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Research article/Araştırma makalesi

Some Toxic Effects of Ammonium Sulfate Fertilizer to *Rana macrocnemis* Tadpoles From Rize (Black Sea Region), Turkey

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Abstract

The aim of this study was to investigate the toxic effects of ammonium sulfate in *Rana macrocnemis* tadpoles. So, the animals were exposed to six different concentrations of ammonium sulfate (50, 75, 100, 150, 250 and 500 mg/L) for 96 hours. We detected morphological and behavioral changes during acute test. Increased concentration of ammonium sulfate-induced scoliosis was observed in the exposed animals. Besides, there was a loss of pigmentation and the movements of the tadpoles decreased with the increasing ammonium sulfate concentrations. In addition to that, we found out statistical differences between control and treatment groups in terms of final body length and weight. Despite increasing concentrations of ammonium sulfate caused increased mortality, there were no significant differences among treatments groups (50, 75, 100, 150, 250 and 500 mg/L) according to Kaplan-Meier survival test. Our results demonstrate that acute exposure to ammonium sulfate causes dose-related morphological and behavioral damage to *Rana macrocnemis* tadpoles in short periods of time.

Key words: *Rana macrocnemis*, acute toxicology, fertilizer, morphological changes, behavioral changes

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Amonyum sülfat gübresinin Rize (Karadeniz Bölgesi), Türkiye'den *Rana macrocnemis* larvaları üzerindeki bazı toksik etkiler

Özet

Bu çalışmanın amacı, *Rana macrocnemis* larvaları üzerinde amonyum sülfatın toksik etkilerini araştırmaktır. Böylece, hayvanlar 96 saat boyunca altı farklı amonyum sülfat konsantrasyonuna (50, 75, 100, 150, 250 ve 500 mg /L) maruz bırakıldı. Bu akut test sırasında morfolojik ve davranışsal değişiklikler tespit ettik. Maruz kalan hayvanlarda artan amonyum sülfat konsantrasyonuna bağlı olarak omurga eğriliği gözlemlendi. Ayrıca artan amonyum sülfat konsantrasyonları ile larvalarda pigmentasyon kaybı vardı ve hareketleri azaldı. Buna ek olarak, kontrol ve amonyum sülfat uygulanan gruplar arasındaki nihai vücut uzunluğu ve ağırlığı açısından istatistiksel farklılıklar bulduk. Artan amonyum sülfat konsantrasyonu ile ölümlerin artmasına rağmen, Kaplan-Meier sağkalım testine göre amonyum sülfat uygulanan gruplar arasında anlamlı farklılık bulunmamıştır. Sonuçlarımız, amonyum sülfata akut maruz bırakılan *Rana macrocnemis* larvalarında kısa sürede, doza bağlı morfolojik ve davranışsal hasara neden olduğunu göstermektedir.

Anahtar kelimeler: *Rana macrocnemis*, akut toksikoloji, gübre, morfolojik değişimler, davranışsal değişimler

1. Introduction

Researchers showed that chemical pollution is one of the main reasons for declining amphibian populations (Davidson, 2004; Relyea, 2005). Ammonia is a common aquatic pollutant that enters natural waters with municipal, agricultural, fish-cultural industrial wastes and from energy-development processes and it is also a natural degradation product of nitrogenous organic matter. In the Black Sea region (Turkey), it is often used as a fertilizer in agricultural areas for tea production and the use of fertilizers is becoming increasingly widespread in our country (Coşan et al., 2015). Applications of the fertilizer generally occur during the spring and summer at that time amphibians are more

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vulnerable to environmental factor because of they have their breeding and larval development. Because of their sensitive skin, gills**,** and eggs, amphibians can be easily affected by pollution from aquatic ponds (Duellman and Trueb, 1986; Materna et al., 1995; Venturino et al., 2003).

In temporary ponds, unpredictable abiotic factors (ammonium, nitrate and nitrite level) are major problems for the inhabitants. To adapt to this condition, they must develop extreme tolerances and/or ensure continuity their homeostasis with developing a physiological mechanism. It is important to know that such a mechanism is generally not supported by a single factor. Rather, complex quantitative and qualitative changes in the ecological conditions of the aquatic environment are managing it. Some amphibian species display strong tolerance to ammonium, nitrate and nitrite level and variation in tolerance among different amphibian species (Hecnar, 1995).

