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THE USE OF THE FIRE BELLIED TOAD, *BOMBINA BOMBINA* (AMPHIBIA: ANURA) AS AN ECOTOXICOLOGICAL TEST ORGANISM

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Abstract

Amphibians are being increasingly used for toxicity screening purposes and are often considered as sensitive indicator organisms of possible adverse effects of environmental contaminants because of their permeable skins and bi-phasic life cycle. As a reliable and realistic amphibian model, employing ecological relevant species of Türkiye for evaluating the potential risks of xenobiotics does not exist, this toxicological study has been aimed to present a modified amphibian toxicity test model with *Bombina bombina* that acute, subchronic and chronic effects of Cu^{+2} have been evaluated. The modified ecotoxicological test model with native species of Türkiye was described and termed as "BOMBITOX" for the first time. Fertilized eggs obtained by intralymphatic administration of hCG. Observations and quantitative estimations were done on the breeding biology of *B. bombina* in captivty. Early embryonic development of *B. bombina* was inspected at 21±1 °C. Toxicity tests were performed at 15 h light/9 h dark cycle and 21 ± 1 °C. Test solutions were renewed every other day. This study revealed that mean clutch size was 104 eggs, the mean egg sizes with and without jelly capsule were 2.05 and 1.50 mm respectively, the embryos generally hatched 6^{th} day and 22^{nd} stage, and the average hatching length was 7.76 mm. The staged series with morphological characteristics and time data specific to *B. bombina* early embryonic development are determined. Cu^{+2} concentrations representing LC_{10}, LC_{50}, and LC_{90} from 24 h to 14 days were also obtained and plotted as toxicity profile curves for embryo and larval toxicity tests. NOEC values were 0.04, 0.005 and 0.02, 0.01 mg/L for 96 h, 7 days embryo and larval toxicity test, respectively. Cu^{+2} caused reduced growth rates and delay in developmental stages achieved. Shedding of epithelial cells, edema and malformations such as axial abnormalities, tail flexure, and wavy tail fin were observed in the exposed groups of embryo and larval toxicity tests. TI values for embryo toxicity tests was calculated as 2.06. The experimental animals exposed to Cu^{+2} showed toxicity signs such as hyperactivity symptoms and poor swimming ability. It can be concluded that BOMBITOX, the modified ecotoxicological test model with native species of Türkiye, was found to be useful in testing developmental toxicity of heavy metals and meet both ecologic and economic criteria.

Keywords: BOMBITOX; modified ecotoxicological test model; fire-bellied toads*; Bombina bombina*, Cu^{+2}; heavy metal toxicity; breeding biology; early embryonic development.

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1. Introduction

During the past 50 years, the extent of ecotoxicological literature has expanded and level of the research has become increasingly more complex and informative. Although vertebrates have been the topic of a good portion of this kind of researches, the studies of amphibian ecotoxicology constitute only 2.9% of all vertebrate ecotoxicology studies. Most of the topics focused on fish, bird and mammal ecotoxicology. It has been accepted that amphibian ecotoxicity has not been extensively studied because of the fact that amphibians are of relatively little economic importance in comparison to fish and other wildlife animals [1, 2, 3, 4]. Due to the lack of amphibian ecotoxicological literature, amphibian toxicity data are either not included in the development of numeric water quality criteria for the protection of aquatic life or are dreadfully underrepresented in comparison to other vertebrate organisms, including fish [5, 6, 7]. Using toxicity data from sensitive species that may not be present in a wetland or play a minor ecological role may lead to misleading outcomes such as over-protective and under-protective cleanup levels. Wetland habitats generally serve as a sink for many chemical compounds. Thus, potential exposure to environmental contaminants in wetland systems may be higher than in surrounding upland areas, especially during the critical early life stages of amphibian development commonly spent in wetland habitats. So, amphibian embryos can be used in toxicity studies as indicators of environmental quality for wildlife protection purposes [8].

Since the 1970s, severe decline in many amphibian species from a variety of taxa has been reported. Global declines in amphibian populations are caused by a number of anthropogenic activities including habitat loss, the introduction of exotic species, exposure to environmental contaminants, climate change, increased acid precipitation, and increased UV radiation associated with ozone depletion [9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19]. Therefore, the discovery of amphibian declines and malformations have increased the research and attention on amphibian ecotoxicology and ecological significance. Recently, in spite of little economic importance, amphibians are being increasingly used for toxicity screening purposes. They are often considered as sensitive indicator organisms of possible adverse effects of environmental contaminants because of their permeable skins and bi-phasic life cycle. As keystone species, amphibians may play a disparately large role in wetland community structure, and may not be readily replaceable in the event of a sudden decline or loss in their population size. Furthermore, the unique life history and physiology of amphibians cannot be represented by a surrogate group of organisms within the literature. Thus, the effects of pollution upon this class of animals are important to an understanding of ecosystem health [20, 21].

