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Comparative Toxicity of Ammonium and Perchlorate to Amphibians

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Perchlorate (ClO_4^{-}) is a competitive inhibitor of iodide uptake and can impair thyroid function and hormone production. In humans perchlorate is preferentially selected over iodide by the iodide symporter in thyroidal cells (Greer et al. 2002), thereby inhibiting the production of thyroxines. Recently, perchlorate has merited attention because it has been found in drinking water in many parts of the United States, especially in the western half of the country where it can pose a risk to human health (Von Burg 1995; Urbansky and Schock 1999). Perchlorate in surface waters, however, can also have serious effects on wildlife. Amphibians in particular rely on proper functioning of the thyroid for metamorphosis. (Rosenkilde 1985) and laboratory and field studies on perchlorate in water have shown significant delays in metamorphosis or alterations of thyroid physiology and histology (Miranda et al. 1992; Carr et al. 2003; Sparling et al. 2003).

Environmental perchlorate comes from both anthropogenic and several natural sources. Ammonium perchlorate is used as an oxidizer in solid-fuel rocket propellents by the military and in fireworks and matches (Von Burg 1995). Perchlorate also occurs in some natural fertilizers (Susarla et al.1999a; Susarla et al. 1999b; Urbansky et al. 2000). Because of the link between perchlorate and rocket fuels, however, many toxicity tests have used ammonium perchlorate (Wolff 1998; Thuett et al. 2002; Carr et al. 2003; Goleman et al. 2003; Patiño et al. 2003). Ammonium (NH_4^{+}) comes from bacterial decomposition of nitrogenous wastes, industrial sources, fertilized fields, and livestock operations. Background concentrations of the compound in soil range from 1 to 5 ppm. Ammonium is highly toxic to amphibians (Hecnar 1995; Oldham et al. 1997; Jofre and Karasov 1999) and test results on perchlorate may be influenced by this compound. Urbansky and Schock (1999) state that dissociation of perchlorate from its salts such as ammonium is virtually complete upon mixture with water. The purpose of this study is to distinguish the acute toxic effects of aqueous ammonium from that of perchlorate using the northern leopard frog (*Rana pipiens*).

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MATERIALS AND METHODS

Egg masses of *Rana pipiens* were obtained from the U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Duluth, MN and were maintained in 80 L aquaria until hatching, three days after receipt. Larvae were maintained at 21° C until they reached the free-swimming stage (Gosner 25, Gosner 1960). They were then transferred to 400 ml glass jars containing 300 ml of reconstituted soft water (ASTM 1988). Four tadpoles were placed in each jar and jars were randomly assigned to treatment. Each treatment was replicated three times for NH_4-perchlorate and twice for NH_4^+-bicarbonate.

We used NH_4-perchlorate (NH_4ClO_4, CAS 7790-98-9, 99.9% pure, Sigma Aldrich) for one set of treatments. NH_4-perchlorate is composed of 15.33% ammonium and 84.67% perchlorate by mass so concentrations were set at 0 (controls), 2.7, 7.4, 20.1, 54.7, 149, and 400 mg/L NH_4-perchlorate which translated to the following perchlorate (ammonium) equivalents: 0 (0), 2.29 (0.41), 6.3 (1.13), 17.0 (3.08), 46.3 (8.4), and 338.7 (61.3) mg/L. To determine toxicity due to ammonium alone we used NH_4-bicarbonate (NH_4HCO_3, CAS 1066-33-7, 99.9% pure, Sigma Aldrich) and set the ammonium concentrations equal to those of the NH_4-perchlorate jars with the exception that 0.41 mg/L ammonium was not used in the NH_4-bicarbonate trial due to insufficient number of tadpoles. It was not our intent to determine the toxicity of other perchlorate salts such as potassium or sodium perchlorate so we do not have data from other perchlorate compounds for comparison. Stock solutions were made of both chemicals and the appropriate volume of solution was added to respective jars. At the start of the study dissolved oxygen was 8.42 mg/L and pH was 6.8. Air was not pumped into the jars but dissolved oxygen at the end of the study ranged from 6.85 to 7.41 mg/L. The study was a static exposure with no change of water over the test period. Jars were loosely capped to decrease evaporation and volatization of ammonium. Ammonium and perchlorate levels were checked with ion-specific probes and found to be within 10% of nominal concentrations. At the end of seven days perchlorate concentrations did not show any appreciable decrease but there was approximately a 15% decrease in ammonium concentrations.

