Research Article

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Toxicity of trifluralin on the embryos and larvae of the red-bellied toad, *Bombina bombina*

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Abstract: Many amphibian populations are in decline throughout the world, and one of the most important proposed causes for this decline is agrochemicals, especially pesticides. This study considers the effects of 120 h exposure of embryos and larvae of the red-bellied toad, *Bombina bombina*, to trifluralin, an herbicide, with renewal acute toxicity tests at 22 ± 1 °C. Adult males and females were induced to breed by gonadotropin injection to obtain test organisms. Lethal concentration values were determined through PROBIT analysis. The 120 h LC_{50} of trifluralin for the larvae was 11.80 mg L^{-1}, while the LC_{50} and EC_{50} for the embryos were 9.40 and 5.58 mg L^{-1}, respectively. Trifluralin concentrations representing LC_{10}, LC_{50}, and LC_{90} from 24 to 120 h were plotted as toxicity profile curves for larval-toxicity tests. The teratogenic index for trifluralin was 1.69, which points out a significant teratogenic risk. Abnormal behaviors, improper gut coiling, delayed development, reduced growth rate, edema, and malformations such as axial abnormalities, tail flexure, and wavy tail fins were observed in the embryos and larvae exposed to trifluralin.

Key words: Herbicide, trifluralin, teratogenic effect, lethality, malformation, Amphibia, red-bellied toad, *Bombina bombina*

Trifluralinin kırmızılı kurbağa, *Bombina bombina*'nın embriyo ve larvaları üzerindeki toksisitesi

Özet: Pek çok amfibi popülasyonunda dünya çapında azalmalar mevcuttur ve bu azalmaya neden olduğu düşünülen sebeplerin en önemlilerinden birini zirai kimyasallar, özellikle de pestisitler oluşturmaktadır. Bu çalışmada, trifluraline 22 ± 1 °C sıcaklıkta, yenilenen akut toksisite testleriyle 120 saat maruz bırakılan kırmızılı kurbağa, *Bombina bombina* embriyo ve larvalarında bu herbisitin oluşturduğu etkiler ortaya konmuştur. Test organizmaları, ergin erkek ve dişilerin gonodotropin enjeksiyonuyla uyarılması sonucu elde edilmiştir. Letal konsantrasyon değerleri PROBİT analiziyle belirlenmiştir. Trifluralinin embriyolar için hesaplanan 120 saatlik LC_{50} ve EC_{50} değerleri sırasıyla 9,40 ve 5,58 mg L^{-1} iken, larvalar için LC_{50} değeri 11,80 mg L^{-1} dir. Larval toksisite testi sonunda, 24 saatten 120 saate kadar olan LC_{10}, LC_{50} ve LC_{90} değerleri toksisite profil eğrilerini gösteren bir grafik halinde verilmiştir. Trifluralin için teratojenik indeks değeri 1,69 bulunmuştur ve bu değer trifluralinin önemli seviyede teratojenik etki potansiyeline sahip olduğunu göstermektedir. Trifluraline maruz bırakılan embriyo ve larvalarda; anormal davranışlar, barsak kıvrımlanmasında anomali, gelişimde gecikme, büyüme oranlarında azalma, ödem ve axial anomaliler, kuyrukta kıvrılma ile kuyruk yüzgecinde dalgalanma şeklinde birtakım morfolojik anomaliler gözlenmiştir.

Anahtar sözcükler: Herbisit, trifluralin, teratojenik etki, ölüm, şekil bozuklukları, amfibi, kırmızılı kurbağa, *Bombina bombina*

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Introduction

An increasing number of studies have reported declines in amphibian populations within the last 2 decades (Blaustein and Wake, 1990; Bishop and Pettit, 1992; Blaustein and Wake, 1995; Materna et al., 1995; Houlahan et al., 2000; Gardner, 2001; Blaustein et al., 2003; Wake and Vredenburg, 2008). Numerous factors are contributing to these declines, including habitat destruction, pathogens, introduced nonnative species, ultraviolet radiation, and contaminants (Sparling et al., 2001; Blaustein and Kiesecker, 2002). Chemical contamination as a consequence of pesticide application has also been assumed as a contributing factor for amphibian population decline. Nevertheless, there are limited data regarding the impacts of pesticides on amphibian populations. One of these agrochemicals, trifluralin, is an herbicide and a plant growth regulator, and the intensive use of this pesticide has resulted in its direct release into the environment (according to TOXNET profile from hazardous substances database, *available online*)[1].

