Morphological and histological effects of copper sulfate on the larval development of green toad, *Bufo viridis*

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**Abstract:** Declines in populations of amphibians have been occurring more dramatically over the past 25 years. The declines have various causes. One of the possible causes of amphibian declines is pesticides. Copper sulfate is commonly used as a fungicide in vineyards and gardens in Turkey. In this study, our purpose was to explain the morphologically and histologically acute toxic effects of copper sulfate on *Bufo viridis* tadpoles. *B. viridis* tadpoles at Gosner stage 21 were exposed to different concentrations (0.01, 0.05, and 0.1 mg/L) of copper sulfate for 120 h. The LC₅₀ value of copper sulfate was calculated as 0.058 mg/L. Morphological measurements indicated that copper sulfate concentrations of 0.05 and 0.1 mg/L caused poor larval development and growth. Edema was observed in the histological sections, especially on the cross-sections passing from the head (≥0.01 mg/L CuSO₄). Furthermore, hepatocellular degeneration in the liver, deformation of gastrointestinal epithelial cells, and deformation and anomalies in somite formations were all observed (≥0.01 mg/L CuSO₄). Reduced reaction to stimuli, loss of equilibrium, and shortening of swimming distance were observed in the tadpoles of the 0.05 and 0.1 mg/L CuSO₄ treatment groups.

**Key words:** Amphibian, *Bufo viridis*, copper sulfate, tadpole, development

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Bakır sülfat’ın gece kurbağası, *Bufo viridis* larvalarının gelişimi üzerindeki morfolojik ve histolojik etkileri

Özet: Amfibi populasıyollarındaki azalmalar geçen son 25 yılda daha dramatik bir hal almıştır. Bu azalışın çeşitli nedenleri bulunmaktadır. Amfibilerde görülen bu azalışın nedenleriinden birisi de pestisitlerdir. Bakır sülfat, Türkiye’de bağlarda ve bahçe çöplerinde sık kullanılan birfungisidir. Çalışmada, bakır sülfatın *Bufo viridis* iribaşları üzerindeki akut toksik etkilerinin morfolojik ve histolojik olarak ortaya konulması amaçlanmıştır. Gosner 21. evredeki *B. viridis* iribaşları farklı konsantrasyonlarda (0,01, 0,05, ve 0,1 mg/L) bakır sülfata 120 saat süreyle maruz bırakılmışlardır. Bakır sülfatın LC₅₀ değeri 0,058 mg/L olarak hesaplanmıştır. Morfolojik ölçümler, bakır sülfatın larval gelişim ve büyümede gerilemeye neden olduğunu göstermiştir. Histolojik kesitlerin, özellikle baş bölgesinde geçen enine kesitlerinde ödem gözlenmiştir (≥0,01 mg/L CuSO₄). Bununla birlikte karaciğerde hepatoselüler dejenerasyon, mide ve bağışıklık epitel hücrelerinde hasar, somit taslaklarında deformasyon ve anomaliler gözlenmiştir (≥0,01 mg/L CuSO₄), 0,05 ve 0,1 mg/L CuSO₄ uygulamanın yapıldığı gruplardaki larvalarında, uyaranlara daha az tepki verme, denge kaybı ve yüzme mesafesinin kısaltğı gözlenmiştir.

