

# Impacts of ammonium nitrate on survival of tadpoles of the common skittering frog (*Euphlyctis cyanophlyctis*)

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Amphibians are sensitive to agricultural chemicals such as pesticides and fertilizers. Data on responses of anuran species commonly found in agricultural areas of Pakistan to chemical pollution are lacking. We studied the effects of ammonium nitrate on survival of tadpoles of common skittering frog (*Euphlyctis cyanophlyctis*). Tadpoles were collected from seasonal shallow water ponds formed during rainy season in the vicinities of Rawalpindi and Islamabad cities. Tadpoles were treated with ammonium nitrate at either 62.5, 125, 250, 500 or 1000 mg nitrate / l, and compared with a control group. Individual survival was significantly affected by increased ammonium nitrate treatments ( $P < 0.05$ ), with survival rates after 18 days of exposure ranging from 83% at 62.5 mg  $\text{NO}_3^-$  / l to complete mortality at the highest ammonium nitrate concentration. The logistic regression model with individual survival as a binomial outcome variable and fertilizer concentration as independent variable explained 87% of the variance in survivorship (Nagelkerke  $R^2$ ), and correctly classified 94.0% of the cases ( $\chi^2 = 259$ , 5 d.f.,  $P < 0.0005$ ). Our lab-based findings suggest that ammonium nitrate at high concentrations may affect anuran species of Pakistan in croplands with extensive nitrogen-based fertilizer use.

**Key words:** ammonium nitrate; Dicroglossidae; regression; survivorship; tadpoles.

## **Impacto del nitrato amónico sobre la supervivencia de las larvas de *Euphlyctis cyanophlyctis*.**

Los anfibios son sensibles a los efectos de algunos agroquímicos como plaguicidas y fertilizantes. Investigamos los efectos del nitrato amónico sobre la supervivencia de las larvas de *Euphlyctis cyanophlyctis*. Colectamos los renacuajos de charcas someras estacionales que se forman durante la estación lluviosa en las proximidades de las ciudades de Rawalpindi e Islamabad. Los renacuajos se trataron con nitrato amónico a un de las siguientes concentraciones: 62,5, 125, 250, 500 ó 1000 mg nitrato / l, comparando además con un grupo control. La supervivencia de los individuos se vio afectada de manera significativa por el incremento en las concentraciones de nitrato amónico ( $P < 0,05$ ), con tasas de supervivencia que tras 18 días de exposición variaron entre el 83% a 62,5 mg  $\text{NO}_3^-$  / l hasta la mortalidad total en la concentración más elevada de nitrato amónico. El modelo de regresión logística con la mortalidad de los individuos como respuesta binomial y la concentración de fertilizante como variable independiente explicó el 87% de la varianza de la supervivencia ( $R^2$  Nagelkerke), clasificando correctamente un 94% de los casos ( $\chi^2 = 259$ , 5 g.l.,  $P < 0.0005$ ). Nuestras observaciones en el laboratorio sugieren que las elevadas concentraciones de nitrato amónico po-

drían afectar a las especies de anuros paquistaníes que habitan en zonas agrícolas sometidas a una aplicación intensiva de fertilizantes .

**Key words:** bioacumulación de metales; contaminación; Dicroglossidae; histología; histopatología.

Amphibians have a great significance in ecotoxicological studies, as their naked, permeable skins make them very susceptible to the uptake of anthropogenic chemicals and contaminants in the environment (BLAUSTEIN *et al.*, 2003). Amphibian populations are declining worldwide; it is estimated that 30% (1895 out of 6285) of the amphibians which conservation status has been assessed by the International Union for Conservation of Nature are threatened (IUCN, 2016). Scientists believe that a multitude of interrelated factors are responsible for this decline (ALFORD & RICHARDS, 1999), including climate change, increased exposure to ultraviolet radiation, emergent pathogens, introduced species, habitat destruction, acid rain and agricultural chemicals such as pesticides and fertilizers (BLAUSTEIN *et al.*, 2003; BOONE & BRIDGES, 2003). Actually, the leading role played by agrochemicals on decline of certain amphibian populations has already been demonstrated (DAVIDSON, 2004).