Contaminants can have sublethal effects in amphibians in terms of developmental and behavioral abnormalities such as malformations, developmental delays, hindered growth, behavioral changes like abnormal movements, reduced activity and feeding, swimming disorders, delayed response to stimuli (Bridges, 1997; Diana et al., 2000; Herkovits et al., 2002; Bridges and Boone, 2003). Such a behavioral change is crucial indicator of contamination effects. In the meantime, competition and predation may be damaged by nitrates (Smith et al., 2006).

The present study was designed to determine the acute toxic effects of ammonium sulfate at six different doses in terms of morphology and behavior of *Rana macrocnemis* tadpoles in the laboratory for 96 hours for the first time in literature.

2. Materials and methods

2.1. Experimental Animals

The present study was approved by the animal ethics committee of Karadeniz Technical University, Faculty of Medicine (2013/19). *Rana macrocnemis* tadpoles were obtained from eggs collected from a habitat in Ovit plateau, Rize, Turkey (40˚37'31.76" N, 40˚45'19.99" E, 2500 a.s.l.) that is far away from agricultural areas. The eggs were transported to the laboratory and kept in glass tanks fitted with Reconstituted Soft Water (RSW) solution and air stones. The temperature of the solution was maintained between 19-21°C in the same diluent water. After the eggs hatched, they were fed with cooked lettuce. Fresh food was added and uneaten food was removed from the tanks daily. All tadpoles used in the experiment were at Gosner stage 25 (Gosner, 1960). At the beginning and at the end of the acute test, total body length of the all tadpoles from mouth to tail tip was measured using an electronic digital caliper at the nearest 0.01 mm. Also, we weighed tadpoles before and after the acute test.

2.2. Water Quality

We measured pH and temperature values of water in the field study. Water samples were also taken from the field and ammonium, nitrate and nitrite levels of water samples were analyzed using Merck Spektroquant kit. Water quality criteria according to the Ministry of Forestry and Water Affairs of Turkey were given in Table 1 and we classified our water samples due to these criteria.

Table 1. Inland water classification parameters according to the Ministry of Forestry and Water Affairs of Turkey

	Water quality classes			
Water classification parameters	I. Quality water (First quality water)	II. Quality water (Less contaminated water)	III. Quality water (Contaminated water)	IV. Quality water (High contaminated water)
Temperature (°C)	25	25	30	>30
pH	6.5-8.5	6.5-8.5	6.0-9.0	>6.0-9.0
Oxygen saturation (%)	90	70	40	<40
Dissolved oxygen (mg O_2/L)	8	6	3	<3
Ione of sulfate (mg SO_4^{-2}/L)	200	200	400	>400
Ammonium nitrogen (mg NH_4^+-N/L)	0.2[c]	1[c]	2[c]	>2
Nitrite nitrogen (mg NO_2^--N/L)	0.002	0.01	0.05	>0.05
nitrate nitrogen (mg NO_3—N/L)	5	10	20	>20

2.3. Toxicity Bioassays

Ammonium sulfate, $(NH_4)_2SO_4$ (Sigma), was chosen as the toxic chemical. The water used for the toxicity tests was RSW which was prepared by dissolving 48 mg $NaHCO_3$, 30 mg $CaSO_4.2H_2O$, 61 mg $MgSO_4.7H_2O$ and 2 mg

KCl in deionized water. A stock solution was prepared by dissolving the ammonium sulfate in RSW and treatment concentrations were 50, 75, 100, 150, 250 and 500 mg/L. Each test chamber comprised of an equal volume of experimental test solution (2 L), an equal number of tadpoles (5) and was placed in equal glass tanks (5 L). Tadpoles were exposed to ammonium sulfate solution for 96 hours and 5 replications per concentration were performed. A total of 25 individuals was evaluated for each concentration (n= 150). The control group (n=25) contained only RSW.

Behavioral abnormalities such as swimming disorders, abnormal movements and also, mortalities in different concentrations were recorded daily during the test. We considered them dead when tadpoles did not respond to stimulation and were removed. Dead tadpoles were counted after 24, 48, 72 and 96 h.