**Anahtar sözcükler:** Amfibi, *Bufo viridis*, bakır sülfat, iribaş, gelişim

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Introduction

Decreases in amphibian populations have been observed on the global scale and the causes of these decreases are serious ecological problems. These population decreases have become more dramatic in the last 25 years. It has been reported that amphibians are threatened more than other vertebrate classes (Beebee and Griffiths, 2005). There are many hypotheses to explain this decrease, including habitat deterioration, disease, predation, competition, and the increase in the level of UV radiation as a result of decrease in atmospheric ozone (Collins and Storfer, 2003; Beebee and Griffiths, 2005). Natural habitats are likely to be contaminated in many ways as a result of anthropogenic effects such as agricultural practices (Johansson et al., 2006). Many chemical substances used during these activities have negative effects on aquatic systems (Garcia-Munoz et al., 2009). Thus, the negative effects of chemical pollution on the aquatic ecosystem as a result of agricultural activities have been investigated in recent ecotoxicological studies (Van Dam et al., 1998). When compared with other aquatic vertebrates, amphibians are under a greater risk since they prefer shallow, standing, and temporary ponds around agricultural fields as reproductive habitats (Tyler, 1994; Rowe et al., 2003). They are extremely sensitive to chemical pollutants, particularly in their aquatic biotopes, where they spend their embryonic and larval stages (Murphy et al., 2000). Moreover, they are quite good bioindicators for understanding the effects of environmental changes since they use both aquatic and terrestrial media throughout their lives and their skin is extremely sensitive and permeable (Wyman, 1990; Lips, 1998). In previous studies, it was reported that some pesticides used during agricultural practices caused decreases in amphibian populations (Marco and Blaustein, 1999; Davidson et al., 2001; Sparling et al., 2001; Davidson, 2004; Ezemonye and Ilechie, 2007; Sayum, 2007; Sparling and Fellers, 2007; Bernabo et al., 2008; Kang et al., 2008). The effects of copper sulfate on the tadpoles of *Bufo viridis* are unknown, although there are studies that have investigated the toxic effects of exposure to copper sulfate and copper on the embryos and tadpoles of different anuran species (Porter and Hakanson, 1976; Khangarot and Ray, 1987; Fort and Stover, 1996; Garcia-Munoz et al., 2009).

Copper sulfate (CuSO$_4$·5H$_2$O) is used as a fungicide and an algicide in agricultural practices. Furthermore, copper sulfate, being used in various industries such as the textile, leather, electroplating, tree protection, and oil industries, has many opportunities to contaminate the amphibian habitat. Copper sulfate is classified within the pesticides for general use, as defined by the EPA. It is stated that this pesticide, labeled as class I (highly toxic) in terms of toxicity, may have dangerous effects, particularly on species under threat of extinction in aquatic ecosystems, due to its potential risk of being mixed with surface waters (Extoxnet, 1996). It is said that this pesticide is used quite frequently in Turkey (Öğüt and Küçüköner, 2008).

The green toad, *Bufo viridis*, is included in the conservation category of Least Concern (IUCN, 2008). This terrestrial and nocturnal toad is widely distributed throughout Turkey and is found in all suitable habitats. This species is water-dependent during its reproductive season and stays in water for a long time (Başoğlu et al., 1994). This toad generally uses slow-flowing and standing waters, seasonal ponds, and shallow pits filled with water for egg laying (Kinzelbach and Kasparek, 1992). The eggs and tadpoles of this species were also observed in the seasonal ponds around agricultural fields.

The aim of this study was to determine the morphologically and histologically acute toxic effects of copper sulfate on the development, growth, and survival rates of *Bufo viridis* tadpoles under laboratory conditions.

Materials and methods

Test organisms and acclimatization

The tadpoles for this study were procured in the laboratory from 5 pairs of adult *Bufo viridis* caught in amplexus in March 2009 in a seasonal pond in Çanakkale.

The toad specimens were brought to the laboratory in plastic containers and transferred to polypropylene containers filled with distilled water to a shallow level. They were captured during amplexus and observed until oviposition occurred. Afterwards, eggs were transferred to 30-L glass aquariums filled with distilled water. Tadpoles at stage 21 (Gosner, 1960) were raised...
in glass aquaria. All experiments were carried out at 21 ± 2 °C and in a 14:10-h light-dark cycle. The temperature, pH, and dissolved oxygen levels of the water were measured daily with an Elmetron CO-401 meter. All tadpoles were fed with plant-origin fish feed and boiled lettuce *ad libitum*. Their water was changed once every 2 days and excess food, feces, and dead tadpoles were taken away daily.