Nitrogen-based fertilizers are believed to impact amphibian populations in agricultural areas (OLDHAM *et al.*, 1997; GRIFFIS-KYLE, 2005). Numerous laboratory studies have shown that ammonium nitrate, one of the world's leading nitrogenous fertilizers, can significantly reduce larval survivorship in many species of frogs and toads (BURGETT *et al.*, 2007). Long term exposure to ammonium and nitrate resulting from the dissociation of this compound in the water

is known to alter developmental and behavioural functions of anurans (HECENAR, 1995). Some morphological abnormalities have also been reported in ammonium nitrate-treated tadpoles of species like *Bufo calamita*, *Bufo spinosus* and *Discoglossus galganoi* (ORTIZ *et al.*, 2004). Malformed individuals are more susceptible to predation, have low reproductive success, weak immune system and endocrine system (BRIDGES, 1997, 1999a,b, 2000; OUELLET *et al.*, 1997; BOONE & SEMLITSCH, 2002), all of which considerably reduces their survival chances in the wild.

Ecotoxicological information regarding amphibians from Pakistan is very scarce. Only a few studies about the effects of various pesticides on certain enzymes of adult frogs are available from this country. Pyrethroid, organophosphate and organochlorine pesticides decreased concentrations of enzymes like cholinesterase and alkaline phosphatase in liver, kidney and brain of the common skittering frog (*Euphlyctis cyanophlyctis*) and the bullfrog (*Hoplobatrachus tigerinus*) (KHAN *et al.*, 2006, 2007, 2008; KHAN & YASMEEN, 2008). However, studies reporting the impacts of fertilizers on amphibians from Pakistan are not available. We conducted the present study to investigate if tadpole survivorship in *E. cyanophlyctis*, one of the most common amphibians in croplands from Pakistan, was affected by the widely used nitrogenous fertilizer ammonium nitrate.

## MATERIALS AND METHODS

We carried out the present study in areas of Rawalpindi and Islamabad. The humid subtropical climate prevails in the area, with generally long and very hot summers followed by a monsoon season and mild wet winters. The area features hard substrate and scrub vegetation in most parts. The permanent wetlands of the area include rivers Korang and Soan, and water storage reservoirs such as Rawal Dam, Simly Dam and several other small dams with associated marshes (CHAUDHRY & RASUL, 2004; ASHRAF *et al.*, 2007). Numerous ephemeral ponds and pools are formed during the monsoon season (July-August), during which the breeding activity of anurans reaches its peak. The area possesses few common, non-endangered toad and frog species such as the southeast Asian toad (*Duttaphrynus melanostictus*), the Indus valley toad (*Duttaphrynus stomaticus*), the ant frog (*Microhyla ornata*), *E. cyanophlyctis* and *H. tigerinus* (RAIS *et al.*, 2012).

We selected *E. cyanophlyctis* as model species because it prefers shallow water ponds and edges of other water bodies, is highly aquatic, is the most common frog species in the area, and is also associated with croplands in the plains of Pakistan (KHAN, 2006). No permit is required to collect or dissect this species since it is not included in the applicable catalogues of protected species, i.e. Punjab Wildlife Acts and Rule (1974) and *Islamabad Wildlife Protection, Preservation, Conservation and Management Ordinance* (1979).

We collected *E. cyanophlyctis* tadpoles (N > 200) at developmental stage 25

(GOSNER, 1960) from Rawal Lake Spillway Area (UTM WGS84 coordinates, zone 43: X = 342226.7, Y = 3740053.9) and Loi Bher Wildlife Park (X = 348616.5, Y = 3737423.8); these are non-agricultural areas, which minimized the risk of tadpoles having been exposed to ammonium nitrate prior collection. Tadpoles were collected using dip nets and transferred into buckets filled with water from the same pond. We carried out buckets to the laboratory at the Department of Wildlife Management, Pir Mehr Ali Shah, Arid Agriculture University Rawalpindi, and kept tadpoles for 24 hours in glass aquaria (0.12 m<sup>3</sup>) with water filtering and aeration units.