Tadpoles were observed twice daily over 7 d for mortality, morbidity, or signs of lethargy and abnormal behavior. After the first 24 hr each jar was sprinkled with ca. 0.1 g of crushed rabbit pellets for food and food was added whenever needed. At the end of the 7 d period surviving tadpoles were euthanized with MS-222 and measured for snout vent length with electronic calipers to 0.1 mm.

Probit analysis (SAS® Proc Probit, SAS 1990) was used to examine dose-response relationships in mortality for NH_4-bicarbonate, NH_4-perchlorate and ammonium equivalents, defined as the concentration of ammonium in NH_4-

perchlorate treatments. Snout vent lengths were not normally distributed so their log10 values were used in analyses. Repeated measures ANOVA with jars serving as experimental units was applied to snout-vent lengths to determine if statistically significant ($\alpha=0.05$) differences occurred among treatments.

RESULTS AND DISCUSSION

The number of tadpoles dying by chemical and concentration after 96 hr and 7 d are reported in Table 1. At 96 hr of exposure mortality was observed at 8.4 mg/L and 61.3 mg/L ammonium in NH_4-bicarbonate but only at 61.3 mg/L ammonium NH_4-perchlorate (400 mg/L Nh4-perchlorate). By 7 d substantial mortality had occurred at concentrations $\geq$ 8.4 mg/L ammonium in NH_4-bicarbonate. Similarly, mortality did not occur in the NH_4-perchlorate treatments until 8.4 mg/L ammonium (54.7 mg/L perchlorate).

Because of inconsistencies in mortality rates from one concentration to another, complete dose response curves with corresponding 95% confidence intervals were only available for the analysis of the 7 day NH_4-bicarbonate treatment. However, estimated response curve statistics and LC50 values could be calculated for both chemicals at 96 hr and 7 days.

Table 1. Number of tadpole *Rana pipiens* deaths occurring by 96 hr at 7 days of exposure to NH_4-bicarbonate and NH_4-perchlorate. Deaths in each time period are the accumulated number of deaths to that time. NH_4-bicarbonate concentrations are mg/L ammonium; NH_4-perchlorate are mg/L total molecule; rows represent equivalent ammonium concentrations of both chemicals.

NH_4-Bicarbonate				NH_4-Perchlorate			
Concen	N	96 hrs	7 days	Concen	N	96 hrs	7 days
0	12	0	0	0	12	0	0
				2.7	11	0	0
1.13	8	0	0	7.4	12	0	0
3.08	8	0	0	20.1	12	0	0
8.4	8	4	4	54.7	11	0	1
22.8	8	0	3	149	12	0	1
61.3	8	6	8	400	12	8	12

The response curves had the following descriptions:

1) NH_4-bicarbonate

a) 96 hr: Probability of mortality (PM) = -2.250 + 1.434 $\log_{10}$Con, SE of slope = 1.008, P value for slope=0.155, estimated LC_{50} = 37.1 mg/L

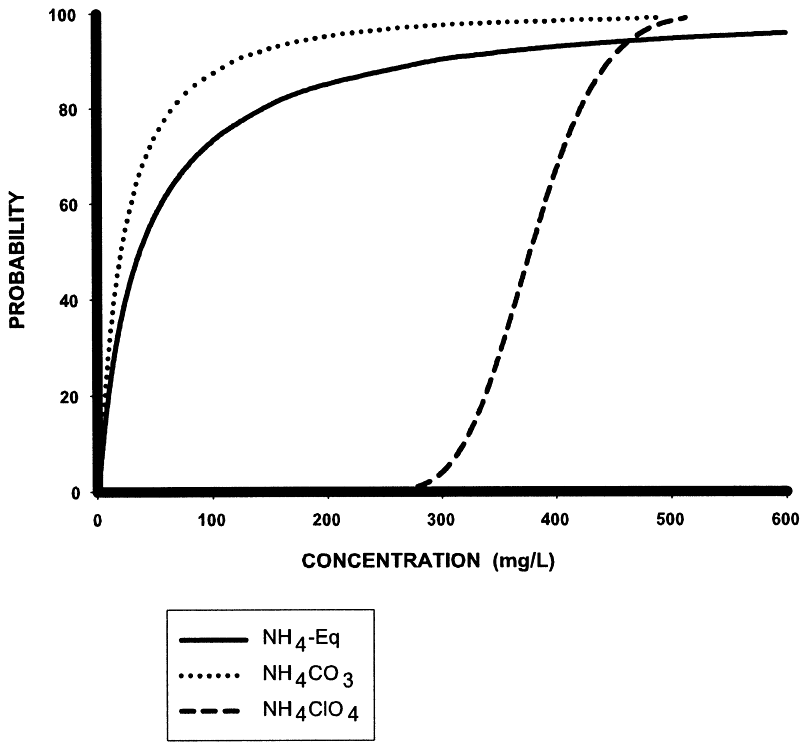

Figure 1. Dose response curves for mortality in *Rana pipiens* larvae exposed to ammonium bicarbonate, ammonium perchlorate, and as ammonium equivalents in ammonium perchlorate.