One of the nontarget biological groups most affected by pesticides is the amphibians (Fulton and Chambers, 1985; Berrill et al., 1994; Sparling et al., 2001). Concern about amphibians is due in part to their value as indicators of environmental stress. They experience both aquatic and terrestrial stressors. Much of the amphibian life cycle occurs in ponds, streams, and temporary pools that are often associated with agricultural areas exposed to pesticide applications. Furthermore, breeding and larval development of most amphibians occurs in spring and summer months, during which heavy application of pesticides on agricultural lands occurs. Amphibians have a moist, permeable skin and unshelled eggs that are directly exposed to soil and water and that can easily absorb toxic substances (Duellman and Trueb, 1994; Blaustein et al., 2003). Amphibians are important components of many ecosystems, acting as prey, predators, and herbivores. Therefore, impacts of contaminants upon these animals are relevant to an understanding of the whole ecosystem's health (Rowe and Dunson, 1994).

To understand the effects of pesticides on amphibians, as a first step, laboratory-based and single-species toxicology tests were conducted in this study. *B. bombina* was chosen for this purpose due to wide geographical distribution in Europe and its being the single representative species of *Bombina* in Turkey. Apart from this, this species has been found to be easy to breed and keep under laboratory conditions throughout the year; it is possible to get a sufficient number of healthy embryos at a low cost for toxicity studies. Trifluralin was selected as the focal pesticide because it is a widely used herbicide to control many annual grasses and broadleaf weeds in a large variety of arable and horticultural crops (EXTOXNET). To my knowledge, no information on the potential teratogenic effect of trifluralin on amphibians has been reported to date.

The purpose of this study was to reveal the toxic effects of trifluralin, one of the herbicides widely used in Turkey, on embryos and larvae of the red-bellied toad, *Bombina bombina*, and to contribute to the knowledge of the effects of pesticides, which is one of the proposed reasons for the decline in amphibians. The evaluation of the toxicity of trifluralin on *B. bombina* was based on mortality, malformation, and inhibition of development and growth.

Materials and methods

Test substance

Formulation grade trifluralin (Trifilin 48 EC, Koruma), an herbicide and a plant growth regulator, was purchased from a retail outlet. Prior to testing, a stock solution was prepared at a nominal concentration of 1000 mg L^{-1} of trifluralin and adequate dilutions were done from the stock solution in order to reach the test concentrations of 0.1, 0.5, 1, 3, 5, 9, and 15 mg L^{-1} for embryo-toxicity tests and 0.5, 1, 2, 4, 8, 16, and 32 mg L^{-1} for larval-toxicity tests. The concentration of formulation grade trifluralin was calculated from the percentage of the active ingredient. Test solutions were freshly prepared before the beginning of the tests using pure spring water.

Test organism

The embryos and larvae of the red-bellied toad, *Bombina bombina*, which has a wide distribution in Europe, were used as test organisms. Adult males and

[1] http://toxnet.nlm.nih.gov/

females of this species were maintained in our amphibian laboratory. Both were induced to breed by intralymphatic administration of gonadotropin in accordance with the standard procedure of the ASTM FETAX Guideline (1992), and the amount of hormones injected was adjusted according to the size of the animals. They were moved as a single pair to a large breeding aquarium filled with dechlorinated tap water in the laboratory, held at 22 ± 1 °C, where egg deposition was completed. Amplexus normally ensued within 6 h and egg deposition about 12 h after injection. The eggs were inspected for fertility and quality. Normally cleaving embryos were selected for use in testing by means of double selection. Experiments were performed on the embryos at the stages of midblastula (stage 8) to early gastrula (stage 10) and at the 21st-22nd fully-aquatic stages of the prefeeding larvae. The stages of the larvae were determined according to the methods of Gosner (1960).