Normality was calculated via Kolmogrov-Smirnov test ($p < 0.05$) and statistical differences between body length and weight changes between groups were analyzed by Kruskal-Wallis test. LC_{10}, LC_{50}, and LC_{70} values were determined using Probit analysis. After exposed to different concentrations of ammonium sulfate, we analyzed survival of tadpoles using with Kaplan-Meier survival and failure time analysis tests. All statistical tests were conducted with SPSS 21.

3. Results

Animals exposed to ammonium sulfate showed different morphological changes. Although disorders can be seen with the naked eyes, the examination of the individuals was performed under the stereomicroscope (Leica LED2500). We could not observe any abnormal behaviors and morphological differences during the test period in the control group. Tadpoles from control groups could act with one stimulus while more than one stimulus is needed by tadpoles from other groups.

Swimming activity decelerates to swimming and reverse swimming started at the concentrations of 50, 75, 100 and 150 mg/L ammonium sulfate. Edema and loss of pigmentation were rarely observed after exposure to 50 mg/L. At 75, 100 and 150 mg/L concentrations, edema and loss of pigmentation were more abundant than 50 mg/L. At 50, 75, 100 and 150 mg/L, only 16%, 8%, 8% and 4% tadpoles were died, respectively. Movements and feedings started to decrease with 50 mg/L. Behavioral and morphological changes observed at 250 and 500 mg/L were characterized by reducing activity and feeding delayed response to stimulation, edema, and loss of pigmentation, bent tails, and scoliosis. Moreover, tadpoles in 500 mg/L did not feed (Figure 1). Abnormal behavior frequencies were given in Table 2.

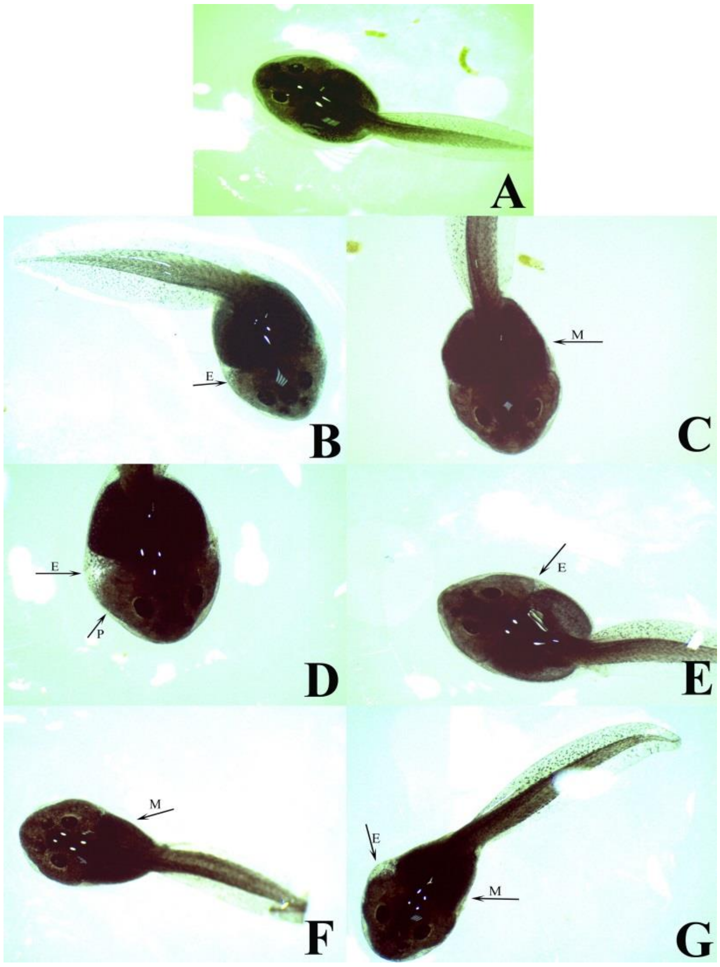

Figure 1. (A) Tadpole exposed to 50 mg/L (B) Tadpoles exposed to 75 mg/L (C) Tadpoles exposed to 100 mg/L (D) Tadpoles exposed to 150 mg/L (E) Tadpoles exposed to 250 mg/L (F) Tadpoles exposed to 500 mg/L. E= edema, M=malformation in body structure P=loss of pigmentation.

