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Bir Azo Boyası Olarak Maxilon Blue 5G'nin Solucanlar Üzerindeki Akut Ekotoksikolojik ve Histopatolojik Etkileri

Mine KOKTURK[1], Fikret ALTINDAG[2*]

ÖZET: Günümüzde boyaların çevre ve yaşam sağlığı üzerindeki etkileri önemli bilimsel konulardır. Maxilon blue 5G'nin toprak yapısı için çok önemli organizmalar olan solucanlar üzerindeki histopatolojik ve ekotoksikolojik çalışmalarını ilk kez bu yazıda sunuyoruz. Solucanlar, Maxilon blue 5G'ye, 1.0-8000 mg L^{-1} aralığında farklı dozlarda, direkt enjeksiyon yöntemiyle 48 saat süreyle maruz bırakıldı. Deneysel analiz, 5000 mg L^{-1} ve 8000 mg L^{-1} Maxilon blue 5G dozajlarının enjeksiyonu ile solucanlarda bazı önemli morfolojik anormalliklerin tespit edildiğini gösterdi. Maxilon Blue 5G'nin solucan deneylerindeki LD_{50} değerleri 48 saat sonra 6324.56 mg L^{-1} olarak hesaplanmıştır ve bu değerler literatür için ilk deneysel bulgulardır. Çalışmanın bulguları, yüksek dozda Maxilon blue 5G enjekte edilen solucanların bağırsaklarında ve tüm vücudunda gözlenen birçok ciddi doku hasarının histopatolojik incelemeleri ile desteklendi.

Anahtar Kelimeler: Solucan, Ekotoksikolojik etkiler, Boyar madde, Histopatoloji, Maxilon blue 5G

Acute Ecotoxicological and Histopathological Effects of Maxilon Blue 5G as an Azo Dye on Earthworms

ABSTRACT: Today, the effects of dyes on the environment and life health are important scientific issues. In this paper, for the first time, we report the histopathological and ecotoxicological studies of Maxilon blue 5G on earthworms as very important organisms for soil structure. Earthworms was exposed to Maxilon blue 5G by direct injection method with different doses in a range of 1.0- 8000 mg L^{-1} for 48 h. The experimental analysis showed that some considerable morphological abnormalities in the earthworms were detected with the injection of 5000 mg L^{-1} and 8000 mg L^{-1} of Maxilon blue 5G dosages. LD_{50} values of Maxilon Blue 5G in earthworms' experiments were calculated as 6324.56 mg L^{-1} after 48 h, and these values are the first experimental findings for the literature. The findings of the study were supported by histopathological investigations that are many severe tissue damages that were observed in the intestine and the whole body of earthworms injected with a high dosage of Maxilon blue 5G.

Keywords: Earthworm, ecotoxicological effects, dyestuff, histopathology, Maxilon blue 5G

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INTRODUCTION

Today, many industrial product components consist of dyestuffs or organic compounds, so dyestuffs are vital and irreplaceable for industrial sectors (Rawat et al., 2016). However, dyestuffs appear as problematic substances, and exhibit adversely affects natural resources. Emitting of dyestuffs into natural sources from industries of paper, textile, printing, and distillery cause numerous environmental problems because these substances pollute the aquatic and soil life (Nas et al., 2019; Tkaczyk et al., 2020). Synthetic dyestuffs consisted of complex and different compositions that exhibit various thermal, optical, and physical/chemical features (Alkan et al., 2008). It has been demonstrated that dyes and degraded dyes products have toxic impacts on various living terrestial and aquatic living organisms and human beings (Puvaneswari et al., 2006; Copaciu et al., 2013) . Especially, azo based dyes possess some recalcitrant and xenobiotic nature that makes a long-life challenge on natural functions and structure for the ecosystem (DeVito, 1993). Normally, the mentioned ecosystems exhibit some altering or remediation effects against dyes, but the products formed from physicochemical and microbial factors are not always less toxic or non-toxic substances (Levine, 1991). According to a study, about 900,000 tons of dyes are manufactured annually, and more than 70% of this amount is azo dyes (Carmen and Daniel, 2012; Balapure et al., 2015). Additionally, a considerable amount of industrial wastes (15-20 %) comprised of dyes and their derivatives are is released into the environment (Kant, 2012). Nearly, 50 % of produced annual dye productions spread to the environments either directly or due to dyestuff production losses (Carmen and Daniel, 2012; Hassaan and El Nemr, 2017). Annually, over 10.000 different dyestuffs with 7.10^5 tons of productions are applied to industrial sectors, and azo based dyes consisted of the main group of these dyes (Langhals, 2004).