Test procedure

Renewal acute toxicity tests were conducted with embryos and larvae in order to evaluate the toxicity of trifluralin on *B. bombina*. Following initial experiments, called range-finding tests, definitive tests were performed. Ten healthy embryos and larvae were randomly selected from different spawn and transferred to each glass Petri dish containing 100 mL of trifluralin solution of the test concentrations for 120 h. Controls were maintained without trifluralin under the same conditions. For each concentration, including the control, 3 replicates were used. The tests were performed at 22 ± 1 °C and the test solutions were renewed every other day. Observations were made at 24 h intervals through 120 h of exposure. Lethality and malformations were the considered endpoints for LC_{50} and EC_{50}, respectively. Every 24 h, the numbers of dead embryos and larvae were recorded; they were removed from the Petri dishes and their external aspects, such as malformations and edema, were recorded. The morphological characteristics (malformations, edema, developmental stage) and behavioral abnormalities (motionlessness, poor swimming ability, spontaneous twitching) of the living ones were also recorded. Malformations were identified according to *Atlas of Abnormalities* (Bantle et al., 1998).

At the end of the experiment, the remaining living larvae were fixed in 3% formaldehyde solution, and their total lengths and body widths (greatest width in dorsal view) were measured under a stereomicroscope with an ocular micrometer and compared to those of the controls. Trifluralin concentrations representing LC_{10}, LC_{50}, and LC_{90} from 24 to 120 h were obtained and plotted as toxicity profile curves (TOPs) for larval-toxicity tests. The teratogenic index (TI), which is a measure of teratogenicity, for trifluralin (LC_{50}/EC_{50}) was determined after 120 h of embryo-toxicity tests.

Statistics

Lethal concentration values (LC_{10}, LC_{50}, and LC_{90}) and the 95% confidence limits were determined through PROBIT analysis, using the software SPSS Version 13.0 for Windows. All values were presented as mean ± standard error (SE). Comparisons were made between the control and treatment groups using one-way analysis of variance (ANOVA) of the software Statgraphics Version 5.0. Values of $P \leq 0.05$ were regarded as statistically significant.

Results

No mortality occurred throughout the 120 h experimental period of embryo-toxicity test in the control group. There were positive relationships between increasing exposure concentration and mortality and malformation of the embryos exposed to trifluralin. The 120 h median lethal and effective concentrations (LC_{50} and EC_{50}) of trifluralin calculated for the embryos of *B. bombina* were 9.40 and 5.58 mg L^{-1}, respectively. The teratogenic index of trifluralin for *B. bombina* embryos was calculated as 1.69. Embryos exposed to trifluralin exhibited malformations in all treatment groups, including the lowest concentration, 0.1 mg L^{-1}. In this study, edema and malformations such as axial abnormalities, tail flexure, and wavy tail fins were recorded in the embryos (Figure 1). Trifluralin-treated embryos exhibited delay in the developmental stage achieved. While the controls progressed to the 23rd stage, trifluralin-treated larvae were able to reach a maximum 22nd stage, depending on the exposure concentration.