This study is about the application of modified amphibian toxicity test model with *B. bombina* that acute, subchronic and chronic effects of Copper (Cu^{+2}) have been evaluated. As a reliable and realistic amphibian model employing native species of Türkiye for evaluating the exposure of xenobiotics does not exist, this toxicological study has been aimed to present a standardized amphibian toxicity test model using ecological relevant species for evaluating the potential risks of copper (Cu^{+2}) to a native amphibian species of Türkiye. *B. bombina* was chosen for this purpose as a bioindicator organism because of the fact that it can easily adapt to laboratory conditions and be hand-fed in our amphibian breeding laboratory. Moreover, this toxicity test can be carried out in short time with low costs.

2. Materials and methods

2.1 Test substance

Copper (II) chloride dihydrate ($CuCl_2.2H_2O$) was used in the toxicity tests. Prior to testing, a stock

solution was prepared as a concentration of 1000 mg/L $CuCl_2.2H_2O$ and adequate dilutions were done from the stock solution in order to reach the test concentrations 0.005, 0.007, 0.01, 0.02, 0.03, 0.04, 0.06, 0.07, 0.09, 0.12, 0.15, 0.20, 0.25 mg Cu^{+2}/L for embryo-toxicity tests and 0.01, 0.02, 0.03, 0.04, 0.06, 0.07, 0.09, 0.12 mg Cu^{+2}/L for larval-toxicity tests. Test solutions were freshly prepared before the beginning of the tests using dechlorinated tap water.

2.2 Test Organism

Adult males and females of *B. bombina* were captured by hand from their natural habitats in Türkiye during the field work and brought to our amphibian breeding laboratory. Here, vivariums were prepared that adults of fire-bellied toad can survive and breed. Captured adults of *B. bombina* held captive in these aquariums (Fig. 1).

Fig. 1. a) Vivariums prepared for fire-bellied toad; b) Adults of *B. bombina* held captive.

Adult males and females were induced to breed by intralymphatic administration of 100 and 125 IU human chorionic gonadotropin (hCG, SIGMA/CAS9002-61-3), respectively. At least 48 hours prior to hCG injection, females were injected 25 IU pregnant mare gonadotropin (PMG, SIGMA/CAS9002-70-4). Then they were moved as a single pair to a large breeding aquarium filled with de-chlorinated tap water in the laboratory held at 21 $\pm$ 1 °C where egg deposition was completed. Amplexus normally ensued within 6 h and egg deposition about 12 h after hCG injection.

2.3 Breeding Biology

Observations and quantitative estimations were done on the breeding biology of *B. bombina* under laboratory conditions. Clutch sizes, total numbers of eggs laid at one time, were counted and the mean number of eggs per batch was calculated. The diameter of the fertilized eggs with and without jelly capsule and the size of hatchlings (head-tail length) were measured with a micrometric ocular and the mean values were calculated. The early embryonic development of *B. bombina* was also monitored within the scope of the breeding biology of the species. By using fertilized eggs obtained in captivity, the embryonic development of *B. bombina* was inspected by means of a stereo microscope in dechlorinated tap water at 21±1 °C. A staged series with morphology and time data for the early embryonic development of fire-bellied toad was presented with photographs. A species-specific table of early embryonic development of *B. bombina* was prepared following Gosner's generalized table.

2.4 Toxicity Tests

Test protocol was approved by the Animal Experiments Local Ethics Committee of Ege University. A

modification of new toxicity test guideline (Larsen et al., 2004) was used in the current study. The embryos and larvae of red-bellied toads, *Bombina bombina*, were used as a model organism in toxicity tests. In this study, the modified amphibian toxicity test model with an ecological relevant species, fire-bellied toad, which makes its use for ecotoxicological researches suited in Türkiye was described and termed as "BOMBITOX" for the first time.

The fertilized eggs obtained in captivity were check out for fertility and quality. Normally cleaving healthy embryos were selected for use in testing by means of double selection. The embryos of midblastula (stage 8) to early gastrula (stage 11) and the 21st - 22nd fully- aquatic stages of pre-feeding larvae were used to start toxicity tests. The stages of the larvae were determined according to Gosner (1960) [23].

