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Effects of Ammonium Nitrate, Sodium Nitrate, and Urea on Red-Legged Frogs, Pacific Treefrogs, and African Clawed Frogs

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The widespread use of nitrogen fertilizers in agricultural areas has been suggested as a contributing factor in the decline of frogs in the United Kingdom (Oldham *et al.* 1997). Nitrogen fertilizers have been implicated in amphibian deaths in Denmark (Wederkinch 1988) and nitrate levels in pond water have been implicated in the disappearance of several species of amphibians in Poland (Berger 1989). Hecnar (1995) reported toxic effects of ammonium nitrate to tadpoles at levels of nitrate-nitrogen commonly exceeded in agricultural areas. Little information is available about the effects of urea on amphibians (Ghate 1985) although its effects on other stream life have been evaluated (e.g. Stay *et al.* 1978). Nitrogen fertilizer use in agricultural landscapes in the United States has increased from less than 0.45 million metric tons to more than 9.98 million metric tons in less than 50 years (Lanyon 1996) and thus should be considered in evaluating amphibian decline.

The Pacific treefrog, *Pseudacris regilla* (Baird and Girard), is the most common frog in the Pacific Northwest and is found in shallow wetland areas which are frequently dry prior to mid-summer (Corkran and Thoms 1996). The red-legged frog *Rana aurora* Baird and Girard, a candidate for threatened or endangered status (Federal Register 1991), frequents ponds and slow streams in forest lowlands west of the Cascades, preferring cooler conditions than *P. regilla* (Corkran and Thoms 1996). The African clawed frog, *Xenopus laevis* Daudin is a common test organism. The purpose of this study was to evaluate mortality and growth in *R. aurora* embryos/larvae exposed to ammonium nitrate and sodium nitrate, and mortality and growth in *P. regilla* tadpoles exposed to urea. *X. laevis* tadpoles were also exposed to urea to obtain comparative toxicity information for this widely-used species.

MATERIALS AND METHODS

R. aurora egg masses were collected from small ponds in the foothills of the Coast Range (Benton County). *P. regilla* tadpoles were raised from egg masses collected in Corvallis, OR. *X. laevis* tadpoles were raised from eggs from an in-house breeding colony. All three species were maintained and tested under a 16:8 light:dark cycle. *P. regilla* were fed ground pelleted rabbit food and *X. laevis* were fed dried Oregon

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Moist fish food pellet (3% of their body weight per day during testing). *R. aurora* were not fed during testing.

Test water was obtained from wells near the Willamette River at Corvallis, OR. Dissolved oxygen and pH were measured by electrode. Total hardness, alkalinity and conductivity were determined by USEPA Methods Nos. 130.2, 310.1 and 120.1, respectively, (USEPA 1979) prior to the start of each test. Water quality parameters (mean ± SE) of well water drawn for the tests were: dissolved oxygen, 8.7 ± 0.2 mg/L; total hardness, 25.5 ± 1.7 mg/L as $CaCO_3$, total alkalinity, 24.2 ± 1.6 mg/L as $CaCO_3$; conductivity, 82.3 ± 3.7µS/cm; median pH, 6.8. Background ammonium-nitrogen concentrations in the well water ranged from 0.005 to 0.010 mg/L NH_4-N.

The tests followed standard procedures as guidelines (ASTM 1997a, 1997b). Test conditions are summarized in Table 1. Animals were exposed to a series of six or seven concentrations of ammonium nitrate, sodium nitrate or urea [expressed as

Table 1. Test conditions.

Parameter	*R. aurora*	*P. regilla*	*X. laevis*
Starting Age/stage	Stage 11-12[a]	12 d[b]	13 d[c]
Starting weight (mg)	--	60	15
Days exposure	16	10	10
Test volume (mL)	100	1000	1000
Replicates/conc.	4	3	3
Animals/replicate	5	5	15
Temperature (°C)	15 ± 1	22 ± 1	22 ± 1
Test compounds	Ammonium nitrate, Sodium nitrate	Urea	Urea

[a]Gosner 1960. [b]6 d post-hatch. [c]11 d post-hatch.

ammonium-nitrogen (NH_4-N), nitrate-nitrogen (NO_3-N) and nitrogen (N), respectively]. *R. aurora* were tested in 100-ml dishes and the other species in 1000-ml beakers. Test solutions were renewed daily, and in the *R. aurora* exposures were analyzed every several days, three times at the beginning and three times at the end of a 24-hr period, for a total of six analyzed samples per concentration. Dead animals were removed at solution renewal.Total length was determined with a digitizer interfaced with a microcomputer. Wet weight was determined by blotting for 1 min prior to weighing. Tests were conducted in a temperature-controlled environmental room.