As mentioned above, releasing dyes to the environment causes several adverse effects for human beings and the other organism (Reile et al., 2020; Moorthy et al., 2021). It can be inferred from the aforementioned information, although dyes have a vast release amount into the environment, limited studies have been conducted to reveal their toxicity effects on living creatures. Therefore, very limited information is known about the carcinogenic and mutagenic effects of dyes and especially related to azo based dyes (Alderete et al., 2021; Haque et al., 2021). In vivo and in vitro studies based on azo dyes to compare genotoxicity and toxicity effects possess some considerable difficulties, so we can say that studies to elucidate toxicities of dyes are very important (Köktürk et al., 2021).

Earthworms are one of the leading ecosystem engineers for terrestrial ecosystems (Lavelle et al., 1998). Some of the earthworms' functions, including casting, burrowing, mixing of soil, and litter, are very effective in the keeping of soil life (Langdon et al., 2003). Therefore, earthworms are very important creatures in soil structure/stability, flowing of air into deep parts of soil, leaking of water in the soil as well as biotic features such as plant community structure, food webs underground, nutrient cycling, microbial activity, mineralization, density and distributions of soil invertebrates (Eisenhauer, 2010). Additionally, earthworms are accepted as the most suitable organism as an indicator in the ecotoxicological evaluation due to their 60-80 % constitution of soil biomass and vital functions (moisture, aeration, and nutrients cycling) in soil, and they are also very sensitive against the very low amount of toxicants (Pino et al., 2015; Yang et al., 2017). That’s why, to detect toxicant substances in soil, earthworm acute toxicity tests are recommended by the authorized environments programs and agencies(Genázio Pereira et al., 2017). To best of this study is that no study has been conducted to detect the toxicological effects of Maxiolon Blue 5G, as an azo based dye, on earthworm yet. Thus, due to the lack of toxicological data on the azo dye Maxilon blue 5G, the present study aimed to evaluate the ability of this dye to induce toxic and histopathological effects on earthworms.

MATERIALS AND METHODS

Chemicals

Maxilon Blue 5G dye ($C_{16}H_{26}N_3O$, MW:266 g mol^{-1}) used in the experiments was purchased from commercial companies, in Bursa in Turkey. Maxilon Blue 5G dye was of analytical grade with a purity > 99.5 %. Dye solutions were prepared using pure water.

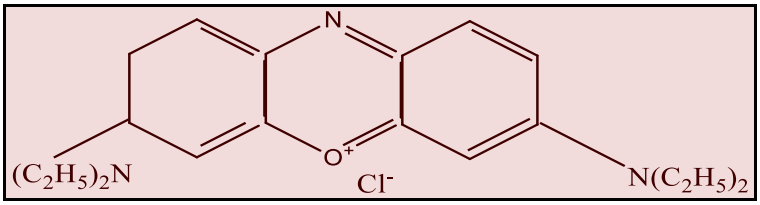

Figure 1. The chemical formula of Maxilon blue 5G

Acute Toxicity Test with Earthworms

Earthworms called *Eisenia fetida* were purchased commercially, and the earthworms used in the study kept under controlled conditions (20 ± 2ºC, 75% humidity and 16:8 hours light-dark) at the research laboratory of Igdır University. These earthworms were fed with cow manure at regular intervals. To adapt to the test conditions, the earthworms used in the experiments were kept under controlled laboratory conditions for two weeks.

