

Review: Toxicity of Glyphosate-based Pesticides to Four North American Frog Species.

PC Code 080803

October 7, 2005

Citation: Howe, C. M, M. Berrill, B. D. Pauli, C. C. Helbing, K. Werry and N. Veldhoen. 2004. *Toxicity of Glyphosate-based Pesticides to Four North American Frog Species*. Society of Environmental Toxicology and Chemistry 23(8): 1928 - 1938.

Reviewers:

Primary Review:

Thomas Steeger, Ph.D., Senior Biologist
Environmental Risk Branch IV, Environmental Fate and Effect Division
U. S. Environmental Protection Agency

Secondary Review:

Rachel K. Evans, Intern
Environmental Risk Branch IV, Environmental Fate and Effects Division

Executive Summary

This paper reports on a series of studies conducted over roughly a seven-year period examining the acute and chronic effects of glyphosate alone, the surfactant polyethoxylated tallowamine (POEA) and glyphosate-based products on four aquatic-phase amphibian (anuran) species. Acute toxicity tests indicate that glyphosate alone was less toxic than POEA or glyphosate formulations containing POEA. Younger amphibian larvae (Gosner stage 20) were less sensitive in terms of acute lethality than older (Gosner stage 25) larvae.

In a chronic study, aquatic phase leopard frog larvae were exposed to glyphosate alone, POEA alone or Roundup® Original for 42 days where treatment tanks were spiked once a week with 0.6 or 1.8 mg glyphosate free acid equivalents per liter (FAE/L) under static renewal conditions. Partial (50%) water changes were conducted every 96 hours. Following the 42-day exposure period, larvae were transferred to clean pond water and observed until they reached Gosner stage 42 (complete metamorphosis to a juvenile) or until 166 days. At completion of metamorphosis, animals were sacrificed, weighed, measured and their gonads taken for histological examination.

Acute toxicity data indicate that POEA alone and glyphosate formulations containing POEA are moderately toxic to aquatic phase amphibians (96-hr LC_{50} range: 2.0 - 8.9 FAE/L); however, the median lethal concentration for glyphosate alone exceeded the highest concentration tested (>17.9 mg FAE/L). Younger (Gosner Stage 20) larvae were less sensitive to glyphosate formulations containing POEA than older (Gosner Stage 25) larvae. At stage 25 though, *R. clamitans* was the most sensitive (96-hr LC_{50} =6.5 mg/L or 2.0 mg FAE/L) and *R. sylvatica* was the most tolerant (96-hr LC_{50} = 16.5 mg/L or 5.1 mg FAE/L).

Treatment with glyphosate alone did not reveal any effects on growth (length), tail morphology, percent surviving to reach Stage 42, time to metamorphosis, gonadal development or thyroid hormone gene expression relative to controls; however, treatment with POEA alone or glyphosate formulations containing POEA reduced the percentage of larvae surviving to reach Stage 42, decreased length at metamorphosis, increased time to metamorphosis, and resulted in mixed-sex gonads. Tail morphology (damage) was significantly higher among larvae treated with POEA and glyphosate formulations containing POEA at 1.8 mg FAE/L. Results for the thyroid hormone mRNA expression were highly variable; however, at Gosner



Review: Toxicity of Glyphosate-based Pesticides to Four North American Frog Species.

PC Code 080803

October 7, 2005

stage 25, larvae treated with Roundup® Original at 1.8 mg FAE/L or with Roundup® Transorb at 0.6 and 1.8 mg FAE/L exhibited significantly higher mRNA expression than controls.

Mortality was relatively high in chronic study control groups (38%) and suggests that environmental conditions were not ideal for promoting survival of leopard frogs. This may have been due to relatively high loading rates coupled with partial water changes every 96 hours. Although effects on metamorphosis and gonadal development appear to be treatment related, poor environmental conditions could affect development. Additionally, although concentrations are expressed in acid equivalents, all of the data are presented in terms of nominal concentrations.

In spite of the uncertainties regarding the extent to which water quality may have affected the study results, the data provide evidence that the surfactant POEA and formulations of glyphosate containing POEA are toxic to amphibian larvae on both an acute and chronic exposure basis. Given the effects on metamorphosis, gonadal morphology and thyroid hormone gene expression, there is some indication that POEA and glyphosate formulations containing POEA can impact endocrine-mediated processes. The relatively high mortality observed in controls though limits the utility of this study in ecological risk assessment.