**Pesticide and bioassay procedure**

Copper sulfate (CuSO$_4$·5H$_2$O, Sigma) was used as the toxic substance in the study. A stock solution was prepared by dissolving the copper sulfate in pure water, and test concentrations of 0.01, 0.05, and 0.1 mg/L were freshly prepared before being used. The specified doses are in agreement with a previous study on a different amphibian species using copper sulfate (Garcia-Munoz et al., 2009). We randomly selected 50 tadpoles (Gosner stage 21) for the control and each treatment. The tadpoles were transferred to polypropylene containers and exposed to the copper sulfate solutions for 120 h. Each test dose was performed in 3 replications. No pesticides were applied to the tadpoles in the control group, and they were allowed to develop under identical environmental conditions as the tadpoles in the test groups. Detailed observations were made every 24 h; the number of dead tadpoles was counted and the LC$_{30}$, LC$_{50}$, and LC$_{70}$ values for these periods were determined. Furthermore, the behavioral changes observed in the tadpoles were recorded.

**Morphological and histological experiments**

Twenty tadpoles from the control and each treatment group, after surviving the 120-h test period, were measured with a digital compass for total body size, width, and tail length. General morphological photographs of the tadpoles were taken under a binocular microscope with a Canon Powershot A60 digital camera. In addition, 5 surviving tadpoles from the control and each treatment group were preserved in 70% alcohol after having been fixed in Bouin’s solution for 10 h. Afterwards, these 5 tadpoles were processed through an alcohol, xylene, and paraffin series and then paraffin blocks were prepared. Cross-sections 8 μm in thickness were obtained from these blocks, stained with hematoxylin and eosin (H&E), and examined histopathologically under a light microscope. Finally, histological photographs of the preparations under examination were taken with a camera adapted to an Olympus BX51 light microscope using DP2-BSW computer software.

**Statistics**

Copper sulfate LC$_{30}$, LC$_{50}$, and LC$_{70}$ values in periods of 24, 48, 72, 96, and 120 h in the study were determined at a 95% confidence interval with Minitab 15 using the Probit analysis method, and a concentration-survival curve was created in order to show the percentages of individuals that survived related to copper sulfate concentrations. Furthermore, a normality test for the morphological measurement data of the tadpoles was performed with the Kolmogorov-Smirnov test, and a comparison was made between the control and treatment groups in terms of total body size as well as width and tail length using the one-way analysis of variance (ANOVA) test. Finally, Box’s M discriminant analysis was done, showing the total difference among groups depending on their doses.

**Results**

During the study period, water temperature, pH, and dissolved oxygen levels were calculated at 24-h periods. Water temperature ranged between 19.8 and 21.3 °C, pH between 6.75 and 7.12, and dissolved oxygen levels between 7.20 and 7.80 mg/L.

No deaths were observed among the tadpoles in the control group within the first 48 h of treatment, whereas 4 dead tadpoles were detected in the control group at the end of 120 h. It was observed that 2 tadpoles died within the first 48 h at 0.01 mg/L, the lowest application dose, while a total of 8 tadpoles died at the end of the treatment. The highest number of deaths was observed at the dose of 0.1 mg/L. After 72 h of treatment, an increase was observed in the number of dead tadpoles at this dose, related to the duration of treatment. The highest number of deaths at this dose was between 96 and 120 h, with 20 tadpoles dying in that time period. A concentration-survival graph showing the percentages of individuals that had survived at the end of 120 h related to dose concentrations is given in Figure 1.