Maxilon Blue 5G Injection to *Eisenia Fetida*

The direct injection route as conducted elsewhere was applied to investigate the toxic effects of Maxilon blue 5G on *Eisenia fetida* by given the dye into the coelomic cavity of the earthworm (Park et al. 2012; Yesudhason et al. 2018). A syringe having a needle with a 0.5 ml micro fine plus was used in the injection of earthworm. Earthworms were placed in wet filter paper to empty all contents of the gut for 24 h before the test. Initially, before injection, earthworms were placed on ice for calm during 15 min. 8 groups ranging control, 1, 10, 100, 1000, 3000, 5000, and 8000 mg L^{-1} (injecting 50 µl) were prepared for experiments. The experiments were performed using 6 earthworms in each group and 3 repetitions. The experiment was performed with adult earthworms with clitellum (300-350 mg). The medium earthworms kept in was set at 75 % humidity and 20 ° C during all the experiments, and filter paper was renewed daily. Dead earthworms were recorded and removed; therefore, the other earthworms were protected from being affected by the dead earthworms. Morphological abnormalities recorded at 48 h both the control and treated groups.

Histopathological Experiments

For histopathological evaluation, randomly four earthworms obtained from each group at 24 and 48 h were fixed in a 10 % formalin. It was embedded in paraffin, after histological tissue was followed. Transverse sections with a thickness of 4µm were taken in the microtome. After the sections were stained with Hematoxylin&Eosin (H&E), they were examined by light microscopy (Nikon Y-IM 7551012, Japan). For histopathological evaluation, an average of 15-17 areas was evaluated by random sampling for each animal in the groups. The findings were semiquantitatively evaluated. according to the average number of lesions observed in the microscopically examined areas.

LD_{50} values for the Maxilon Blue 5G compound was calculated by Probit analysis that is performed by SPSS 20.0 software programme. Each of the toxicity data sets was compared with its corresponding control, followed by one-way ANOVA and Tukey' test. The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Ecotoxicology studies are performed to reveal the basic mechanisms originated from the pollutants that may disrupt the normal physiological state of biological systems and to prevent the negative consequences arising from there (Connon et al., 2012). There are limited studies related to dye ecotoxicology using terrestrial organisms in the literature (Genázio Pereira et al., 2017; Oliveira et al., 2018). In this study, *E. fetida*, as a terrestrial organism model, was used to detect the acute toxicity of Maxilon blue 5G. The experiments of probit analysis were performed by observing died or survived earthworms after 48 h. Additionally, LD_{50} values of Maxilon Blue 5G in earthworms' experiments were calculated as 6324.56 mg L^{-1} after 48 h, and these values are the first experimental findings for the literature. In the study, the survival rate of earthworms was reduced depending on doze and time by the injection process (Figure1). Especially, the survival rated at high dose (8000 mg L^{-1}) was detected to be highly reduced ($p<0.05$) compared to the control group, and the death rate at the same dosage was found to be 80 % at 48 h (Figure 2).

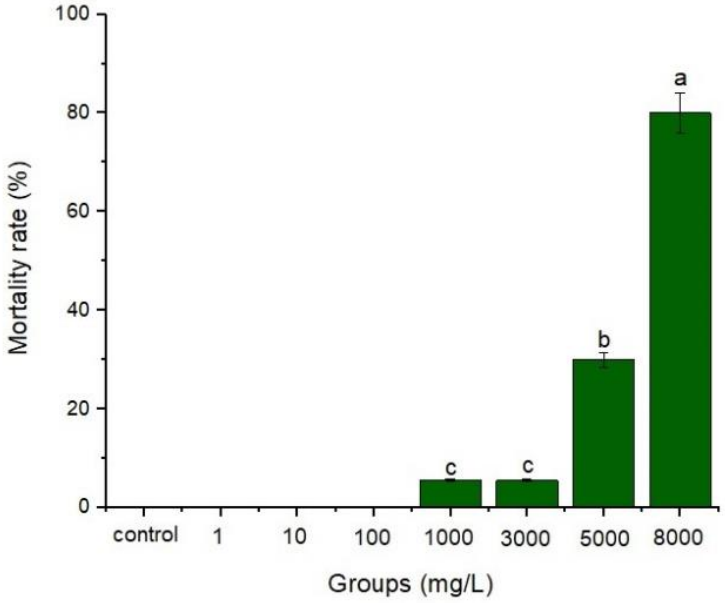

Figure 2. The mortality rate of earthworms during the exposure time of 48 h. Data are expressed as means ± S.D. from three independent experiments. ($p < 0.05$; ANOVA, Tukey's test).