Ten healthy embryos and larvae were randomly selected and transferred to each glass petri dish containing 100 mL Cu^{+2} solution of the test concentrations for 96 h, 7 and 14 days conducting acute, subchronic and chronic toxicity tests respectively. Controls were maintained without Cu^{+2} under the same condition. For each concentration, including the control, three replicates were used. Toxicity tests were performed under controlled laboratory conditions with 15 h light/9 h dark cycle and 21 $\pm$ 1 °C. The test solutions were renewed every other day. Observations were made at 24 h intervals through the exposure period and the numbers of dead organisms were recorded, which were removed from the petri dishes and their external aspects recorded. The morphological and behavioural characteristics of the living ones were also recorded. The evaluation of the toxicity of Cu^{+2} was based on mortality, malformation, and inhibition of development and growth. Lethality and malformations were the considered endpoints for LC_{50} and EC_{50}, respectively. Malformations were identified according to the "Atlas of Abnormalities" [24].

At the end of the experiment, remaining living larvae were fixed in 3% formaldehyde solution, and their total lengths were measured with an ocular micrometer and compared to that of the controls. The Cu^{+2} concentrations representing LC_{10}, LC_{50} and LC_{90} from 24 h to 14 days were obtained and plotted as toxicity profile curves (TOPs) for embryo and larval-toxicity tests. Teratogenic Index (TI), which is a measure of teratogenicity, for Cu^{+2} was determined after subchronic embryo and larval-toxicity tests.

2.5 Statistics

Lethal and effective concentration values (LC_s and EC_s) and the 95% confidence limits were determined through PROBIT analysis, using the software SPSS Version 20.0 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.

3. Results and discussion

3.1 Breeding Biology Data

In our amphibian breeding laboratory, adult males of *B. bombina* injected gonadotrophin were observed to make a very strong typical call "oop-oop-oop-oop". During the call, when a pair of vocal sacs at the floor of the mouth is fully ballooned, the throat of the male takes a rounded shape and becomes larger than the rest of the head (Fig. 2a). Amplexus is inguinal type (Fig. 2b). Eggs with a gelatine capsule, which are laid singly or several together, are adhered to plants in water (Fig. 3). It was determined that the clutch size, number of eggs per batch, in captivity was 104 eggs on average. The mean egg sizes measured as diameters of fertilized eggs with and without jelly capsule were calculated as 2.05 and 1.50 mm, respectively. It was determined that the average time span from fertilization to hatching for *B. bombina* was 6th day and the stage of newly hatched larvae was generally 22nd stage.

However, individual variations have been observed in this species, with some larvae hatching earlier (stages 20 and 21). The average hatching length was 7.76 mm. Fig. 4 shows the photographs of the newly hatched 20th, 21st, and 22nd stage larvae and the 25th stage larvae in which the spiraculum was formed.

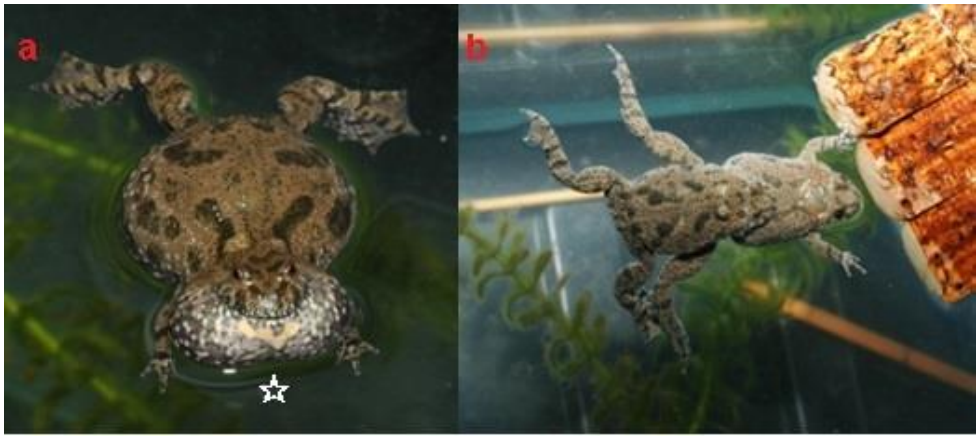

Fig. 2. a) A calling male in captivity * Vocal Sac; b) A pair in amplexus (inguinal type) in captivity.

Fig. 3. Eggs laid and adhered to plant in water in captivity

Fig. 4. The newly hatched 20th, 21st, and 22nd stage larvae (a, b, c) and the 25th stage larvae (d) of *B. bombina*.