Ammonium nitrate solutions were prepared from granular ammonium nitrate. Data on ecologically relevant concentrations of this fertilizer in the study area are not available, but published literature (XU & OLDHAM, 1997; ROUSE *et al.*, 1999; ORTIZ-SANTALIESTRA *et al.* 2006) have reported concentrations of ammonium or nitrate corresponding to 10 to 258 mg / l ammonium nitrate to be ecologically relevant for amphibians elsewhere. We prepared five nominal concentrations of ammonium nitrate: 1000, 500, 250, 125 and 62.5 mg ammonium nitrate / l. A 20 g / l stock solution was prepared in distilled water. To prepare the different experimental concentrations, volumes of 200, 100, 50, 25 and 12.5 ml of the stock solution were added to 4 l of tap water, respectively. Each concentration was replicated thrice, with a batch of 10 tadpoles (2.5 tadpoles per litre) in each replicate. Three additional batches with 10 untreated tadpoles each were used as controls. To control for the presence of excess unionized ammonia, initial pH was meas-

**Table 1:** Results of the repeated measures analysis of the variance to test the evolution over time of ammonium nitrate effects on *Euphlyctis cyanophlyctis* tadpole survival .

Test	Source of variation	Sum of squares	d.f.	F	P
Within-subject effects	Time	10.911	1	56.999	<0.001
	Concentration*Time	7.877	5	8.229	0.001
	Error	2.297	12		
Between-subject effects	Intersection	122.881	1	209.419	<0.001
	Concentration	70.701	5	24.098	<0.001
	Error	7.041	12		

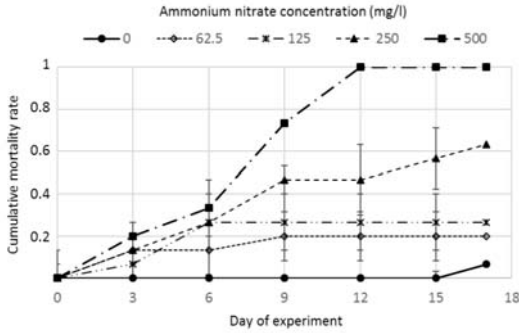
ured in aquaria and found to range from 7.37 to 7.41. The water was not renewed for the entire duration of the experiment, which lasted 18 days; this means that actual ammonium and nitrate concentrations were expected to significantly decrease over time throughout the experiment (HATCH & BLAUSTEIN, 2003), although MARCO *et al.* (1999) did not detect significant variation in nitrate levels after 7 days in experimental aquaria. All reference to experimental levels in the present paper refers to nominal values. The tadpoles were fed with boiled chopped cabbage, fish meal and boil chicken flakes.

Tadpole survivorship was calculated daily as number of surviving tadpoles at that specific day of experiment divided by the initial number of tadpoles. Daily mortality rates were arcsine of square root transformed to fit a normal distribution of residuals. Evolution of daily mortality rates during the experiment was analysed using a repeated measures analysis of the variance (ANOVA), with the ammonium nitrate concentration as fixed factor. Treatments causing significant mortality were identified through Tukey post-hoc tests run after ANOVAs for inter-treatment comparisons conducted with data of each

specific day of experiment. A logistic regression was performed to ascertain the effects of ammonium nitrate concentrations on the likelihood of tadpoles to survive.

## RESULTS

Repeated measures ANOVA showed that tadpole survival significantly decreased over time as ammonium nitrate concentration increased (Table 1). Significant mortality was detected from day 2 of experiment ( $F_{5,12} = 15.827$ ;  $P < 0.001$ ), when all tadpoles exposed to the highest concentration had died. Survival of tadpoles exposed to 500 mg / l ammonium nitrate was significantly lower than that of controls after day 7 of experiment ( $F_{5,12} = 13.132$ ;  $P < 0.001$ ). From day 8 of experiment onwards, we also observed reduced survival compared to controls among tadpoles exposed to 250 and 100 mg / l ( $F_{5,12} = 12.704$ ;  $P < 0.001$ ). The lowest ammonium nitrate tested concentration did not significantly affect survival (Fig. 1). The logistic regression (Nagelkerke  $R^2$ ) explained 87% of the variance in the survivorship and correctly classified 94% of cases. The model was statistically significant ( $\chi^2 = 259$ , 5 d.f.,  $P < 0.0005$ ).