Similar studies conducted with textile dyes (Direct Black 38, Reactive Blue 15) on earthworms have been carried out by some researchers, and due to low mortality, LC_{50} values could not be detected at 24 h and 48 h. However, a study reported that LC_{50} values were detected with the high dosage of textile dyes (Indigo Carmine) at 72 h (Genázio Pereira et al., 2017). In our work, somebody abnormalities like thinning in the body, a rupture in areas near the tail with swelling in some areas, and blood collection in the tail area were observed after injection of Maxilon Blue 5G (Figure 3). The morphological abnormalities compared to the control group after the injection process of 5000 mg L^{-1} and 8000 mg L^{-1} dosages were determined as considerably effective ($p<0,05$). Serious morphological variations like swelling and thinning in some parts of earthworms were detected with toxic dyes in elsewhere (Gopinathan et al., 2015; Genázio Pereira et al., 2017). Histopathological findings are given in Table 1.

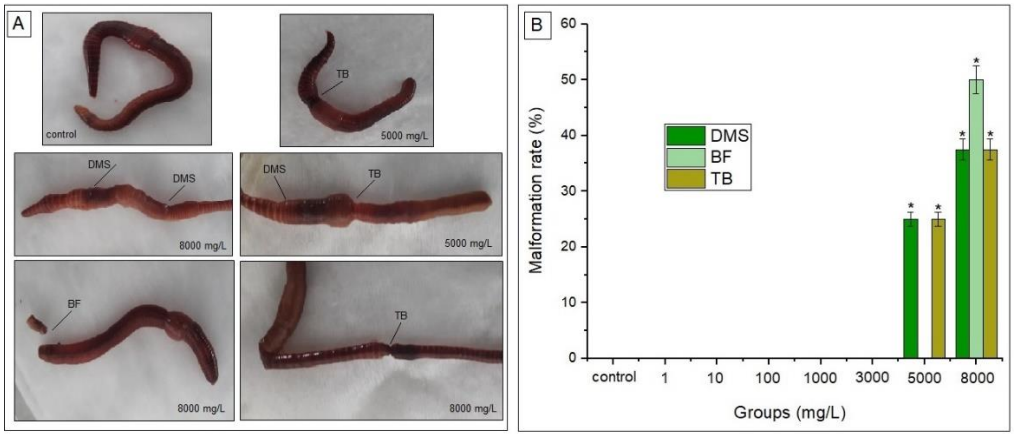

Figure 3. (A) Malformations of earthworms exposed to Maxilon Blue 5G and (B) percentage of observed malformations. *p <0.005 compared with the control group (by one-way ANOVA and Tukey's comparison). Data are expressed as means ± S.D. from three independent experiments. DMS: disruption of metameric segmentation; BF: body fragmentation at posterior region; TB: thinning of the body wall. The malformations were recorded during the exposure time of 48 h.

Control groups were seen in normal histological appearances after 24 h (Figure 4A and 5A) and 48 h (Figure 4B and 5B). At the same time, the group injected with 1 mg L^{-1} of Maxilon Blue 5G (Figure 4C, 5C) and 10 mg L^{-1} (Figure 4D, 5D) exhibited a normal appearance at 48 h. However, another group injected 100 mg L^{-1} of Maxilon Blue 5G exhibited mild atrophy and deformation in the longitudinal muscle layer (Figure 4E), but the intestinal epithelium exhibited normal appearances at 48 h (Figure 5E). After 24 h (Figure 4F) and 48 h (Figure 4G), the group injected 1000 mg L^{-1} of Maxilon Blue 5G was observed with mild atrophy and deformation in the longitudinal muscle layer, and intestinal epithelium has a normal histological appearance (Figure 5F and 5G). The group injected 3000 mg L^{-1} of Maxilon Blue 5G exhibited moderated atrophy and deformation in the longitudinal muscle layer, and mild degeneration and necrosis were observed in the epidermis both at 24 h (Figure 4H) and at 48 h (Figure 4I), the intestinal epithelium exhibited mild degeneration and necrosis both at 24 h (Figure 5H) and at 48 h (Figure 5I). The group proceeded using 5000 mg L^{-1} of Maxilon Blue 5G was observed with moderately atrophy and deformation in the longitudinal muscle layer, mild atrophy and deformation in the circular muscle layer, mild degeneration and necrosis in the epidermis (Figure 4J), and moderate degeneration and necrosis in the intestinal epithelium (Figure 5J) at 24 h. However, the same group proceeded using 5000 mg L^{-1} of Maxilon Blue 5G at 48 h exhibited severe atrophy and deformation in the longitudinal muscle layer, moderate atrophy and deformation in the circular muscle layer, moderate degeneration and necrosis in the epidermis (Figure 4K), and moderate degeneration and necrosis in the intestinal epithelium, mild hyperemia in vessels (Figure 5K). The latest group injected with 8000 mg L^{-1} of Maxilon Blue 5G was observed with severe atrophy and deformation in the longitudinal muscle layer, moderate atrophy and deformation in the circular muscle layer, moderate degeneration and necrosis in the epidermis (Figure 4L), and moderate degeneration and necrosis in the intestinal epithelium, mild hyperemia in vessels (Figure 5L) at 24 h. But after 48 h, the same group exhibited severe atrophy and deformation in the longitudinal and circular muscle layer, severe degeneration, and necrosis in the epidermis (Figure 4M). Moderate degeneration and necrosis in the intestinal epithelium, mild hyperemia in vessels (Figure 5M).