ammonium.
b) 7 days: PM = -2.823 + 2.3648 $\log_{10}$ Con, SE on slope = 0.671, 95% CI on slope = 1.049 to 43.679 mg/L, P value for slope = 0.0004, estimated LC_{50} = 15.6 mg/L ammonium, 95% CI = 8.8-30.4 mg/L.

2) NH_4-perchlorate

a) 96 hr: PM = -45.105 + 17.500 $\log_{10}$ Con, P value for slope > 0.500, estimated LC_{50} = 378 mg/L NH_4-perchlorate.
7 days: PM = -4.295 + 1.875 $\log_{10}$ Con, P value for slope = 0.124, 95% CI on slope = 0.51- 4.26 mg/L, estimated LC_{50} = 195 mg/L NH_4-perchlorate.

3) If the NH_4-perchlorate treatment is based on ammonium concentrations only then

a) 96 hr: PM = -30.802 + 17.473 $\log_{10}$ Con, P value for slope > 0.50,

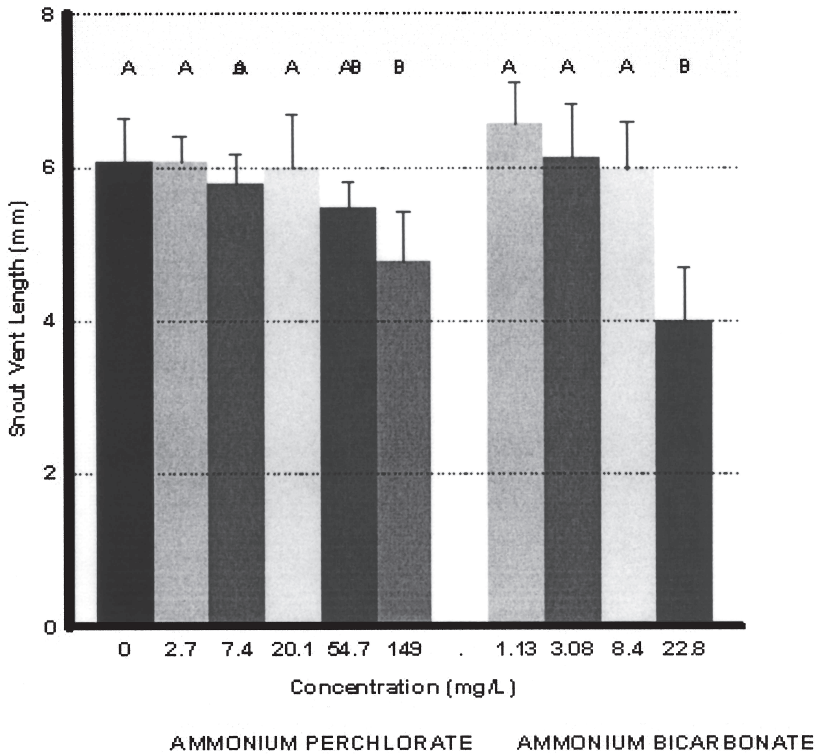

Figure 2. Snout vent lengths for *Rana pipiens* larvae exposed to ammonium perchlorate and ammonium bicarbonate for 7 days. Bars with the same letters within a chemical cannot be distinguished at p = 0.05.

estimated LC_{50} = 57.9 mg/L ammonium.

b) 7 days: PM = -2.767 + 1.875 $\log_{10}$ Con, P value for slope = 0.125, estimated LC_{50} = 29.9 mg/L ammonium.

Where Con=Concentration (mg/L).