The percent purity of the technical grade ammonium nitrate and sodium nitrate was 99.0%. The purity of the urea was >99.9%. Stock solutions were prepared with well water. Ammonium nitrogen and NO_3-N were analyzed with a Hach DR/700 photometer. Samples from each replicate were pooled and analyzed within 2 hr after collection; approximately 10% were run in replicate. The specific recovery of NH_4-N in test water was 98.1 ± 0.6% (mean ± SE, n = 6). The mean coefficient of variation for paired duplicate samples of NH_4-N was 0.6 % (n = 7 pairs). The specific recovery of NO_3-N in test water was 107.4 ± 2.3% (mean ± SE, n = 9). The mean coefficient of variation of duplicate or triplicate sets of NO_3-N samples was 3.6 % (n = 11 sets). The mean percent difference between quality assurance split samples analyzed with both the Hach calorimeter and a Lachat Quikchem 8000 flow injection analyzer was 6.0 ± 0.1% (mean ± SE, n = 2) for NH_4-N and 4.6 ± 2.3% (mean ± SE, n = 2) for NO_3-N. Test solutions were not analyzed for urea, concentrations given are nominal.

RESULTS AND DISCUSSION

There was no mortality in the controls from the ammonium nitrate, sodium nitrate and urea tests (Tables 2, 3). All *R. aurora* embryos died at concentrations of ≥ 105 mg/L NH_4-N in the ammonium nitrate test and at 918 mg/L NO_3-N in the sodium nitrate test (Table 2). The 16-d LC50s (median lethal concentration) were 71.9 mg/L NH_4-N and 636.3 mg/L NO_3-N (Table 4). The levels of NO_3-N present in solutions containing sufficient NH_4-N to cause adverse effects were insufficient to have an effect on survival or growth. This indicates the ammonium ion to be more toxic and to be the primary cause of mortality in ammonium nitrate exposures. Similar findings were reported by Schuytema and Nebeker (1999a, 1999b) in exposures of *P. regilla* and *X. laevis* embryos and tadpoles to various ammonium compounds and sodium nitrate. It is also probable that acute toxicity of ammonium nitrate reported by Hecnar (1995) to various frog species was due to the ammonium ion. Significant ($p \leq 10.05$) decreases in length or weight of *R. aurora* embryos occurred at concentrations of NH_4-N ≥ 13.2 mg/L and at concentrations of NO_3-N <29.1 and ≥ 235 mg/L (Table 2). LOAEL (Lowest Observed Adverse Effects Level) and NOAEL (No Observed Adverse Effects Level) values based on length and weight for *R. aurora* embryos (Table 4) exposed to ammonium nitrate were 13.2 and 6.4 mg/L N H_4-N, respectively. The LOAEL and NOAEL values in the sodium nitrate exposures were <29.1 mg/L NO_3-N for length, and 235.0 and 116.8 mg/L NO_3-N, respectively for weight.

Responses to urea were very similar between *P. regilla* and *X. laevis.* All died at a concentration of 15,000 mg/L N, with 7% or less mortality at lower concentrations (Table 3). The 10-d LC50 values were 8396 and 9108 mg/L N for *P. regilla* and *X. laevis*, respectively (Table 4). Significant ($p \leq 10.05$) effects on length and weight occurred at concentrations of 6000 mg/L N (Table 3). LOAEL and NOAEL values for both length and weight for *P. regilla* were 6000 and 2400 mg/L N, respectively (Table 4). The LOAEL value based on length for *X. laevis* was >6000 mg/L N;

Table 2. Mortality, total length and wet weight of *R. aurora* embryos/larvae exposed to ammonium nitrate and sodium nitrate for 16 days (test terminated at 4 d post-hatch).

Measured conc. (mean ± SE)	% mortality	Length, mm (mean ± SE)	Wet weight, mg (mean ± SE)
Ammonium nitrate (mg/L NH_4-N)			
312.9 ± 13.4	100	--	--
105.5 ± 2.4	100	--	--
52.7 ± 1.2	5	10.7 ± 0.1[b]	14.0 ± 1.7[b]
25.4 ± 0.5	0	12.0 ± 0.1[b]	16.7 ± 1.0[b]
13.2 ± 0.3	0	12.7 ± 0.1[b]	20.4 ± 0.4[b]
6.4 ± 0.1	0	13.3 ± 0.1	23.0 ± 1.0
3.2 ± 0.1	0	13.3 ± 0.1	24.3 ± 1.0
0.05 ± 0.02[a]	0	13.4 ± 0.1	25.8 ± 0.7
Sodium nitrate (mg/L NO_3-N)			
3058.0 ± 222.0	100	--	--
918.0 ± 16.7	100	--	--
472.1 ± 13.2	5	12.1 ± 0.1[b]	20.4 ± 0.3[b]
235.0 ± 5.0	0	13.0 ± 0.1[b]	23.0 ± 0.1[b]
116.8 ± 3.8	0	13.2 ± 0.1[b]	24.6 ± 0.3
57.9 ± 1.6	0	13.2 ± 0.1[b]	23.7 ± 0.3
29.1 ± 0.9	0	13.5 ± 0.1[b]	26.0 ± 0.9
0.6 ± 0.2[a]	0	13.9 ± 0.1	27.2 ± 2.5

[a]Control. [b]Significantly less than control ($p \leq 0.05$).