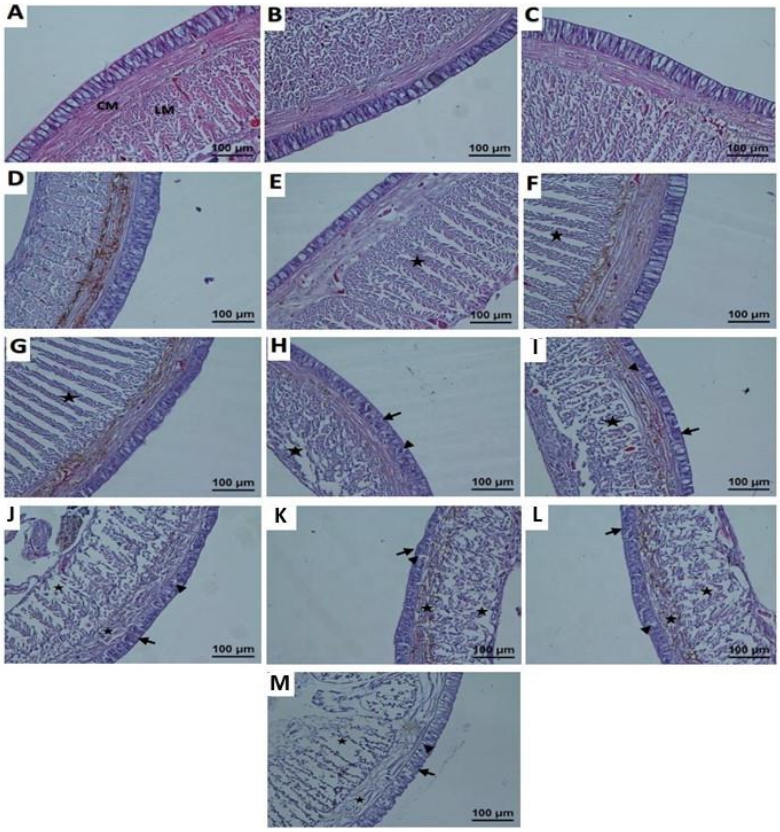

Figure 4. Cross-sections of worms. **LM:** Longitudinal muscle**, CM:** Circular muscle, **E:** Epidermis. **Control 24 h (A), Control 48 h (B), 1 mg L^{-1} 48 h** (C), **10 mg L^{-1} 48 h (D) groups** in normal histological appearance. **100 mg L^{-1} 48 h group (E):** Mild atrophy and deformation (star) in the longitudinal muscle layer. **1000 mg L^{-1} 24 h group (F):** Mild atrophy and deformation (star) in the longitudinal muscle layer. **1000 mg L^{-1} 48 h group (G):** Mild atrophy and deformation (star) in the longitudinal muscle layer. **3000 mg L^{-1} 24 h group (H):** Moderate atrophy and deformation (star) in the longitudinal muscle layer, mild degeneration (arrowhead), and necrosis (arrow) in the epidermis. **3000 mg L^{-1} 48 h group** (I): Moderate atrophy and deformation (star) in the longitudinal muscle layer, mild degeneration (arrowhead), and necrosis (arrow) in the epidermis. **5000 mg L^{-1} 24 h group (J):** Moderate atrophy and deformation in the longitudinal muscle layer, mild atrophy and deformation the circular muscle layer (star), mild degeneration (arrowhead), and necrosis (arrow) in the epidermis. **5000 mg L^{-1} 48 h group (K):** Severe atrophy and deformation in the longitudinal muscle layer, mild atrophy and deformation the circular muscle layer (star), mild degeneration (arrowhead), and necrosis (arrow) in the epidermis. **8000 mg L^{-1} 48 h group (L):** Severe atrophy and deformation in the longitudinal muscle layer, mild atrophy and deformation the circular muscle layer (star), mild degeneration (arrowhead), and necrosis (arrow) in the epidermis. **8000 mg L^{-1} 48 h group (M):** Severe atrophy and deformation (star) in the longitudinal and circular muscle layer, Severe degeneration (arrowhead), and necrosis (arrow) in the epidermis. H-E. x20.

