G and RB-203 dyes on heart and brain tissues deserves further examination.

Oxidative stress is a complex biological process that results from an imbalance between the production of reactive oxygen species (ROS) and antioxidant defense systems (van Velzen et al., 2017). ROS can attack deoxyribose phosphate backbones and nucleobases of DNA nucleotides, generating a wide variety of base- and sugar-modified products (Dalle-Donne et al., 2006).Oxidative damage induced by reactive oxygen species is a major factor in the progression of many neurodegenerative and oncological diseases. DNA lesions and 8-OHdG are known as biomarkers of oxidative damage (Cooke et al., 2003; Hussein et al., 2017). There is a limited number of studies on mechanisms of the DNA damage of textile dyes (Oliveira *et al.* 2018, Carvalho da Cruz Brambilla *et al.* 2019). We detected a dose-dependent increase in 8-OHdG levels in the brain tissues of zebrafish larvae treated with MB-5G or RB-203. This increase may activate the sympathetic nervous system by stimulating the hypothalamus and cardiac activities (Jia et al., 2015). The neurotoxic effects of dyes depend on the sensitivity of the brain to oxidative stress. High oxygen consumption rate, high polyunsaturated fatty acids content, regional high iron levels, and proportionately low antioxidant capacity are among the key factors that determine the reaction of the brain to oxidative stress (Noseworthy and Bray,

1998; Meireles et al., 2018). The mechanism of the effect of RB-203 and MB-5G dyes remains largely unknown. However, it is possible that the high 8-OHdG activity in the brain is not only caused by high doses of the dyes but also due to the cationic state of MB-5G that may cause toxicity by binding to the negatively charged DNA with high affinity (Alkan et al., 2008; Dezhampanah et al., 2019). It is also known that binding molecules with a cationic structure can interact with DNA by forming hydrogen bonds with base pairs in small grooves in the DNA structure (Rehman et al., 2014). There is evidence that textile dyes and products of electrolysis can generate DNA damage by interfering with the double helix structure (Uliana et al., 2013). Future studies will not only clarify the molecular mechanisms underlying the toxic effect of these dyes but will also pave the way for mitigating these undesirable impacts in the marine environment.

Conclusion

The findings of this paper are summarized as follows:

- MB-5G and RB-203 dyes exert toxicity and genotoxicity during zebrafish embryonic development.
- Based on the LC_{50} values, MB-5G appears to be more toxic than RB-203.
- RB-203 and MB-5G dyes can induce DNA damage due to oxidative stress, causing irreversible damage to embryos/larvae.
- RB-203 and MB-5G result in developmental abnormalities such as yolk sac edema, pericardial edema, curved body axis, tail malformation, and microphthalmia, strongly suggesting that they have detrimental effects on reproductive success.
- The embryotoxic effect of the dyes indicates a potential teratogenic effect.
- The synthetic textile dyes that are discharged into the water pose a threat to aquatic organisms and their habitats. Thus they must be considered as major pollutants and subject to strict environmental protection regulations.

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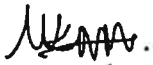
Declaration of Interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Authors	Signatures
Mine KÖKTÜRK	
Fikret ALTINDAĞ	
Gunes OZHAN	
Mehmet Harbi ÇALIMLI	
Mehmet Salih NAS