LOAEL and NOAEL values for wet weight were 6000 and 2400 mg/L N, respectively. Urea is a commercial synthetic amide of carbonic acid in wide use in solid and liquid fertilizers and for direct application. It is also commonly applied to fields in liquid form combined with other fertilizers such as in urea ammonium nitrate solutions, or broadcast in granulated form as urea ammonium sulfate or urea ammonium phosphate (Meister 1995). The present tests have indicated urea in its pure form to affect tadpoles only at extremely high concentrations. Growth effects

Table 3. Mortality, total length and wet weight of *P. regilla* and *X. laevis* tadpoles exposed to urea for 10 days.

Urea, mg/L N (nominal)	% mortality	Length, mm (mean ± SE)	Wet weight, mg (mean ± SE)
P. regilla			
15,000	100	--	--
6,000	7	18.3 ± 0.1[b]	88.3 ± 8.7[b]
2,400	7	19.1 ± 0.3	108.9 ± 8.5
960	0	18.9 ± 0.2	100.1 ± 4.1
384	0	19.0 ± 0.4	108.5 ± 5.3
154	0	19.8 ± 0.5	114.6 ± 10.4
0[a]	0	20.3 ± 0.6	125.1 ± 10.2
X. laevis			
15,000	100	--	--
6,000	2	12.2 ± 0.1	19.5 ± 0.2[b]
2,400	2	12.2 ± 0.1	22.2 ± 1.0
960	0	12.6 ± 0.1	26.6 ± 1.5
384	0	12.2 ± 0.4	25.1 ± 2.2
154	0	12.5 ± 0.3	26.4 ± 0.6
0[a]	0	12.5 ± 0.2	25.5 ± 0.5

[a]Control. [b]Significantly less than control ($p \leq 0.05$).

of urea were observed at 6000 mg/L N, equivalent to 12,800 mg/L of whole compound. Ghate (1985) found no teratogenic effects in embryos of the frog *Microhyla ornata* exposed to 1000 mg/L urea (equivalent to 467 mg/L N) for 4 d; little information appears to be available on the effects of urea on North American amphibians. Stay *et al.* (1978) investigated the effects on stream organisms of fertilizing second growth Douglas fir in the Cascade mountains of western Oregon with urea at the rate of 225 kg N/ha. They found no significant effects on algal growth potential, stream macroinvertebrates, or caged rainbow trout fingerlings.

Soil type, rainfall, and amount and type of fertilizer applied can all affect the NH_4-N and NO_3-N content of agricultural runoff, and levels high enough to cause potential harm to amphibians can occur. For instance, runoff from fescue fields contained as

Table 4. LC50, LOAEL and NOAEL values for embryos and tadpoles exposed to ammonium nitrate, sodium nitrate and urea.

Test	Days	LC50[a] (95% CI)	LOAEL[a]	NOAEL[a]
R. aurora embryo				
Ammonium nitrate	16	71.9 (67.1 - 77.1)	13.2[bc]	6.4[bc]
Sodium nitrate	16	636.3 (595.4 - 680.0)	<29.1[b] 235.0[c]	<29.1[b] 116.8[c]
P. regilla tadpole				
Urea	10	8396 (7105 - 9921)	6000[bc]	2400[bc]
X. laevis tadpole				
Urea	10	9108 (8604 - 9642)	>6000[b] 6000[c]	>6000[b] 2400[c]

[a]Values for ammonium nitrate expressed as mg/L measured NH_4-N; values for sodium nitrate expressed as mg/L measured NO_3-N; values for urea expressed as mg/L nominal N. [b]Based on length. [c]Based on wet weight.

much as 42 mg/L NH_4-N (Edwards and Daniel 1994) and corn field runoff contained as much as 21 mg/L NH_4-N and 78 mg/L NO_3-N for over 30 d (McDowell and McGregor 1979). Tilewater drainage nitrate nitrogen from mixed crops in Minnesota ranged from 13 to 40 mg/L for over 5 yr (Randall *et al.* 1997). Hecnar (1995) believed the water quality guidelines of 10 mg/L NO_3-N were not protective of some amphibian species.

Increased levels of nitrogen in amphibian habitats could result from poor fertilizer handling practices. Liquid nitrogenous fertilizers composed of urea and/or ammonium nitrate and containing 20 -30% N can be applied directly to agricultural lands (Simpson 1986). A 20% solution (equivalent to 200,000 mg/L N) is extremely more concentrated than that shown to have deleterious effects on amphibians. Localized pooling or runoff for a sufficiently long period into areas used by spawning amphibians could cause adverse effects.

The ammonium ion appears to be the main contributor of toxicity of ammonium nitrate to amphibians. While urea appears to be relatively non-toxic to amphibians at normally used concentrations, its effects when combined with ammonium compounds remains to be tested.

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