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Effects of Ammonium Sulfate on Growth of Larval Northwestern Salamanders, Red-Legged and Pacific Treefrog Tadpoles, and Juvenile Fathead Minnows

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Ammonium-nitrogen fertilizers are used in large quantities in agricultural areas of the United States, including the grass-seed fields of the Willamette Valley of western Oregon, and are a potential threat to larval amphibians living in the treated areas (Edwards and Daniel 1994, McDowell and McGregor 1979, Bonn *et al*. 1996, Boyer and Grue 1995). Nitrogen fertilizer use over the past 50 years in the United States has increased from less than 0.45 million metric tons per year to more than 9.98 million metric tons per year (Lanyon 1996). Ammonia has been found to be toxic to fish, invertebrates and amphibians in acute tests, but little data are available from chronic tests, especially for larval amphibians (Thurston *et al*. cited in USEPA 1985, U.S. EPA 1986). Hecnar (1995) showed that four amphibian species were affected by ammonium nitrate at levels that are commonly exceeded in agricultural areas globally. Acute tests gave 96-hr LC50's ranging from 13.6 to 39.3 mg/L NO_3-N. In chronic tests chorus frog and leopard frog tadpoles had significantly lower survival at 10 mg/L NO_3-N. Schuytema and Nebeker (1999a) have shown that the toxicity of ammonium nitrate, ammonium sulfate and ammonium chloride in static-renewal tests is essentially the same for the Pacific treefrog, *Pseudacris regilla*, due to the ammonium ion. Where the use of ammonium compounds is widespread chronic effects could be important Schuytema and Nebeker (1999a, 1999b) have conducted 10-day static-renewal tests with embryos and tadpoles of *P. regilla* and have shown significant effects as low as 6.9 mg/L NH_4-N for embryos and 24.6 mg/L for tadpoles. The purpose of this study was to determine effects of ammonium sulfate in flow-through tests, a representative of several ammonium compounds used to add nitrogen to the soil, on growth of three native amphibian species and one introduced fish species. The four species are all residents of the Willamette Valley of western Oregon. The Northwestern salamander *Ambystoma gracile* and the Pacific treefrog *Pseudacris regilla* continue to thrive in lowland areas. Historically the red-legged frog *Rana aurora* was more common than it is today, and the fathead minnow is a relative newcomer to the valley.

MATERIALS AND METHODS

Eggs of the Northwestern salamander *Ambystoma gracile* (Baird), the Pacific treefrog *Pseudacris regilla* (Baird and Girard), and the red-legged frog *Rana aurora* Baird and Girard were collected locally from non-agricultural areas (Wilson wildlife refuge and foothill watersheds) and hatched in the EPA laboratory facility at Corvallis, OR. Fathead minnows, *Pimephales promelas* Rafinesque, were hatched and reared in a laboratory colony. All four species were subsequently reared in aquaria with a continuous flow of temperature-controlled fresh water ranging from 10 to 20° C. They were gradually raised from collection temperature, e.g. 10° C, to test temperature of 20 ±21° C. Temperature was continuously recorded. They were held at a photoperiod of 14: 10 1ight:dark. Newly hatched *A. gracile* were fed newly hatched brine shrimp, and then daphnids *(Daphnia pulex* and *D. magna)* and annelid worms *(Lumbriculus variegatus)* as they grew larger. Both *P. regilla* and *R. aurora* tadpoles were fed pelleted rabbit feed, with small amounts of newly hatched brine shrimp and frozen fish food. Fish were fed newly hatched brine shrimp and then frozen fish food.