This study revealed that clutch characteristics determined in captivity were in accordance with the limited previously reported data on *B. bombina* [25]. It was reported that females deposit 60–200 eggs in numerous small egg clusters and the embryos hatch within 4 to 10 days [26]. Present study showed that mean clutch size was 104 eggs, the mean egg sizes with and without jelly capsule were 2.05 and

1.50 mm respectively, the embryos generally hatched 6th day and 22nd stage, and the average hatchling length was 7.76 mm. The hatching time reported by [27] as 22 days for *B. bombina* is quite an exaggeration when compared to the finding of present study, which was found to be an average of 6 days. In contrast, [26] reported the mean hatching time as 6.2 days which supports our finding. In terms of clutch size and ovum diameter, the results of present study are consistent with previously reported data [26]. However, the clutch size of *B. bombina* was reported as 30.6 and 32.5 by [25, 28], respectively, which is considerably lower than the average value determined as 104 in the current study [28] stated that this number may show variations depending on the size and age of the female. The ovum diameter stated as 1.49 in these studies [25, 28] is consistent with the finding of current study as 1.50.

3.1.1 Early Embryonic Development of B. bombina

The staged series with morphological characteristics and time data specific to *B. bombina* early embryonic development are shown in Table 1. These stages are presented with photographs in Fig. 5. According to these data, the early embryonic development of this species is in accordance with the standard developmental table developed by Gosner (1960) [23] for anurans.

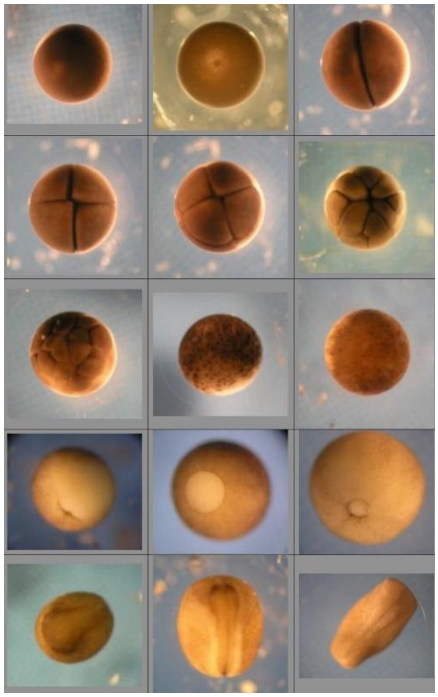

Fig. 5. A staged series with morphology data for *B. bombina* early embryonic development

Table 1. Early embryonic stages of *B. bombina* follow Gosner (1960). The ages were given in hours at 21 ± 1°C.

Stage	Characteristics	Age in hour
1	Fertilization	0
2	Second polar body released	0.30
3	First cleavage (meridional); 2 blastomeres	1.45
4	Second cleavage (meridional); 4 blastomeres	2.30

5	Third cleavage (latitudinal); 8 blastomeres	3.00
6	Fourth cleavage (meridional); 16 blastomeres	3.30
7	Fifth cleavage (latitudinal); 32 blastomeres	4.00
8	Mid-cleavage; early blastula	7.00
9	Late cleavage; late blastula	8.30
10	Involution at dorsal lip of blastopore; beginning of gastrulation	12.45
11	Mid-gastrula; large yolk plug	18.45
12	Late gastrula; small yolk plug	23.45
13	Dorsal flattening; formation of neural plate	26.15
14	Early neurula stage; neural folds approach each other	31.00
15	Mid neurula stage; neural folds coalesce; body begins to elongate	33.30
16	Formation of the neural tube; body elongated	34.45

Various studies have shown that very different patterns can be seen in the embryonic development of many anuran species [29, 30, 31, 32, 33, 34, 35, 36, 37]. Data on the early embryonic development of *B. bombina*, which are important in terms of revealing interspecific variations of embryonic development in anurans, have been inadequate. Current study has made an important contribution to this area by giving the staged series with morphological characteristics, time data and photographs specific to early embryonic development of *B. bombina*.