Objective: To assess the acute and chronic toxicity of several glyphosate-based formulations and determine if certain formulations pose a lower risk to growth and development of North American amphibians.

Compare the acute toxicity to and developmental effects on amphibians of the active ingredient glyphosate, the polyethoxylated tallow amine (POEA; MON0818) surfactant, and six glyphosate-based formulations (Roundup® Original, Roundup® Bioactive, Touchdown®, Glyfos® Bio, Glyfos® AU and Roundup® Transorb) examining differences in species and developmental stage sensitivity.

Compare chronic effects of various formulations on development of the Northern leopard frog (*Rana pipiens*) by examining changes in the rates of growth and development.

Disruption of thyroid axis measured by examining length and rate of development and thyroid hormone receptor β gene expression.

Assessed gonadal differentiation at metamorphic climax.

Methods: Two egg broods for each of four species: green frogs (*Rana clamitans*), wood frogs (*R. sylvatica*), leopard frogs (*R. pipiens*) and American toads (*Bufo americanus*) collected within 24 hours of being laid from ponds within the Otonabee River watershed. Tadpoles reared in sand-filter, aerated water from Otonabee River water.

Animals fed cooked lettuce or spinach *ad libitum*. Food replaced daily and uneaten material removed.

Review: Toxicity of Glyphosate-based Pesticides to Four North American Frog Species.

PC Code 080803

October 7, 2005

Environmental Conditions

Light/dark cycle: 12/12 hrs. Temperature selected to simulate pond temperature:

R. sylvatica, *B. americanus* and *R. pipiens* $15 \pm 1^\circ\text{C}$

R. clamitans $20 \pm 1^\circ\text{C}$

Water chemistry data apparently based on historical rather than measured values; however, the methods section implies that actual measurements were recorded during the study even though no data are reported.
pH 7.8 (measured 7.8 - 8.3)

Total ammonia 2.4 mg/L

DOC 11.6 mg/L

metals <0.03 mg/L

no contamination with organic pollutants

Test Materials

Technical grade glyphosate (Monsanto isopropylamine salt)

POEA (Monsanto MON 0818; 69 - 73%)

Roundup® Original (Monsanto isopropylamine salt of glyphosate + 15% POEA), Vision® (Monsanto isopropylamine salt of glyphosate + 15% POEA)

Roundup® Transorb (Monsanto isopropylamine salt of glyphosate + surfactant blend containing 15% POEA)

Roundup® Biactive (Monsanto isopropylamine salt of glyphosate + 10- 20% of an unspecified surfactant)

Glyfos® AU and Glyfos® BIO (Cheminova isopropylamine salt of glyphosate + 3 - 7% POEA)

Touchdown® 480 (Syngenta trimethylsulfonium salt of glyphosate + 10 - 20% unspecified surfactant)

Limit of quantitation 0.05 mg/L; 88% procedural recovery for glyphosate and its primary degradate aminomethyl phosphonic acid (AMPA).

Acute Toxicity Study

In 2001, *R. clamitans* (20) at Gosner stage 25 placed in 1-L glass beaker using each of the test materials at four concentration levels up to a maximum of 18 mg formulation glyphosate acid equivalents (FAE)/L with three replicates per concentration for determination of 96-hr LC_{50} under static exposure conditions.

In 1994, 10 tadpoles of *B. americanus*, *R. clamitans*, *R. pipiens* and *R. sylvatica* at two life stages (Gosner stages 20 and 25) exposed to Roundup® Original at 12.9, 19.3 and 25.8 mg/L (4, 6, and 8 mg FAE/L) with three replicates per treatment concentration. Tadpoles were exposed to formulated glyphosate in a 1-L glass beaker half-filled with river water for 96-hours.

Chronic Toxicity Study

In 2000, 20 *R. pipiens* at Gosner stage 25 maintained in a 5-gallon aquarium (approximately 1 tadpole/350 mL of water) with filtered river water until Gosner stage 42 (metamorphic climax). Aquaria were treated with each of the formulated product at 0.6 and 1.8 mg FAE/L (believed to represent average and maximum environmental exposure level) once a week in a static renewal system for a total of 42 days of exposure followed by rearing in clean water. Every 96 hours, half of the water in the exposure tank was changed. Rate of development (number of days to reach Gosner stage 42 was recorded. Controls exhibited a 38% mortality rate which according to the author was not unusual for "long-term chronic experiments with these animals."