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Table 1. Tests and water quality parameters conducted at 20° C with larvae of the Northwestern salamander, *Ambystoma gracile,* tadpoles of the frogs *Pseudacris [=Hyla] regilla* and *Rana aurora,* and juvenile fathead minnows, *Pimephales promelas.*

Test and[a] species	Age and life stage at test start	Total[b] hardness ($\bar{x} \pm$ SD) mg/L	Total alkalinity ($\bar{x} \pm$ SD) mg/L	Conductivity ($\bar{x} \pm$ SD) μS/cm	Median pH
1 *Ambystoma gracile*	5-wk-old larvae	34 ± 0	34 ± 0	104.4 ± 1.2	7.2
2 *Pseudacris regilla*	6-wk-old tadpoles	72 ± 4.2	63 ± 2.8	188.8 ± 7.3	7.3
3 *Pseudacris regilla*	9-wk-old tadpoles[c]	34 ± 0	34 ± 0	104.4 ± 1.2	7.2
4 *Rana aurora*	4-wk-old tadpoles	28.5 ± 7.8	30.0 ± 7.1	87.9 ± 17.8	7.2
5 *Pimephales promelas*	6-wk-old juveniles	72 ± 4.2	63 ± 2.8	188.8 ± 7.3	7.3
6 *Pimephales promelas*	9-wk-old juveniles	28.5 ± 7.8	30.0 ± 7.1	87.9 ± 17.8	7.2

[a] Test duration was 10 days for all tests; three replicate tanks/conc., 4 larvae, 5 tadpoles, and 5 fish/tank

[b] Water quality differences due to seasonal changes in well water. Dissolved oxygen near saturation (continuous-flow thru test chambers)

[c] From different eggs masses than 6-wk-olds

The test species were exposed to ammonium sulfate in 18 10-L aquaria with a continuously-flowing water diluter system (Nebeker *et al.* 1994, 1998) that automatically delivered six concentrations of chemical and controls to three aquaria per concentration, at a flow rate of 125 ml/min/aquarium. Rearing and test water was obtained from wells near the Willamette River at Corvallis, Oregon. Dissolved oxygen, measured by electrode, was maintained near saturation by the flowing water in the test chambers; pH was also measured by electrode. Hardness, alkalinity, and conductivity (Table 1) were measured by U.S. EPA methods 130.2, 310.1, and 120.1, respectively (US EPA 1979). Background well water NH_4-N ranged from 0.005-0.010 mg/L, and from 0.00 to 0.03 in control tanks. The *A. gracile* test (Table 2) used four 5-week old larvae in each of three replicate aquaria at test initiation and the test was run for 10 days. They were fed newly hatched brine shrimp *ad libitum* daily. Aquaria were siphoned clean daily. Two *P. regilla tests* were conducted (Table 3) using five animals per aquarium. The first test was with 6-week old tadpoles. The second test used tadpoles that were 9-weeks old and from different egg masses than the 6-week old tadpoles. They were fed pelleted rabbit food and aquaria were cleaned every other day. For the *R. aurora* 10-day test (Table 4) five 4-week old tadpoles were used to start the test. The tadpoles were fed pelleted rabbit food *ad libitum.* Test aquaria were cleaned every other day. Two juvenile fathead minnow tests were conducted (Table 5) using five animals in each of three replicate aquaria. The first test used 6-week old animals; the second test used 9-week old animals. They were fed newly-hatched brine shrimp *ad libitum* during testing.

Table 2. Effects of ammonium sulfate on survival and growth of larval Northwestern salamanders *(Ambystoma gracile)* (3 replicates/conc., 4 animals/replicate).

Mean $\pm$ SD measured ammonium-nitrogen concentration (mg/L)	Number alive at 10 days	Mean $\pm$ SD total length (mm)	Mean $\pm$ SD wet weight (mg)
211.5 $\pm$16	12	27.6 $\pm$1.2[a]	155.0 $\pm$23[b]
126.5 $\pm$5[c]	12	28.5 $\pm$3.3	154.7 $\pm$14[b]
81.5 $\pm$5[d]	12	29.4 $\pm$1.0	181.7 $\pm$13
52.5 $\pm$5	12	28.9 $\pm$0.3	180.3 $\pm$12
32.4 $\pm$3	12	29.1 $\pm$2.2	181.0 $\pm$29
0	12	30.3 $\pm$0.3	214.0 $\pm$13

[a] Length significantly less than controls ($p \leq 0.05$)
[b] Weight significantly less than controls ($p \leq 0.05$)
[c] LOAEL= lowest observed adverse effect level
[d] NOAEL= no observed adverse effect level