Table 2. Behavioral changes of *R. macrocnemis* tadpoles exposed to different concentrations of ammonium sulfate

	Treatments (mg/L)						
Abnormal behavior	0	50	75	100	150	250	500
Reverse swimming	-	4	-	-	1	-	10
Decelerate to swimming	-	4	1	1	2	2	15
No. of surviving tadpoles	25	21	23	23	24	23	7
Frequency of abnormality (%)	-	19.05	4.35	4.35	12.5	8.70	100

Despite increasing concentrations of ammonium sulfate caused increased mortality, there were no significant differences among treatments according to Kaplan-Meier survival test ($\chi2 = 1.24$, $p > 0.05$) (Figure 2).

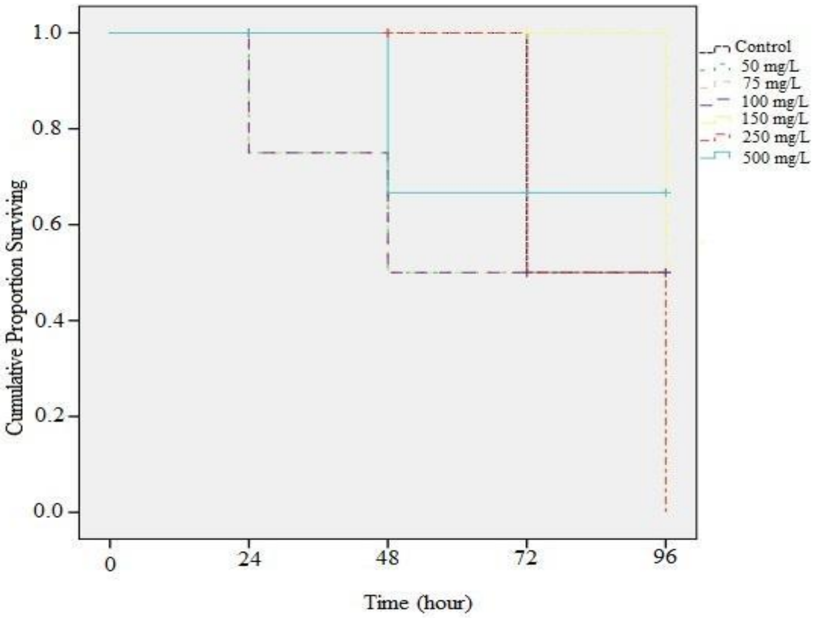

Figure 2. Cumulative survival (Kaplan-Meier) of *R. macrocnemis* tadpoles after exposure to ammonium sulfate at different concentrations.

After 96 h, the values of the lethal concentrations were found as LC_{10}=117.670 mg/L, LC_{50}=436.623 mg/L and LC_{70}=567.141 mg/L (Table 3).

Table 3. Lethal concentrations of ammonium sulfate exposure for *R. macrocnemis*

Lethal concentrations	Concentrations (mg/L)	%95 Confidence Interval	
		Lower Bound	Upper Bound
LC_{10}	117.670	29.381	179.832
LC_{50}	436.623	357.884	575.724
LC_{70}	567.141	461.938	768.087

The total length and weight of the tadpoles were measured both before and after the acute test (Table 4). Statistical differences were detected between control and treatment groups in terms of body length and weight in exposed tadpoles (Kruskal Wallis, body length; χ^2= 14.556, df= 6, p<0.05, weight; χ^2= 19.473, df=6, p<0.05). Body length reduced significantly because of the exposure to 250 and 500 mg/L concentrations of ammonium sulfate.

Table 4. Comparison of morphological measurements of before and after treatment.

		Before treatment			**After treatment**		
	Concentrations (mg/L)	Mean±S.D.	Minimum	Maximum	Mean±S.D.	Minimum	Maximum
Body length	0	13.69±1.92	11.28	16.80	16.20±2.38	12.12	19.88
	50	13.87±1.60	12.15	16.55	17.39±3.83	11.51	24.12
	75	13.89±1.31	12.11	16.88	15.10±4.22	0.00	19.18
	100	13.92±1.39	12.34	16.71	16.54±2.26	12.88	22.96
	150	13.87±1.30	12.09	16.17	16.40±1.96	12.03	19.97
	250	13.80±1.15	11.63	15.86	15.73±2.34	11.65	20.28
	500	14.12±1.30	12.02	16.08	13.61±2.20	8.85	16.52
Weight	0	0.40±0.02	0.01	0.08	0.06±0.02	0.02	0.09
	50	0.40±0.02	0.02	0.07	0.08±0.06	0.02	0.29
	75	0.39±0.01	0.03	0.06	0.05±0.02	0.02	0.10
	100	0.41±0.01	0.03	0.05	0.05±0.02	0.03	0.11
	150	0.40±0.01	0.03	0.05	0.07±0.10	0.02	0.54
	250	0.40±0.01	0.03	0.05	0.04±0.02	0.02	0.08
	500	0.40±0.00	0.03	0.04	0.03±0.01	0.02	0.06