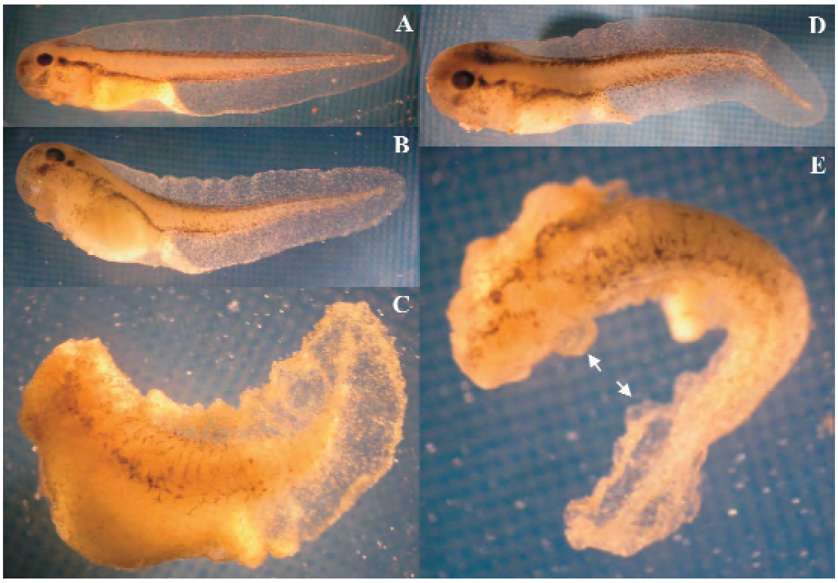

Figure 1. (A) Lateral view of a control larva (Stage 23); (B) and (C) lateral views, (D) dorsa-lateral view, and (E) dorsal view of trifluralin-treated larvae at the end of the 120 h experimental period of embryo-toxicity test. (B) Minor axial abnormality in which the tail is slightly dorsally curved (Stage 22). (C) Dorsal tail flexure and wavy tail fin. Also note that delayed development is evident (Stage 19). (D) More complex axial abnormality in which the distal portion of the tail is flexed ventrally (Stage 22). (E) Severe lateral tail flexure and wavy tail fin (Stage 21). Note the edema (è). These malformations were observed in all treatment groups with different frequencies.

While no mortality occurred throughout the 120 h experimental period of the larval-toxicity test in the groups of the control and the lowest concentration (0.5 mg L^{-1}), all experimental larvae died at the highest concentration (32 mg L^{-1}). The 120 h LC_{10}, LC_{50}, and LC_{90} values of trifluralin calculated for the larvae of *B. bombina* were 5.79, 11.80, and 17.80 mg L^{-1}, respectively. Figure 2 shows the toxicity profile curves of trifluralin for the larvae of *B. bombina* from 24 to 120 h of the larval-toxicity tests. Edema, wavy tail fin, and improper gut coiling were observed in the trifluralin-treated larvae, except for the 0.5, 1, and 2 mg L^{-1} groups (Figure 3). These larvae also exhibited delayed development. While the controls were in the 25th stage, trifluralin-treated larvae were able to reach a maximum 24th stage, depending on the exposure concentration. Trifluralin also caused reduced growth rates of the larvae of the treatment groups. When compared with the control animals, there were statistically significant decreases in the total length and body width of larvae treated with trifluralin, except for the larvae in the 0.5 mg L^{-1}, and the 0.5 and 1 mg L^{-1} groups, respectively (Figures 4 and 5).

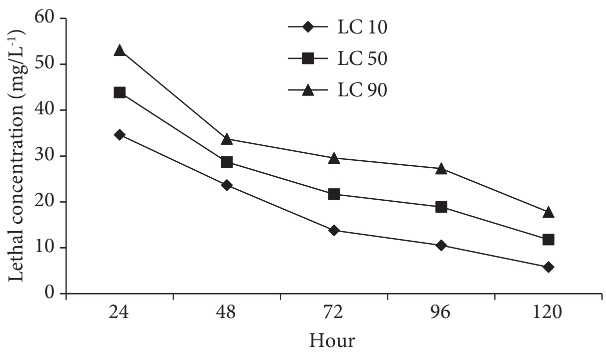

Figure 2. Toxicity profile curves of trifluralin for the larvae of *B. bombina* (120 h larval-toxicity test, with larvae at 21st and 22nd stages).