Ammonium sulfate used for testing was 99.2% pure (reagent grade; Mallinckrodt Baker, Inc., Phillipsburg, NJ). Stock ammonium sulfate test solutions pumped to the diluter test facility were prepared using well water. Water flow, test solution flow, temperature and diluter performance were checked several times daily. Water samples for measurement of ammonium sulfate were taken from each concentration (aquaria in the diluter) on each of four days during the first *P. regilla* and the first *P. promelas* test, and on two days during the test with the second *P. regilla, P. promelas, R.. aurora* and *A. gracile* tests. Samples were analyzed with a Hach DR/700 digital photometer. Samples from pooled replicates were analyzed within two hours after collection; approximately 10% were run in duplicate. The detection limit for ammonium-nitrogen from the ammonium sulfate (NH_4-N), using the Hach method 42.05.1 (Hach 1995) a nesslerization technique, was 0.01 mg/L. The mean percent difference between quality assurance split samples analyzed with both the Hach colorimeter and a Lachat Quikchem 8000 Flow Injection Analyzer was 1.8±0.01%.

Ten-day Lowest Observed Adverse Effect Level (LOAEL) values, the lowest concentration producing adverse effects (growth impairment) significantly different from the controls ($p \leq 0.05$), and No Observed Adverse Effect Level (NOAEL) values, the highest concentration producing no adverse effects significantly different from the controls, were determined with the Dunnett's multiple comparison procedure (Computer Science Corp. 1988).

RESULTS AND DISCUSSION

The growth of *Ambystoma gracile* larvae was impaired at and above 126.5 mg/L ammonium nitrogen (NH_4-N), with wet weight being more sensitive than total length (Table 2). The LOAEL was 126.5 mg/L NH_4-N based on wet weight. The NOAEL value was 81.5 mg/L NH_4-N based on wet weight (Table 6). This species is one of the few native amphibians of the Pacific Northwest to continue to survive in lowland lakes and sluggish streams that have well established populations of non-native fishes and bullfrogs (Leonard *et al.* 1993). It should not be affected by these fertilizers as it is not as sensitive to ammonium nitrogen in fertilizers as the treefrog, which appears to thrive in agricultural areas of western Oregon. Watt and Oldham (1995) exposed newt larvae *(Triturus vulgaris)* to ammonium nitrate and

Table 3. Effects of ammonium sulfate on survival and growth of Pacific treefrog tadpoles *(Pseudacris regilla)* (3 replicates/conc., 5 animals/replicate).

Mean $\pm$ SD measured ammonium-nitrogen concentration (mg/L)	Number alive at 10 days	Mean $\pm$ SD total length (mm)	Mean $\pm$ SD wet weight (mg)
Six-wk-old tadpoles			
211.2 ± 17.5	13	16.7 ± 2.5^{a}	85.3 ± 44^{b}
118.0 ± 9.8	15	17.9 ± 1.1^{a}	89.7 ± 7^{b}
66.6 ± 7.7	15	22.6 ± 2.2^{a}	170.3 ± 39^{b}
37.0 ± 4.6^{c}	15	22.9 ± 1.5^{a}	171.3 ± 25^{b}
17.4 ± 3.8^{d}	15	25.9 ± 0.6	239.7 ± 17
0	13	28.4 ± 2.8	306.7 ± 59
Nine-wk-old tadpoles			
211.5 ± 16.3	15	26.1 ± 1.9^{a}	301.7 ± 73^{b}
126.5 ± 4.9	14	28.5 ± 1.4^{a}	381.7 ± 48^{b}
81.5 ± 7.8	15	31.0 ± 2.1^{a}	488.0 ± 115^{b}
52.5 ± 4.9^{c}	15	29.2 ± 2.2^{a}	456.7 ± 62^{b}
32.4 ± 3.3^{d}	14	32.5 ± 1.2	585.7 ± 53
0	15	35.0 ± 0.9	724.7 ± 48

[a] Length significantly less than controls ($p \leq 0.05$)
[b] Weight significantly less than controls ($p \leq 0.05$)
[c] LOAEL= Lowest observed adverse effect level
[d] NOAEL= No observed adverse effect level

found effects on growth at 200 mg/L ammonium nitrate (35 mg/L NH_4-N).