After exposure of the tadpoles to the pesticide for 120 h, LC$_{30}$, LC$_{50}$, and LC$_{70}$ values were calculated as 0.03, 0.058, and 0.086 mg/L, respectively. The LC$_{30}$, LC$_{50}$, and LC$_{70}$ values at the end of 24, 48, 72, 96, and 120 h are presented in Figure 2.
Morphological and histological effects of copper sulfate on the larval development of green toad, *Bufo viridis*

**Statistical differences between the control and treatment groups were calculated according to morphological measurements (total body size: $Z = 0.850$, $P = 0.465$; $F_{3.76} = 71.189$, $P < 0.001$; width: $Z = 0.643$, $P = 0.803$; $F_{3.76} = 54.971$, $P < 0.001$; tail length: $Z = 0.773$, $P = 0.589$; $F_{3.76} = 49.515$, $P < 0.001$). When compared with the control group, the measurements of total body size, width, and tail length for the tadpoles in each of the 3 treatment groups were lower (Figure 3).**

There were differences among the groups according to morphological measurements (Wilks' lambda = 0.187, $X = 124.830$, $P < 0.05$). The function graph of the total variation of doses among the groups is given in Figure 4. As is also understood from the discriminant graph, variation occurs in Function 1. Function 1 represents 98.9% of the total variation (Table).
No anomalies were observed in the tadpoles of the control group (Figure 5). In contrast, edema was observed in the treatment tadpoles, especially those that were exposed to 0.1 mg/L of copper sulfate (Figure 6).

The histological cross-sections of the tadpoles included in the control and treatment groups were examined. No anomalies to support the morphological findings were observed in the tadpoles of the control group. In the cross-sections of the medulla oblongata, notochord, pronephric

<table>
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Figure 4. According to morphological measurements, the total differences of optional application doses between groups.

Figure 5. (a) Lateral view and (b) dorsolateral view of a control-group tadpole at the end of the 120-h experimental period (stage 23).

Figure 6. (a) Dorsolateral view and (b) ventral view of a tadpole treated with 0.1 mg/L copper sulfate at the end of the 120-h experimental period (stage 22) (edema shown with arrows).
tubules, liver, stomach, and intestines for the tadpoles in the treatment groups, it was observed that the distance between organs increased due to edema, and that there were deformations in the epithelial cells of the stomach, intestines, and pronephric tubules. Furthermore, deformation was observed in the somites of the tadpoles that were exposed to 0.05 and 0.1 mg/L of copper sulfate (Figure 7).

No histological anomalies were observed in the cross-sections of the livers of the tadpoles in the control group. However, it was observed that the tissue was more deformed in the liver cross-sections of the tadpoles that were exposed to 0.1 mg/L of copper sulfate, the highest dose. Degeneration of hepatocytes and an increase in intercellular areas were the other important histopathological findings observed (Figure 8).

An examination was performed on the cross-sections of the pronephric tubules that are responsible for the function of excretion at the larval stage, when metamorphosis continues. No histopathological findings were encountered in the cross-sections of the pronephric tubules of the tadpoles in the control group. However, deformations in the epithelium of the pronephric tubules, barely seen in the tadpoles that were exposed to 0.01 mg/L of copper sulfate, the lowest dose, were more obvious at doses of 0.05 mg/L, the middle dose, and 0.1 mg/L, the highest dose (Figure 9).

In the cross-sections of the somites, poor development and deformations were observed in the somite formations of all tadpoles included in the treatment groups. This effect was most obvious at the dose of 0.1 mg/L (Figure 10).

![Figure 7. Transverse sections of the medulla oblongata and internal organs in (a) control-group tadpole, (b) tadpole treated with 0.01 mg/L CuSO₄, (c) tadpole treated with 0.05 mg/L CuSO₄, and (d) tadpole treated with 0.1 mg/L CuSO₄ (Mo: medulla oblongata, S: somite, N: notochord, Pt: pronephric tubules, O: esophagus, L: liver, St: stomach, I: intestine, edema shown with asterisks), H&E staining.](image)
Figure 8. Transverse sections of the liver of (a) control-group tadpole and (b) tadpole treated with 0.1 mg/L CuSO$_4$ (deformation of liver shown with asterisks, degeneration of hepatocytes shown with arrows), H&E staining.