3.2 Toxicity Tests (BOMBITOX)

Lethal concentrations of Cu^{+2} for the embryos and larvae of *B. bombina* were calculated according to data from acute toxicity tests (Table 2). Although no mortality occurred throughout the 96-h experimental period of acute toxicity tests in the control groups, there were positive relationships between increasing exposure concentration and mortality of the embryos and larvae exposed to Cu^{+2}. Copper is an essential nutrient at low concentrations; however, it is toxic to aquatic organisms at higher concentrations [40]. While NOEC value determined from 96 h embryo toxicity test was 0.04, the value that of 7 days embryo toxicity test was 0.005 mg/L. NOEC values determined from 96 h and 7 days larval toxicity tests were 0.02 and 0.01 mg/L, respectively. All animals in exposed groups above the concentrations of 0.04 and 0.005 mg/L died in the end of 7^{th} and 12^{th} days of embryo toxicity test, respectively. At the end of the 14^{th} day of embryo toxicity test, only 1 larva was alive in the group of 0.005 mg/L. All animals in the group of 0.12 mg/L concentration died in the 11^{th} day of larval toxicity test. Cu^{+2} concentrations representing LC_{10}, LC_{50}, and LC_{90} for 24 h to 14 days were also obtained and plotted as toxicity profile curves (TOPs) for embryo (Fig. 6) and larval (Fig. 7) toxicity tests. The results from embryo toxicity tests showed LC_{10}, LC_{50}, and LC_{90} for 24 and 48 h were the same as 0.276, 0.497 and 0.717, respectively. However sharp decreases in the concentrations of Cu^{+2} (LC_{10}, LC_{50}, and LC_{90}) that exerts the same adverse effects were observed in 72^{nd} hour of treatment (Fig. 6). In larval toxicity test, LC values decreased gradually with the exposure time (Fig. 7). It can be concluded from TOPs that the exposure threshold for copper exerting lethal effects is at the 72^{nd} h of treatment, especially for embryo toxicity test.

Table 2. Lethal concentrations of Cu^{+2} on the embryos and larvae of *B. bombina* from acute toxicity tests.

96 h	LC_{10} *(95% C.I.)*	LC_{50} *(95% C.I.)*	LC_{90} *(95% C.I.)*
Embryo Toxicity Test	0,047	0,150	0,253

	(0,024-0,065)	*(0,133-0,173)*	*(0,222-0,293)*
Larval Toxicity Test	0,039	0,085	0,130
	(0,024-0,049)	*(0,074-0,099)*	*(0,113-0,161)*

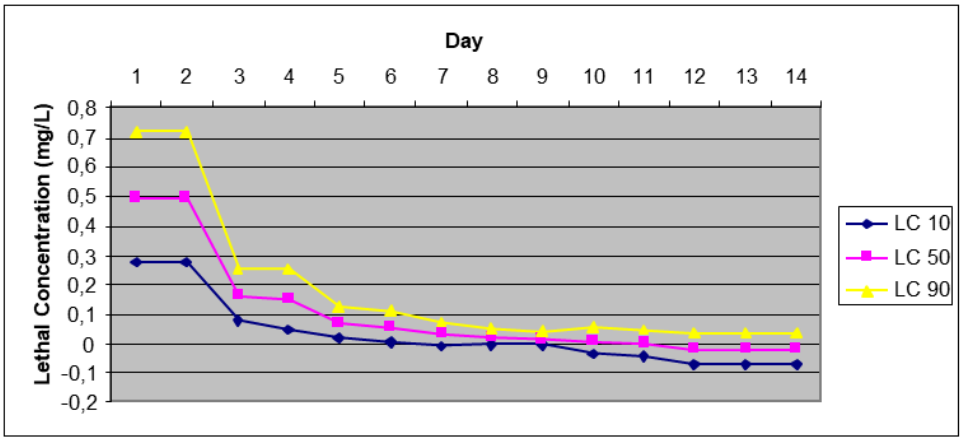

Fig.6. Toxicity profile curves of Cu^{+2} for *B. bombina* from embryo-toxicity tests.

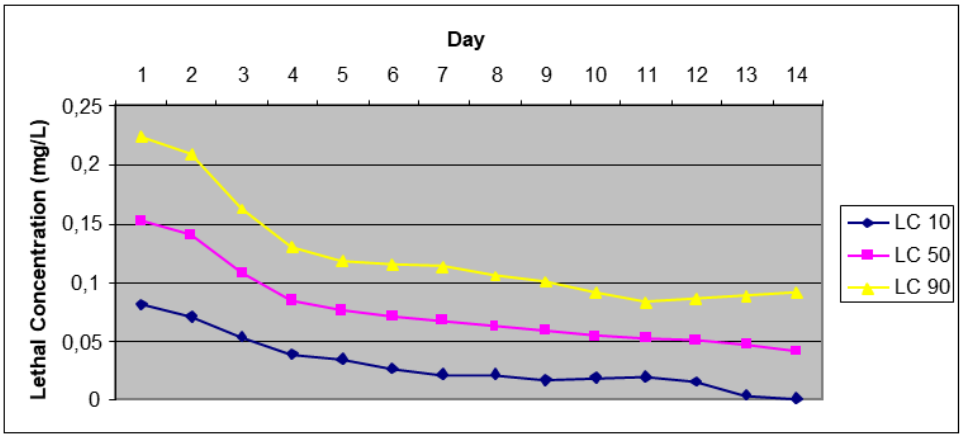

Fig. 7. Toxicity profile curves of Cu^{+2} for *B. bombina* from larval-toxicity tests.