**Figure 1:** Evolution during the experiment of cumulative mortality rates (mean  $\pm$  standard error) of *Euphlyctis cyanophlyctis* tadpoles exposed to different ammonium nitrate concentrations. Data corresponding to the 1000 mg / l treatment are not shown as 100% mortality was observed at day 2 of experiment.

## DISCUSSION

The present study reveals that increasing ammonium nitrate concentrations reduce survival of *E. cyanophlyctis* tadpoles. Amphibian eggs and larvae living in agricultural regions are impacted by ammonium nitrate pollution originated from the use of this substance as fertilizer (Rouse *et al.*, 1999), and several studies have provided evidence of the lethal effect of this chemical on amphibian aquatic stages. HECNAR (1995) reported that fertilizers and other chemicals used in agriculture are severe threats to amphibian populations after observing mortality of tadpoles of American toad (*Anaxyrus americanus*), chorus frog (*Pseudacris triseriata*), northern leopard frog (*Lithobates pipiens*) and green frog (*Lithobates clamitans*) exposed to ammonium nitrate at concentrations between 77.71 and 224.57 mg  $\text{NO}_3\text{NH}_4$  / l. After long-term exposures, survival of *P. triseriata* and *L. pipiens* was significantly reduced

also at 57.14 mg  $\text{NO}_3\text{N}$  / l. ORTIZ-SANTALIESTRA *et al.* (2006) concluded that environmentally relevant levels of ammonium nitrate could induce mortality and might affect population dynamics of several amphibian species in agricultural environments. BURGETT *et al.* (2007) reported that ammonium nitrate at concentrations above 50 mg / l (lower than the lowest used in the present study) decreased survivorship of wood frog (*Lithobates sylvaticus*) tadpoles. OROMI *et al.* (2009) suggested that if the levels of nitrate in water surpassed 100 mg / l (corresponding to 129 mg  $\text{NO}_3\text{NH}_4$  / l), the survival probabilities of the European common frog (*Rana temporaria*) larvae might be reduced. The results of the present study are in accordance with the aforementioned findings on ammonium nitrate-associated lethality.

Besides lethal effects, ammonium nitrate has been suggested to alter amphibian endocrine system, affecting development and other functions (GUILLETTE & EDWARDS, 2005; EDWARDS *et al.*, 2006; ORTON *et al.*, 2006), and also to affect central nervous system and impair anti-predatory ability of tadpoles (ORTIZ-SANTALIESTRA *et al.*, 2010). Although we did not analyse sublethal effects because of the high mortality recorded in almost all tested ammonium nitrate concentrations, the potential of these sublethal effects occurring at lower concentrations to affect larval survival probabilities in the wild needs further investigation.

Common skittering frog (*E. cyanophlyctis*) is one of the most common frogs in Pakistan, and is abundant also in agricultural areas. Our lab-based findings suggest that ammonium nitrate in high con-

centrations might affect this anuran species in croplands with extensive nitrogen-based fertilizer use. We must notice that tadpoles were exposed to a single pulse of ammonium nitrate, resembling what can happen in the wild after runoff or wash of applied fertilizers towards the breeding ponds. Since amphibians breed in water, ammonium and nitrate occurring when ammonium nitrate is dissolved may get absorbed by anuran eggs and permeable skin of tadpoles and adults, or may enter into their bodies directly by oral water uptake. The results of the present paper enhance the necessity of recommending that detailed studies are carried out on the monitoring of ammonium nitrate levels in Pakistan's croplands, and that field data are collected to understand how pollution by nitrogenous fertilizers affect anurans in croplands of the country.

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