The experimental animals exposed to trifluralin showed toxicity signs in their responses during the experimental period. These signs were characterized by motionlessness and poor swimming ability. Most

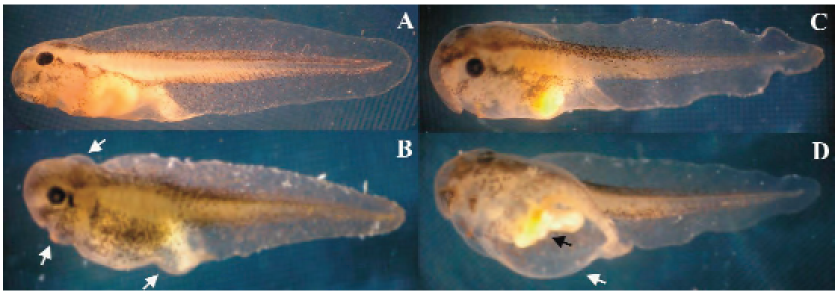

Figure 3. (A) Lateral view of a control larva (Stage 25); (B) and (C) dorsa-lateral view, and (D) ventral-lateral view of 4, 8, and 16 mg L^{-1} trifluralin-treated larvae (Stage 24) at the end of the 120 h experimental period of larval-toxicity test. (B) Note the edema (è). (C) Wavy tail fin. (D) Wavy tail fin, abdominal edema (è), and improper gut coiling (black è). Failure to achieve normal gut development often accompanies such abdominal edema. These malformations were observed in the 4, 8, and 16 mg L^{-1} treatment groups with different frequencies.

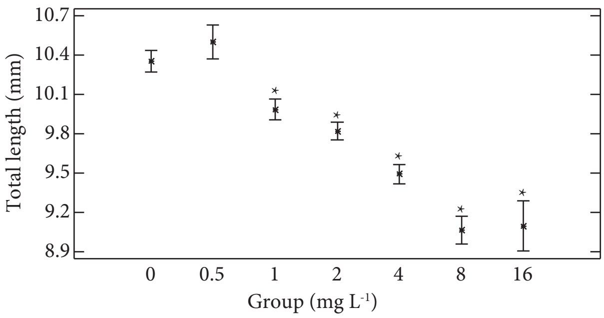

Figure 4. Mean total length of the larvae of control (0) and treatment groups at the end of the 120 h experimental period of the larval-toxicity test.
* Statistically significant difference from control ($P \leq 0.05$).

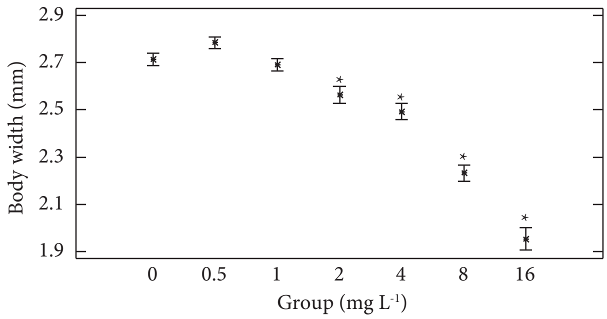

Figure 5. Mean body width of the larvae of the control (0) and treatment groups at the end of the 120 h experimental period of the larval-toxicity test.
* Statistically significant difference from control ($P \leq 0.05$).

of the larvae were observed lying and/or swimming on their sides or backs.

Discussion and conclusion

This experimental study revealed the toxicity of trifluralin to the embryos and larvae of *B. bombina* with a renewal acute toxicity test. A chemical's acute toxicity is typically characterized by an endpoint such as a median lethal concentration (LC_{50}) calculated at the end of the toxicity test. In this study, the 120 h LC_{50} values of trifluralin for the embryos and the larvae of *B. bombina* were determined as 9.40 and 11.80 mg L^{-1}, respectively. Regarding these results, the embryos were more susceptible to trifluralin than the larvae. It was also reported in a previous study that the sensitivity to a pesticide called dimethoate was greater in the early life stage of an anuran species, *Hyla arborea* (Sayım and Kaya, 2006). The fact that early life stages were more susceptible could be related to the active morphogenetic and cellular differentiation processes that occur at that time (Pérez-Coll and Herkovits, 2004). The TI value determined in this study for trifluralin (1.69) indicates a significant teratogenic risk of this herbicide. TI values higher than 1.5 signify a large separation of mortality and malformation concentration ranges, and therefore, its potential to exert malformations in the absence of high lethality (ASTM, 1992). The LC_{10}, LC_{50}, and LC_{90} values of trifluralin calculated for the larvae of *B. bombina* from 24 to 120 h were plotted as toxicity profile curves, which provide a better understanding of concentration and time-dependent thresholds. The toxicity of a chemical depends on the chemical's concentration and effects expressed throughout exposure (Pérez-Coll and Herkovits, 2004). Hence, threshold values accounting for these parameters would be more useful for a characterization of toxicity endpoints for a hazard/risk assessment targeted for a specific chemical of concern (Pérez-Coll and Herkovits, 2004).