Although all the tadpoles were fed during the test, the mean weight of tadpoles belonging to concentrations 250 and 500 mg/L decreased. Also, we found a negative correlation between weight and concentrations ($r = -0.159$, $p = 0.047$). Exposure of *R. macrocnemis* tadpoles to ammonium sulfate treatments reduced larval food consumption and caused a decrease in final larval mass. In addition to that, when the animal reduced the feeding, they exhibited restricted movements.

According to the values, measurement values of NO_2, NO_3, NH_4 and SO_4 were ranged as 0.009 mg/L, 0.2-0.6 mg/L, 0.43-4.31 mg/L and 2-4 mg/L, respectively. Our measurements from water samples demonstrated that dissolved oxygen concentrations, temperature and pH range from 8.19 to 10.13 (mg O_2/L); 8.4 to 15.8 °C and 6.86 to 7.28, respectively. Under the criteria of the Ministry of Forestry and Water Affairs, our water samples were classified as contaminated water.

4. Conclusions and discussion

Amphibians have different sensitivity to ammonium derivations. Berger (1989) stated that the significant mortality was observed among *Rana esculenta* exposed to 20 mg NH_4NO_3/L after 96 h. Nevertheless, Watt and Jarvis (1997) did not observe any mortality as a result of the exposure to 387.5 mg $NO_3NH_4NO_3$/L in *Triturus helveticus*. *Pseudacris regilla* and *Pimephales promelas* tadpoles were exposed to different ammonium sulfate concentration ranging from 17.4 to 212.2 mg/L and 32.4 to 211.5 mg/L, respectively. Also in the same study, *Rana aurara* tadpoles were exposed to ammonium sulfate ranging from 28.8 to 227 mg/L (Nebeker and Schuytema, 2000). They showed that these three amphibians had a different toleration to the ammonium. *Pimephales promelas* was the most tolerant than the others and. The 6-wk-old juveniles had LOAEL and NOAEL values of 118.0 and 66.6 mg/L NH4 -N based on length and weight. Ortiz-Santaliestra et al. (2006) detected a lethal effect in amphibians of exposure to the highest ammonium nitrate level (225.8 mg NH_4NO_3/L) in 96 h. Significant mortality was detected at concentrations of 180 mg NH4NO3/L from *B. bufo*, *B. calamita* and *P. ibericus*. However, in the highest concentration exposure, there was no mortality detected in *P. perezi* (García-Muñoz et al. 2011). Johansson et al. (2001) stated that in the ammonium treatments, mortality increased with both time and increasing concentration. In our acute tests demonstrated that *R. macrocnemis* tolerated, even high concentrations of ammonium sulfate, while similar concentrations of ammonium sulfate detected highly lethal in other amphibians.

We showed that ammonium sulfate has induced morphological and behavioral effects on *R. macrocnemis* tadpoles. In the present study, unusual behaviors, including loss of balance, swimming in circles and no activity (unless disturbed) were observed in animals. The magnitude of the changes was correlated with the concentration of ammonium sulfate including sub-lethal doses. The major changes observed in all concentrations were scoliosis, edema, and loss of pigmentation. In a similar study, Hecnar (1995) found that tadpoles of *Bufo americanus*, *Pseudacris triseriata*, *Rana pipiens* and *Rana clamitans* exposed to ammonium nitrate exhibited unusual behavior like loss of balance, swimming in circles, edema, loss of pigmentation, and scoliosis, which supports our study. Karaoğlu (2011) also reported similar damages to *Pelodytes caucasicus* tadpoles exposed to ammonium nitrate. Hecnar (1995) explained swelled up bodies in terms of nitrite interference with osmoregulation.