Pseudacris regilla tadpoles were most sensitive of the four species tested, with 6-wk-old tadpoles being more sensitive that 9-wk-old tadpoles (Table 3). The 6-wk-old animals had LOAEL and NOAEL values of 37.0 and 17.4 mg/L NH_4-N, respectively, based on total length and wet weight (Table 6). The 9-wk-old tadpoles had LOAEL and NOAEL values of 52.5 and 32.4 mg/L NH_4N, respectively, again based on total length and wet weight (Table 6). The older animals may have had higher LOAEL values because the softer water (Table 1) may have affected the toxicity of the ammonium sulfate.This species is very abundant in Willamette valley wetlands on and near agricultural areas and does not appear to be affected by fertilizer practices in the valley. Wigington (personal comm.) showed that NH_4-N concentrations in a small stream receiving runoff from grass seed fields in the Willamette Valley, fertilized with 220 kg N/ha, were usually < 1.0 mg/L NH_4-N. The results of these flow-through tests are similar to results from static-renewal tests with treefrog tadpoles *(P. regilla)* conducted by Schuytema and Nebeker (1999b), where tadpoles had 10-day LOAEL and NOAEL values of 49.0 and 24.6 mg/L NH_4-N based on length, and 24.6 and 12.3 mg/L

Table 4. Effects of ammonium sulfate on survival and growth of red-legged frog tadpoles *(Rana aurora)* (3 replicates/conc., 5 animals/replicate).

Mean $\pm$ SD measured ammonium-nitrogen concentration (mg/L)	Number alive at 10 days	Mean $\pm$ SD total length (mm)	Mean $\pm$ SD wet weight (mg/L)
227.0 $\pm$ 1.4	14	29.9 $\pm$ 4.9[a]	318.7 $\pm$ 176[b]
134.0 $\pm$ 7.1[c]	15	32.6 $\pm$ 1.4[a]	396.0 $\pm$ 80[b]
82.7 $\pm$ 3.2[d]	15	36.4 $\pm$ 3.9	554.0 $\pm$ 168
50.5 $\pm$ 4.9	15	38.4 $\pm$ 0	635.3 $\pm$ 33
28.8 $\pm$ 5.2	15	41.3 $\pm$ 1.5	814.0 $\pm$ 134[e]
0	15	40.3 $\pm$ 0.6	747.0 $\pm$ 87[e]

[a] Length significantly less than controls ($p \leq 0.05$)
[b] Weight significantly less that controls ($p \leq 0.05$)
[c] LOAEL= lowest observed adverse effect level
[d] NOAEL= no observed adverse effect level
[e] Hind legs visible

based on weight. *P. regilla* embryos were more sensitive with 10-day LOAEL and NOAEL values of 23.1 and 11.7 mg/L NH_4-N based on length; values of 11.7 and 6.1 mg/L NH_4-N were lower based on weight (Schuytema and Nebeker 1999a). Hecnar (1995) determined 96-hr LC50 values for *Pseudacris triseriata, Rana pipiens, R. clamitans,* and *Bufo americanus* ranging from 13.6-39.3 mg/L NO_3-N (calculated NH_4-N of 13.5-38.9 mg/L), similar to LOAEL values in the present study.

The tadpoles of *Rana aurora* were intermediate in their sensitivity compared to the other three species tested, with effects on growth occurring at and above 134 mg/L NH_4-N, the same concentrations that caused growth impairment in the salamander *A. gracile* (Table 4). The 10-day LOAEL and NOAEL values based on total length and wet weight were 134.0 and 82.7 mg/L NH_4-N (Table 6). Longer tests would probably give lower NOAEL values, especially if they included metamorphosis. The widespread use of fertilizers in the Willamette Valley grassfields where this species used to be common was thought to be one possible cause of its decline, but the fact that it is no more sensitive than other valley species such as *the* common salamander *A. gracile* or the abundant treefrog *P. regilla* indicates that other factors are likely more important in its decline, such as bullfrog introduction and competition (Olson *et al.* 1997).