In addition to acute effects such as mortality, chronic exposure to copper can lead to adverse effects on survival, growth, reproduction as well as alterations of brain function, enzyme activity, blood chemistry, and metabolism [38]. The results of the subchronic toxicity tests of the current study revealed that Cu^{+2} caused reduced growth rates of the embryos and larvae of the treatment groups. When compared to the control animals, there were statistically significant decreases in the mean total lengths of embryos and larvae treated with Cu^{+2}, except for 0.01, 0.02 and 0.03 mg/L groups of larval toxicity tests (Figs. 8 and 9). According to the results of the current study, Cu^{+2} treated embryos also exhibited delay in the developmental stages achieved. While the controls progressed to 20^{th}-21^{st} stages in acute (96 h) embryo toxicity tests, Cu^{+2} treated larvae in the groups between the 0.06 - 0.25 mg/L concentrations were able to reach 18^{th} stage. In subchronic (7-days) embryo toxicity tests, Cu^{+2} treated larvae

in the groups between the 0.02 - 0.09 mg/L were able to reach 19th-21st stages depending on the exposure concentration, while the controls progressed to 25th stage.

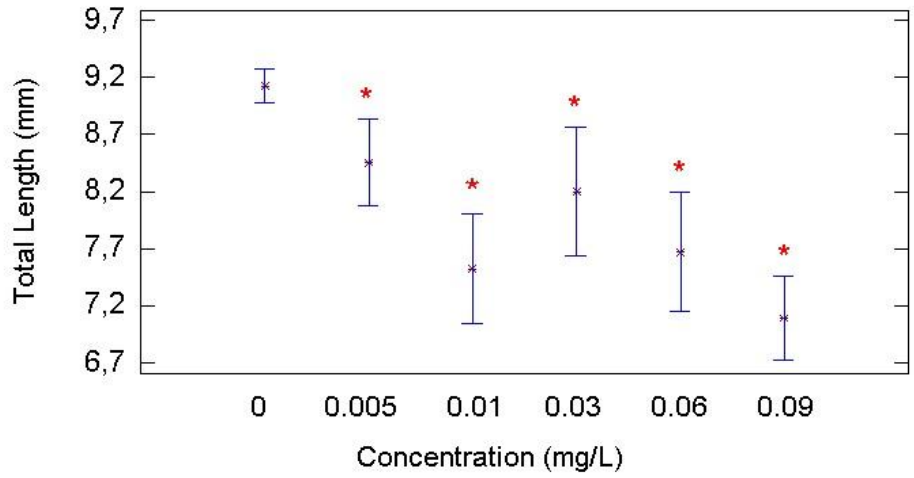

Fig. 8. Mean total length of the larvae of control (0) and treatment groups at the end of the 7 days experimental period of the embryo toxicity test * Statistically significant difference from control (P ≤ 0.05).

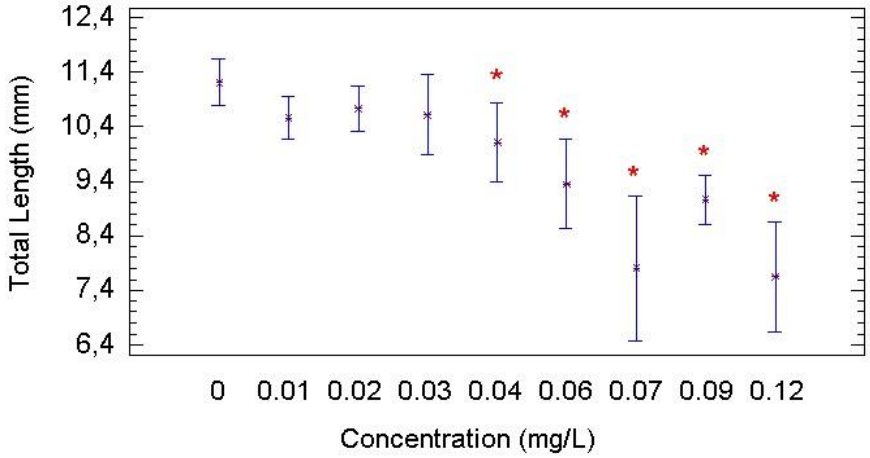

Fig. 9. Mean total length of the larvae of control (0) and treatment groups at the end of the 7 days experimental period of the larval toxicity test. * Statistically significant difference from control (P ≤ 0.05).