Table 1. Evaluation of histopathological formations of soil worms in different doses of Maxilon Blue 5G in the whole body and intestinal tissue

Histopathological parameters	**Control 24 h**	**Control 48 h**	**1 mg/L 48 h**	**10 mg/L 48 h**	**100 mg/L 48 h**	**1000 mg/L 24 h**	**1000 mg/L 48 h**	**3000 mg/L 24 h**	**3000 mg/L 48 h**	**5000 mg/L 24 h**	**5000 mg/L 48 h**	**8000 mg/L 24 h**	**8000 mg/L 48 h**
Deformation in longitudinal muscle layer, atrophy	-	-	-	-	-	+	+	++	++	++	+++	+++	+++
Deformation, atrophy in the circular muscle layer	-	-	-	-	-	-	-	-	-	+	++	++	+++
Degeneration in the epidermis	-	-	-	-	-	-	-	+	+	+	++	++	+++
Necrosis in the epidermis	-	-	-	-	-	-	-	+	+	+	++	++	+++
Degeneration in intestinal epithelium	-	-	-	-	-	-	-	+	+	++	++	++	++
Necrosis in intestinal epithelium	-	-	-	-	-	-	-	+	+	++	++	++	++
Hyperemia	-	-	-	-	-	-	-	-	-	-	+	+	+