These LC50 values for ammonium and perchlorate are consistent with other studies. For example, 10-d LC50s for Pacific treefrogs (*Pseudacris regilla*) with NH_4-nitrate were 25 - 32.4 mg/L ammonium (Schuytema and Nebeker 1999). Perchlorate when presented with less toxic anions (e.g. Na, K) is not particularly toxic. Although metamorphosis was significantly delayed in *Hyla versicolor* at 50 mg/L perchlorate as potassium perchlorate, survival over 120 days exceeded 90% (Sparling et al. 2003). In green frogs (*Rana clamitans*) the calculated 96 hr LC50 for sodium perchlorate was 5500 mg/L (Dean et al. 2004). For zebrafish (*Danio* *rerio*) it was 1401 mg/L (Liu et al. 2005). Environmental concentrations below 9.3 mg/L were considered safe for aquatic organisms (Dean et al. 2004).

It appears that lethal mortality of NH_4-perchlorate is entirely due to ammonium and not to perchlorate (Fig.1). Although there is no significant difference in dose-response characteristics between toxicity due to ammonium in NH_4-bicarbonate and that due to NH_4-perchlorate, perchlorate may slightly reduce toxicity of ammonium.

During the 7 days of the study we also observed two sublethal effects. One effect was an apparent loss of coordination and orientation in tadpoles exposed to either chemical. During day 2 tadpoles exposed to 61.3 mg/L ammonium in NH_4-perchlorate (61.3 mg/L) swam in tight circles when probed with a glass rod. Approximately 7 hr later similar behavior was observed among tadpoles exposed to 61.3 mg/L ammonium in NH_4-bicarbonate and 22.8 mg/L ammonium in NH_4-perchlorate (149 mg/L). By Day 3 surviving tadpoles at 61.3 mg/L ammonium in NH_4-perchlorate (400 mg/L) displayed lordosis or dorsal/ventral flexion of the spine. At this time two tadpoles at 22.8 mg/L ammonium in NH_4-bicarbonate showed uncoordinated swimming. This uncoordinated behavior was confined to ammonium concentrations $\geq$ 22.8 mg/L (NH_4-perchlorate $\geq$ 149 mg/L). The lack of coordination, malformations and poorer swimming ability may be related to an effect of perchlorate on muscle (Ma et al. 1993; Wolff 1998; Huang 1998). However, NH_4-nitrate can also cause deformities at relatively low concentrations (Jofre and Karasov 1999)

The other sublethal effect was reduced growth among tadpoles exposed to either chemical (Fig. 2). For NH_4-bicarbonate, snout vent lengths differed among concentrations (F=6.15; df=4,6; p=0.257) with tadpoles at 22.8 mg/L ammonium being smaller than at any other concentration. For NH_4-perchlorate, snout vent lengths were also different among concentrations (F=11.31; df=5,12; p=0.0003). Tadpoles at 22.8 mg/L ammonium in NH_4-perchlorate (149 mg/L) were shorter than at other concentrations except 8.4 mg/L ammonium (54.7 mg/L). When concentrations were based on ammonium equivalents and NH_4-bicarbonate and NH_4-perchlorate were compared, significant main and interaction effects were noted. Snout vent lengths differed significantly among concentrations (F=25.67; df=3,12; p< 0.0001) and in the interaction between concentration and chemical (F=4.40; df=3,12; p=0.0262) but not between chemicals. The severity of the decrease in snout vent lengths as concentrations increased was less for larvae exposed to NH_4-perchlorate than to NH_4-bicarbonate. In a study where the maximum concentration of potassium perchlorate was 50 mg/L, no adverse effects were observed on growth (Sparling et al. 2003). NH_4-nitrate can cause reduced growth in amphibians at low concentrations (Hecnar 1995; Jofre and Karasov 1999)

In summary, this study and other published reports show that perchlorate ion has a low lethal toxicity to amphibians. Concentrations necessary to produce mortality in those amphibians tested are far above environmentally realistic values. Any evidence of acute or chronic toxicity must be evaluated in terms of the associated cation. Ammonium, which is highly toxic by itself, can account for all of the toxicity seen in this study. However, environmental perchlorate should not be considered safe to amphibians. Concentrations in the low mg/L range have been repeatedly shown to have negative effects on thyroid histology, thyroxine production and metamorphosis. Further work on thyroid disruption in amphibians and possible synergistic effects with other ions is warranted.

REFERENCES

ASTM (1988). Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians. ASTM, West Conshohocken PA.

Carr JA, Urquidi LJ, Goleman WL, Hu F, Smith PN, Theodorakis CW (2003) Ammonium perchlorate disruption of thyroid function in natural amphibian populations: Assessment and potential impact. In: Linder G, Krest S, Sparling DW, Little EE (ed) Multiple stressor effects in relation to declining amphibian populations. ASTM, West Conshohocken, PA, p 130-142.