In this study, shedding of epithelial cells, edema and malformations such as axial abnormalities, tail flexure, and wavy tail fin were observed in the exposed groups of embryo and larval toxicity tests (Figs. 10 and 11). TI values of Cu^{+2} for embryo toxicity test was calculated as 2.06. This kind of studies indicated that the production of such deformities could be a sensitive indicator of pollution by certain chemicals [39, 40]. The experimental animals exposed to Cu^{+2} showed toxicity signs in their responses during the experimental period of the present study. These signs were characterized by hyperactivity symptoms and poor swimming ability. Most of them were observed laying and/or swimming on their sides or backs. These

results from the toxicity tests are in accordance with the results found in previous studies about the toxic effects of Cu^{+2} on amphibians [39, 40, 41, 42, 43, 44].

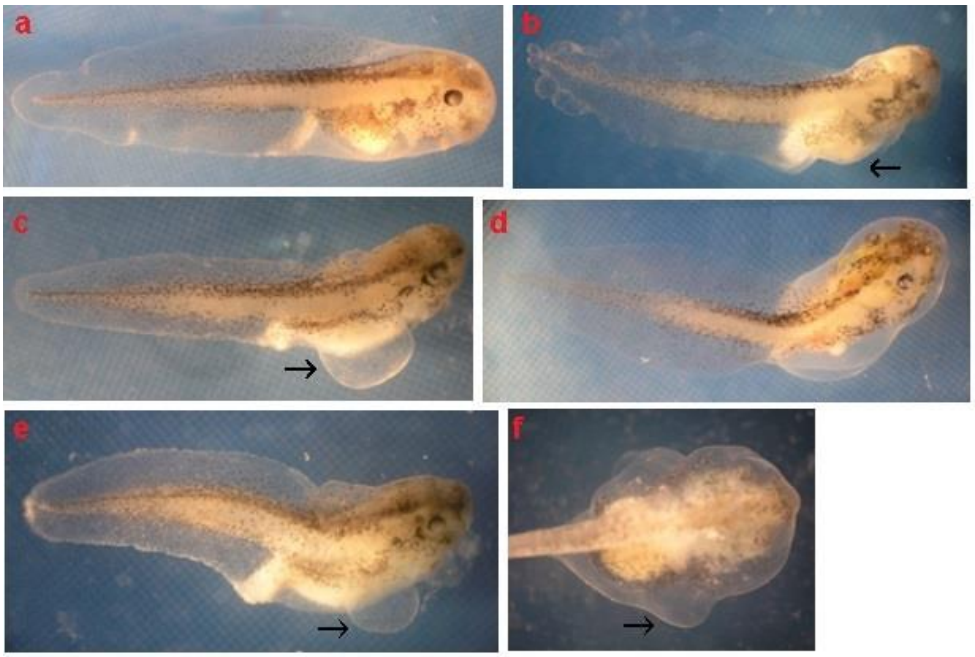

Fig. 10. (a) Lateral view of a control larva (Stage 25); (b) and (c) lateral views; (d) dorsolateral view; (e) lateral view; and (f) ventral view of Cu^{+2} treated larvae (Stage 19-21) at the end of 7 days embryo-toxicity test. Axial abnormality (b, d, e). Note the edema (→).

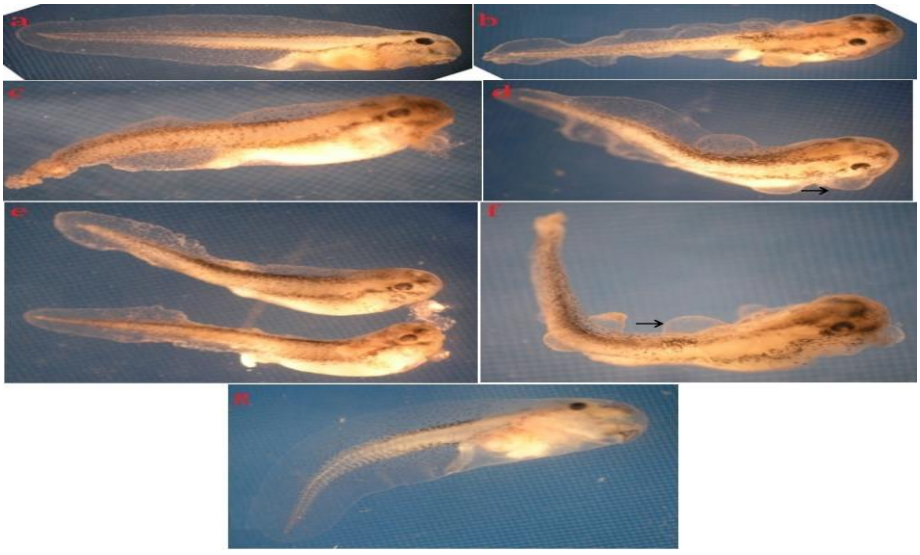

Fig. 11. (a) Lateral view of a control larva (Stage 25); (b), (d) and (f) dorsolateral views; and (c), (e) and (g) lateral views of Cu^{+2}-treated larvae at the end of 7 and 14 days larval-toxicity test. Wavy tail fin (b), Axial and Tail fin abnormality (c, d, e), Tail flexure (f, g). Note the edema (→).