Pimephales promelas (9-wk-old animals) was the most tolerant of the four species tested, although the 6-wk-old animals had the same sensitivity as the salamanders and red-legged frogs (Table 5).The 6-wk-old juveniles had LOAEL and NOAEL values of 118.0 and 66.6 mg/L NH_4-N based on length and weight (Table 6). The 9-wk-old animals had LOAEL and NOAEL values of 227.0 and 134.0 mg/L NH_4-N based on length and weight. The older animals may have had higher LOAEL values because the softer water (Table 1) may have affected the toxicity of the ammonium sulfate. This species is not native to Oregon but is found in a few localities the Willamette valley. Swigert and Spacie (cited in U.S. EPA 1985) conducted an early life stage test with fathead minnows and showed significant reductions in growth at $\geq$0.33 mg/L unionized NH, (~9 mg/L NH_4-N). Thurston *et al.* (cited in U.S. EPA 1985) found significant effects on growth at 30 days of fathead minnows in full life

Table 5. Effects of ammonium sulfate on survival and growth of juvenile fathead minnows *(Pimephales promelas)* (3 replicates/conc., 5 animals/replicate).

Mean ± SD measured ammonium-nitrogen concentration (mg/L)	Number alive at 10 days	Mean ± SD total length (mm)	Mean ± SD wet weight (mg/L)
Six-wk-old fish			
211.2 ± 17.5	6	13.1 ± 0.1[a]	18.7 ± 5.3[b]
118.0 ± 9.8[c]	14	12.7 ± 0.4[a]	21.7 ± 3.0[b]
66.6 ± 7.7[d]	13	13.6 ± 0.8	23.3 ± 2.9
37.0 ± 4.6	15	13.3 ± 0.2	23.7 ± 3.5
17.4 ± 3.8	15	14.1 ± 1.1	32.0 ± 9.6
0	15	14.3 ± 0.2	34.3 ± 3.1
Nine-wk-old fish			
227.0 ± 1.4[c]	14	14.3 ± 1.1[a]	27.0 ± 10.1[b]
134.0 ± 7.1[d]	15	16.7 ± 1.4	48.7 ± 15.8
82.7 ± 3.2	15	16.1 ± 0.9	41.7 ± 9.1
56.5 ± 4.9	15	17.5 ± 1.3	52.0 ± 12.5
28.8 ± 5.2	15	17.1 ± 1.2	51.0 ± 13.9
0	15	17.7 ± 0.3	55.7 ± 5.1

[a] Length significantly less than controls ($p \leq 0.05$)
[b] Weight significantly less that controls ($p \leq 0.05$)
[c] LOAEL= lowest observed adverse effect level
[d] NOAEL= no observed adverse effect level

cycle tests at 0.9 mg/L unionized NH, (~18 mg/L NH_4-N), values lower than those found in this study but logical considering that early life stage tests and 30-day tests will usually give lower values than juvenile growth tests (Nebeker *et al.* 1974).

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Table 6. LOAEL and NOAEL values (mg/L ammonium-nitrogen) for *Ambystoma gracile, Pseudacris regilla, Rana aurora* and *Pimephales promelas* (10-day exposures).

Test species (age and life stage)	LOAEL[a]	NOAEL[b]
Ambystoma gracile (5-wk-old larvae)	126.5	81.5
Pseudacris regilla (6-wk-old tadpoles)	37.0	17.4
Pseudacris regilla (9-wk-old tadpoles)	52.5	32.4
Rana aurora (4-wk-old tadpoles)	134.0	82.7
Pimephales promelas (6-wk-old juveniles)	118.0	66.6
Pimephales promelas (9-wk-old juveniles)	227.0	134.0

[a] LOAEL = lowest observed adverse effect level = the lowest concentration producing adverse effects on growth significantly different from controls ($p \leq 0.05$)
[b] NOAEL = no observed adverse effect level = the highest concentration producing no significant adverse effects on growth compared to controls

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