Baker and Waights (1993), who obtained a smaller size of *Bufo bufo* larvae exposed to 40 and 100 mg $NaNO_3$/L, compared them with those in the control group. *Pelobates cultripes* tadpoles exposed to 90.3 mg N-NO_3NH_4/L were 23% shorter than those in the control group (Ortiz-Santaliestra et al., 2006). Also in the same study, body length of *Bufo calamita* tadpoles exposed to 90.3 mg N-NO_3NH_4/L was 26.6% smaller than those in the control group. The opposite effect of low ammonium nitrate levels on larval size of the common toad, *Bufo bufo*, was defined (Xu and Oldham, 1997) in literature. Bigger tadpoles were observed in concentrations of 22.6 mg N-NO_3NH_4/L compared with those in the control group. At the end of our acute test, the mean body size of tadpoles exposed to ammonium sulfate differed. Bigger tadpoles were determined in concentrations of 50, 100 and 150 mg/L compared to control groups. This suggests that adaptation to local nitrate levels is possible. Also, the body length of tadpoles exposed to 75, 250 and 500 mg/L concentrations was smaller than control groups. In spite of these conclusions, there is a negative correlation between body length and ammonium sulfate concentrations according to Pearson correlation test. Despite ammonium sulfate concentrations increasing, body length of tadpoles is still decreasing.

We found the LC_{50} (96 h) value of ammonium sulfate to was 436.623 mg/L. The results indicated that the acute lethality of the ammonium sulfate for the *Rana macrocnemis* tadpoles was very high. In *Triturus helvatica*, high mortality was seen as a result of the concentration of ammonium nitrate at 70 mg/L (Watt and Jarvis, 1997). Hecnar (1995) revealed that medium lethal concentrations of four amphibian species were between 27.2 and 78.6 mg/ after 96 h acute tests. Schuytema and Nebeker (1999) determined the LC_{50} of ammonium nitrate, ammonium sulfate and ammonium chloride for the tadpoles of *Pseudacris regilla* and *Xenopus laevis*. For *P. regilla* tadpoles, LC_{50} of ammonium nitrate and ammonium sulfate were 55.2 and 89.7 mg/L, respectively. For *X. laevis* tadpoles, LC_{50} varied between 45-64 mg/L among the three ammonium compounds. Karaoğlu (2011) compared the LC_{50} values of ammonium nitrate for *Pelodytes caucasicus* tadpoles taken from the clean and soiled water, which were 204 mg/L and 170 mg/L for soiled and clean water, respectively. Our results showed that *R. macrocnemis* tadpoles were more tolerant to ammonium compounds than other tadpoles.

Typical sulfate levels in fresh spring water are in the vicinity of 20 mg/L and range from 0 to 630 mg/L in rivers according to the World Health Organization (2004). In European countries, NO3-N concentration in groundwater value is 23 mg/L and in the USA it is 45 mg/L (Savcı, 2012). We measured some water parameters of ponds where we took our eggs from. We found that values of NO_2, NO_3, NH_4 and SO_4 were ranged as 0.009 mg/L, 0.2-0.6 mg/L, 0.43-4.31 mg/L and 2-4 mg/L, respectively. Under the inland water criteria of the Ministry of Forestry and Water Affairs of Turkey, our water samples were classified as contaminated water. Amphibian eggs are more tolerant to ammonia than fish eggs. They are protected by their egg capsules or gelatinous matrix from ammonia. In addition to this, this situation may increase genetic or physiological tolerance of tadpoles.

Factors like dissolved oxygen concentration, temperature and salinity are a cofactor in ammonia toxicity (Dawning and Merkens 1955; Bower and Bidwell 1978). Ammonia was affected by pH, particularly. The acute toxicity of NH_3 increased with pH decreased. Our measurements from water samples demonstrated that dissolved oxygen concentrations, temperature, and pH range from 8.19 to 10.13 (mg O_2/L); 8.4 to 15.8 °C and 6.86 to 7.28, respectively. The values of these parameters are not enough to contaminate water according to the Ministry of Forestry and Water Affairs of Turkey inland water criteria.

The results of this research suggest that ammonium sulfate used on croplands in Rize, Turkey appears to pose a little acute risk of mortality of *Rana macrocnemis*. In this study, we considered neither chronic exposure nor sub-lethal toxicity. Our study represents a first attempt to quantify the toxicity of ammonium sulfate to *Rana macrocnemis*, but more data are needed to be sure of ammonium sulfate affection.

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