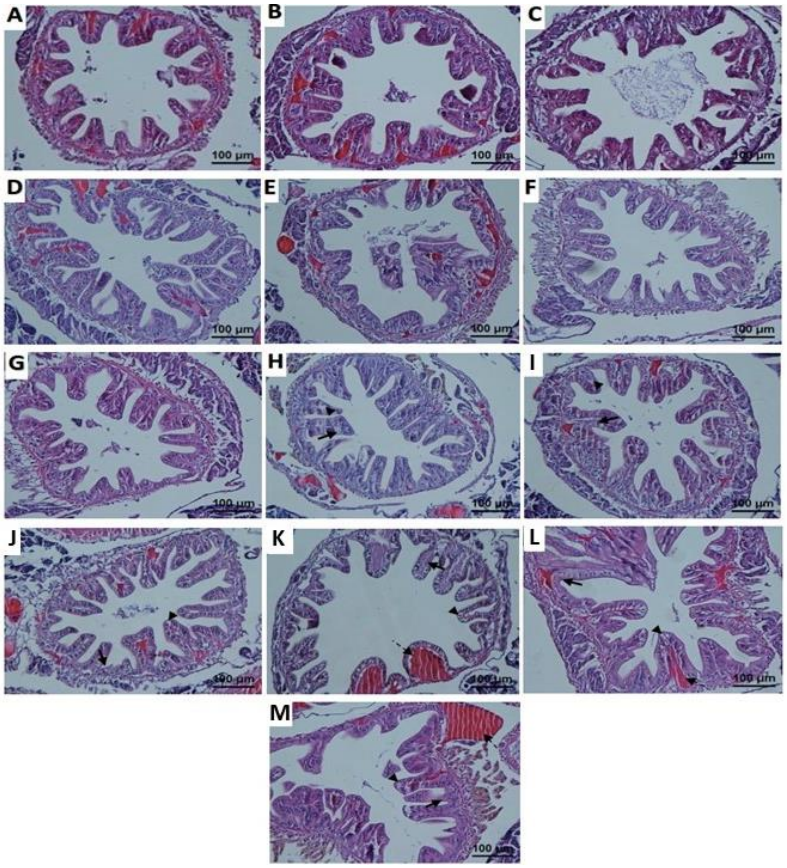

Figure 5. Cross-sections of worms. In the **Control 48 h (B), 1 mg L^{-1} 48 h (C), 10 mg L^{-1} 48 h (D), 100 mg L^{-1} 48 h (E), 1000 mg L^{-1} 24 h (F) and 1000 mg L^{-1} 48 h (G) groups,** the intestinal tract has a normal histological appearance. **3000 mg L^{-1} 24 h group (H):** Mild degeneration (arrowhead) and necrosis (arrow) in the intestinal epithelium. **3000 mg L^{-1} 48 h group (I):** Mild degeneration (arrowhead) and necrosis (arrow) in the intestinal epithelium. **5000 mg L^{-1} 24 h group (J):** Moderate degeneration (arrowhead) and necrosis (arrow) in the intestinal epithelium. **5000 mg L^{-1} 48 h group (K):** Moderate degeneration (arrowhead) and necrosis (arrow) in the intestinal epithelium, mild hyperemia in the vessels (dashed arrow). **8000 mg L^{-1} 48 h group (L):** Moderate degeneration (arrowhead) and necrosis (arrow) in the intestinal epithelium, mild hyperemia in the vessels (dashed arrow). **8000 mg L^{-1} 48 h group (M):** Moderate degeneration (arrowhead) and necrosis (arrow) in the intestinal epithelium, mild hyperemia in the vessels (dashed arrow). H-E. x20.

The intestine is a vital organ responsible for the metabolism of various compounds such as azo dyes (Sweeney et al., 1994). Intestinal microflora of humans and animal species can decrease azo groups of xenobiotics (Cerniglia et al., 1982). The decline reaction is responsible for the manufacture of aromatic amines with carcinogenic and mutagenic effects (Govindwar et al., 2014; Beer et al., 2019). Bacterial degradation of azo dyes has generally been reported when tearing of nitrogen bonds is initiated by the biotransformation process of the azo reductase enzyme (Zanoni et al., 2013; Franco et al., 2018). In the light of the above data and literature information, we can evaluate that the observing the degenerations of intestinal epithelium in earthworms exposed to 5000 mg L^{-1} and 8000 mg L^{-1} of Maxilon Blue 5G showed the reduction of Maxilon blue 5G by intestinal microflora that conversion the dye into harmful amines. It was predicted that lower levels of intestinal degeneration in soil worms treated with Maxilon Blue 5G than in other tissues might be due to the success of enteric bacteria in the intestine's ability to remediate chemicals (Nayak et al., 2018; Banerjee et al., 2019). The main process of muscle atrophy is myofiber decline, which is the result of high protein deterioration (Washington et al., 2011; Cai et al., 2018). This demolition process can be induced by chronic inflammation and acute metabolic change (Albadarin and Mangwandi, 2015).

CONCLUSION

Summarized, Maxilon blue 5G as the azo dye is extensively used in the textile industry, and its presence in nature is increasing day by day. Our study proved that Maxilon Blue 5G could cause cell injuries such as degeneration and necrosis besides deformation and atrophy of muscles in earthworms. With this report, we revealed that severe injury of tissue and cells such as necrosis in epidermis tissue, hyperemia in vessels, necrosis in the intestinal epithelium, deformation, and atrophy of muscles in earthworms exposed to Maxilon Blue 5G dye. The findings of the current study can be explained as the main reasons for observing atrophy in the muscle layer of earthworms exposed with high (3000, 5000 and 8000 mg L^{-1}) dye dosage is that the forming of oxidative stress or nitrosative stress due to enhanced the number of reactive oxygen species (ROS) or reactive nitrogen species (RNS) in tissues by Maxilon Blue 5G. Further studies to determine the mechanism of action of Maxilon blue 5G dye and their effects on other target organisms are necessary to predict the future effects of this dye and to take precautions

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Conflict of Interest

The authors declared that there is no conflict of interest

Author's Contributions

Mine Köktürk planned the study, performed experiments, commented on the data, writed and edited the manuscript. **Fikret Altındağ** performed the histopathological examination, commented the histopathological findings.

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