Amphibian embryos are useful indicators of environmental pollution and they are employed in toxicity studies of single substances, complex mixtures and environmental samples. Their ecological

significance represented by their role in the trophic system and occupancy of unique habitats is well documented [45, 46].

Ecotoxicological tests with amphibians generally use African clawed frog, *Xenopus laevis* as a test organism [47]. The first amphibian test to be standardized and internationally recognized was a developmental toxicity test: FETAX (Frog Embryo Teratogenesis Assay-Xenopus) using embryos of Xenopus sp, as a test organism [47]. It was first developed by [48] and is well suited as a screening assay for the potential developmental toxicity of both single chemicals, and complex chemical mixtures on embryos of *X. laevis*. ASTM (American Society for Testing and Materials) publishes the guideline for conducting the FETAX [47]. This study procedure includes the exposure of *X. laevis* embryos to a test solution to which some test material has been added. The test is conducted on mid-blastula stage whose development is followed through organogenesis. The teratogenic potential of a compound is determined after analysis of mortality and malformations. The whole test is performed at embryonic, non-protected stages of development and is an alternative to the use of mammalian species for the assessment of developmental toxicity [49]. However, FETAX has a number of deficiencies which make its use for ecotoxicological risk assessment less suited in Europe and Türkiye. Because of representing a very special limited ecosystem in Africa, *X. laevis* has a disadvantage in the evaluation of the xenobiotics' effect on amphibians in a traditional risk assessment context. There are some uncertainties associated with using this bioassay in a risk assessment study in Türkiye, since it employs a non-native species for Türkiye. There are limited comparative sensitivity data available between native Turkish species and Xenopus sp. The sensitivity of embryo and tadpole stages of a particular species may vary significantly [50]. Moreover the effect of many substances is found to be highly dependent on the temperatures, and therefore varies with the latitude and the season. Thus, the effect of xenobiotics on populations in the northern latitudes as well as spring-breeding species may be different from the effect found in test with an African frog.

Another test using amphibians is termed as AMPHITOX which is a set of customized toxicity tests using early life stages of *Bufo arenarum* embryos for acute, short term chronic, and chronic exposure period [8]. This test procedure allows selecting the most appropriate exposure period and end points according to toxicity of a sample and purpose of a study. Recently a new toxicity test guideline, related to FETAX with the fire-bellied toad, *Bombina bombina* as test organism, was developed [22] because of the fact that *X. laevis* is not ecological relevant for Europe. *B. bombina* has a wide distribution in East, Middle and North Europe and also present in Türkiye (Turkish Thrace and northwestern Anatolia). Apart from being a native species of Türkiye, this species has been found to be easy to breed and keep under conditions of the amphibian breeding laboratory throughout the year and get a sufficient number of healthy embryos at low cost. It has been known that much of the ecotoxicological work has been represented by species that were easily to breed in captivity. It has also been known that fire-bellied toad is declining in especially the north-western part of its range. In an ecosystem, the presence or absence of *B. bombina* is ecologically significant and the presence of this species indicates a relatively large diversity of amphibians, algae, bacteria and presumably also invertebrates [22]. Therefore *B. bombina* is considered as bioindicator organism and so the effects of xenobiotics on this animal are also important in terms of representing ecosystem health. Test organisms to be used for ecotoxicological studies have to satisfy both ecological and economic criteria. From the ecological point of view, a test organism should be as representative as possible of a specific ecosystem and of a specific trophic level. The relevant economic criteria are fulfilled if sufficient quantities of the organism can be made available for standardized tests without difficulty and at acceptable cost.

4. Conclusion

This study revealed that *B. bombina*, as a native species, is an ideal indicator test organism that can be used in ecotoxicology studies in Türkiye in terms of adaptability to laboratory conditions and high-

susceptibility to xenobiotics. From the present study, it can be concluded that BOMBITOX, the modified ecotoxicological test model with native species of Türkiye, was found to be useful in testing developmental toxicity of pollutants and meet both ecologic and economic criteria.

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