Dean KE, Palachek RM, Noel JM, Warbritton R, Aufderheide J, Wireman J (2004) Development of freshwater water-quality criteria for perchlorate. Environ Toxicol Chem 23:1441-1451.

Goleman WL, Urquidi LJ, Anderson TA, Smith EE, Kendall RJ, Carr JA (2003) Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis*. Environ Toxicol Chem 21:424-430.

Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183-190.

Greer MA, Goodman G, Pleus RC, Greer SE (2002) Health effects assessment for environmental perchlorate contamination: The dose response for inhibition of thyroidal radioiodine uptake in humans. Environ Health Perspect 110:927-937.

Hecnar SJ (1995) Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from southern Ontario. Environ Toxicol Chem 14:2131-2138.

Huang CLH (1998) The influence of perchlorate ions on complex charging transients in amphibian striated muscle. J Physiol 506:699-714.

Jofre MB, Karasov WH (1999) Direct effects of ammonia on three species of North American anuran amphibians. Environ Toxicol Chem 18:1806-1812.

Liu F, Kendall RJ, Theodorakis CW (2005) Joint toxicity of sodium arsenate and sodium perchlorate to zebrafish *Danio rerio* larvae. Environ Toxicol Chem 24:1505-1507.

Ma J, Anderson K, Shirokov R, Levis R, Gonzalez A, Karhanek M, Hosey MM, Meissner G, Rios E (1993) Effects of perchlorate on the molecules of excitation-contraction coupling of skeletal and cardiac muscle. J Gen Physiol 102:423-448.

Miranda LA, Pisano A, Paz D (1992) Effect of potassium perchlorate on thyroid activity of *Bufo arenarum* larvae. Comm Biol 10:125-135.
Oldham RS, Latham DM, Hilton-Brown D, Towns M, Cooke AS, Burn A (1997) The effect of ammonium nitrate fertiliser on frog (*Rana temporaria*) survival. Ag Ecosys Environ 61:69-74.
Patiño R, Wainscott MR, Cruz-Li EI, Balakrishnan S, McMurry C, Blazer VS, Anderson TA (2003) Effects of ammonium perchlorate on the reproductive performance and thyroid follicle histology of zebrafish. Environ Toxicol Chem 22:115-121.
Rosenkilde P (1985) The role of hormones in the regulation of amphibian metamorphosis. In: Balls M, Bounes M (ed) Metamorphosis. 8th Symposium British Society Developmental Biology. Clarendon Press, Oxford, p 222-259.
Schuytema GS, Nebeker AV (1999) Comparative toxicity of ammonium and nitrate compounds to Pacific treefrog and African clawed frog tadpoles. Environ Toxicol Chem 18:2251-2257.
SAS (1990) SAS/STAT User's Guide, Vol. 1, Ver. 6, 4th Edition. SAS Institute, Cary, NC.
Sparling DW, Harvey G,.Nzengung VA (2003) Interaction between perchlorate and iodine in the metamorphosis of *Hyla versicolor* . In: Linder G, Krest S, Sparling DW, Little EE (ed) Multiple stressor effects in relation to declining amphibian populations. ASTM, West Conshohocken, PA, p 143-158.
Susarla S, Bacchus ST, Wolfe NL, McCutcheon SC (1999a) Phytotransformation of perchlorate using parrot-feather. Soil Ground Cleanup February/March:20-23.
Susarla S, Collette TW, Garrison AW, Wolfe NL, McCutcheon SC (1999b) Perchlorate identification in fertilizers. Environ Sci Technol 33:3469-3472.
Thuett KA, Roots EH, Mitchell LP, Gentles BA, Anderson T, Kendall R, Smith EE (2002) Effects of in utero and lactational ammonium perchlorate exposure on thyroid gland histology and thyroid and sex hormones in developing deer mice (*Peromyscus maniculatus*) through postnatal day 21. J Toxicol Environ Health 65:2119-2130.
Urbansky ET, Magnuson ML, Kelty CA, Brown SK (2000) Perchlorate uptake by salt cedar (*Tamarix ramosissima*) in the Las Vegas Wash riparian ecosystem. Sci Total Environ 256:227-232.
Urbansky ET, Schock MR (1999) Issues in managing the risks associated with perchlorate in drinking water. J Environ Manage 56:79-95.
Von Burg R (1995) Perchlorates. J Appl Toxicol 15:237-241.
Wolff J (1998) Perchlorate and the thyroid gland. Am Soc Pharmacol Exper Therap 50:89-105.