At the end of the experimental period of the current study, delayed development and reduced body size were observed in the embryos and larvae treated with trifluralin. Similarly delayed development, reduced growth rates, and mortality of anuran tadpoles have been documented after exposures of eggs and larvae to different pesticides (Mohanty-Hejmandi and Dutta, 1981; Mizgireuv et al., 1984; Sayım and Akyurtlaklı, 1999; Sayım and Kaya, 2006; Sayım, 2008). Such a delay in larval growth can have a significant detrimental impact later in the life of a frog by decreasing its survival, its adult size (Berven, 1990), its sexual maturation rate (Smith, 1987), mate selection (Forester and Czarnowsky, 1985), and locomotion ability for predator evasion (Goater et al., 1993). Delay in development may place larvae at a greater risk of predation and dehydration as temporary ponds dry out throughout the year. Moreover, such delays increase the exposure of larvae to pesticide runoff.

In this study, different abnormalities, such as axial abnormalities, tail flexure, wavy tail fin, edema, and improper gut coiling, were observed in the embryos and larvae exposed to trifluralin. According to the TOXNET profile from a hazardous substances database (*available online*)[2], trifluralin is classified as class C, a possible human carcinogen, and it is strongly mutagenic in plants, producing a 3-4 times increase in spontaneous mitoses and chromosomal aberrations. Malformations exerted by trifluralin could be related to its mutagenic and carcinogenic effects, and the kind of abnormalities reported in this study are of importance as being new contributions reflecting its teratogenic, mutagenic, and carcinogenic potential, as well. These kinds of malformations, such as axial abnormalities and tail flexure, are usually related to malformations of the notochord (Bantle et al., 1998). The edema observed in the embryos and larvae exposed to trifluralin could be related to an osmoregulatory dysfunction exerted by this chemical. Similarly, axial abnormalities, tail deformations, edema, and abnormal gut coiling were observed in anuran larvae exposed to pesticides in previous studies (Pawar et al., 1983; Bishop, 1992; Snawder and Chambers, 1993; Gaizick et al., 2001; Bonfanti et al., 2004; Sayım and Kaya, 2006). Failure to achieve normal gut development often accompanies severe abdominal edema (Bantle et al., 1998). Cooke (1981) summarized several investigations regarding exposure to environmental pollutants and the occurrence of deformities in amphibian larvae, and these studies indicated that the production of such deformities could be a sensitive indicator of pollution by certain chemicals.

During the present study, toxicity signs, such motionlessness and poor swimming ability, were observed in the behavior of the larvae exposed to trifluralin. Similar behavioral effects of laboratory exposures to other pesticides in amphibian larvae were reported in previous studies (Bishop, 1992; Sayım and Akyurtlaklı, 1999; Sayım and Kaya, 2006). These behavioral effects are not surprising, as most of these pesticides are neurotoxic. It is also known that behavioral changes of those types increase the chances of predation on amphibian larvae (Bishop and Pettit, 1992).

In this study, by means of renewal acute toxicity tests with the embryos and larvae of *B. bombina*, lethality, malformations, delayed development, reduced body size, and abnormal behaviors exerted by trifluralin were reported. Thus, according to the results of the present study, this herbicide should also be considered a contributing cause of declines in

[2] http://toxnet.nlm.nih.gov/

amphibian populations. It may also alter the structure (species richness, density, and biological diversity) and functional activities of an ecosystem. Therefore, biological, cultural, and environmental control measures and host plant resistance mechanisms should be improved and used in order to reduce the effects of this kind of chemical.

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