Figure 9. Transverse sections of the pronephric tubules of (a) control-group tadpole, (b) tadpole treated with 0.01 mg/L CuSO$_4$, (c) tadpole treated with 0.05 mg/L CuSO$_4$, and (d) tadpole treated with 0.1 mg/L CuSO$_4$, (deformation of pronephric tubules shown with asterisks), H&E staining.
Reduced reaction to stimuli, short swimming distance, and loss of equilibrium were important behavioral changes observed in the tadpoles exposed to copper sulfate in comparison to the tadpoles in the control group.

Discussion
As can be clearly understood from the results of this study, copper sulfate is a pesticide that has the effect of decreasing the survival percentages of *Bufo viridis* tadpoles. It has been stated that the use of pesticides during agricultural practices is one of the most significant causes of the decreases observed in amphibian species on the global scale (Sparling et al., 2001). Considering this, the serious declines detected in the survival percentages of the tadpoles that were exposed to increasing concentrations of copper sulfate lead us to conclude that populations of this species may be negatively affected as a result of such an exposure that is likely to occur spontaneously in nature.

At the end of the 120-h experiment, the LC_{50} value of copper sulfate for *Bufo viridis* tadpoles was calculated as 0.058 mg/L. The lowest LC_{50} value detected in the 96-h period of the study was 0.09 mg/L, which is similar to 0.08 mg/L, the dose detected in a study on the toxic effects of copper sulfate application for 96 h on tadpoles of the species *Epidalea (Bufo) calamita* (Garcia-Munoz et al., 2009).

In similar acute toxicity studies that investigated the toxic effects of pesticides on toads, it was reported that morphological anomalies (head and tail deformations, edema, ventral blistering, crooked body) were observed at the embryonic and larval stages of development (Nebeker and Schuytema, 2000; Osano et al., 2002; Sayım and Kaya, 2006). The fact that the morphological anomalies that were seen at the end of the application period, such as edema on the necks of the tadpoles, were more apparent...
with increasing doses of copper sulfate suggests the susceptibility of the tadpoles of this species to copper sulfate toxicity. Likewise, it was reported that developmental anomalies and deformations were observed in larvae in a 96-h acute toxicity study using copper on the larvae of the toad species *Xenopus laevis* (Fort and Stover, 1996).

During embryonic and larval development, all organs and systems interact with each other. The deformations observed in the gastrointestinal epithelial cells, hepatocytes, and pronephric tubule epithelial cells in the histological sections of tadpoles in the treatment groups are considered to be due to pesticide interaction. Among these effects, the deformations in the general structure and cells of liver and pronephric tubules were especially apparent in the tadpoles treated with copper sulfate in increasing concentrations. Depending on the level of exposure to the pesticide, deformations and poor development were observed in the somite structures of the tadpoles in the treatment groups. Since these negative effects may also prevent the development of the skeletal and muscular systems of the tadpoles, the immobility and shortened swimming distances observed in the tadpoles of the treatment groups in comparison to the tadpoles in the control group may be attributed to this effect.

The immobility, loss of equilibrium, and late reaction to stimuli observed in the tadpoles of the treatment groups during the study period were important behavioral changes detected. These behavioral changes are similar to changes that were reported in previous studies and were said to depend on exposure to pesticides (Widder and Bidwell, 2008; Garcia-Munoz et al., 2009).

In conclusion, we are of the opinion that copper sulfate has negative morphological and histological effects on the growth and development of *Bufo viridis* tadpoles. Considering that the pesticides used during agricultural practices may also affect living things other than the target organisms, similar fieldwork and laboratory studies will be increasingly important in the future in order to more clearly put forward the reasons for decreases that are likely to occur on a global scale in amphibian populations as a result of unconscious and uncontrolled use of pesticides.

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**References**


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