Review: Toxicity of Glyphosate-based Pesticides to Four North American Frog Species.

PC Code 080803

October 7, 2005

The study was terminated after 80% or more of the surviving tadpoles reached Gosner stage 42. The experiment was terminated after 166 days. After metamorphosis or at 166 days, animals were euthanized with MS-222, and snout-to-vent length and total length recorded. Animals were fixed for histological assessment.

Twenty-eight days after termination of exposure (42 days of exposure + 28 days=70 days into the study) evaluated using digital imaging for total length, body length, tail length and maximum tail height. Visible damage to the tail was also assessed.

Transverse step section (7 μ m section) of preserved Stage 42 frog gonads used to determine sex. The diameter of 10 randomly selected oocytes measured in gonads of all animals categorized as intersex along with five randomly selected female gonads from the control group.

Natural sex ratios of wild populations of ranid amphibians determined in 1993, 1994, 1996 and 1999 by observing natural frog migrations along a section of road. Secondary sex characteristics (thumb pads on males) used to determine phenotypic sex of spring- and fall-migrating *R. pipiens* and *R. clamitans*.

Gene Expression Analysis. Five "slowly developing" tadpoles that remained at Gosner stage 25 randomly selected from each treatment on day 70 of the experiment along with five tadpoles from each treatment that had reached Gosner stage 42 used for mRNA expression analysis using tadpole tail tips. Steady state levels of ribosomal proteins L8 and TR β mRNA were analyzed.

Results

Acute Toxicity

Median lethal concentrations (LC_{50}) for glyphosate-based formulations are summarized in **Table 2**. For Touchdown[®], Glyphos[®] BIO, Roundup[®] Biactive and glyphosate technical, the LC_{50} exceeded the maximum concentration tested, *i.e.*, $LC_{50} > 17.9$ mg FAE/L. POEA was the most toxic compound tested ($LC_{50}=1.1$ mg/L. For Roundup[®] Original [containing POEA] toxicity values range from LC_{50} of 6.6 to 18.1 mg/L (LC_{50} range: 2.0 - 5.6 mg FAE/L). For Roundup[®] Original (containing POEA), Gosner stage 25 was more sensitive than Gosner stage 20 across all four species of amphibians tested

After 24 hours, the LC_{50} value exceeded the highest concentration tested for stage 20 tadpoles of any of the species exposed to technical grade glyphosate or Roundup[®] Original. At stage 25 though, *R. clamitans* was the most sensitive (96-hr $LC_{50}=6.5$ mg/L or 2.0 mg FAE/L) and *R. sylvatica* was the most tolerant (96-hr $LC_{50}= 16.5$ mg/L or 5.1 mg FAE/L).

According to the study authors, at concentrations approaching the LC_{50} values for POEA, Roundup[®] Original, Roundup[®] Transorb and Glyphos[®] AU, tail damage occurred in 52 to 71% of surviving *R. pipiens* tadpoles within the first 24 hr of exposure (no data presented).

Chronic Toxicity

Average time to metamorphosis was 120 ± 36 days. According to the study authors, the rate at which tadpoles reached metamorphic climax (**Figure 1a**) and the percentage of surviving larvae to reach Gosner stage 42 were significantly reduced (**Figure 1b**) following exposure to POEA, Roundup[®] Original and Roundup[®] Transorb. Tadpoles exposed to POEA, Roundup[®] Original and Roundup[®] Transorb were

Review: Toxicity of Glyphosate-based Pesticides to Four North American Frog Species.

PC Code 080803

October 7, 2005

significantly smaller compared to animals exposed to glyphosate technical or the control animals (**Figure 1c**).

Exposure to POEA, Roundup® Original and Roundup® Transorb resulted in an increased frequency of tail damage; at higher concentrations, 94% of the animal tails were damaged.. Damage occurred through necrosis of the tail tip and in some cases was characterized by flexure of the tail tip, fin damage and abnormal growths at the tail tip; tail damage persisted post-exposure. Tail damage was not observed in tadpoles exposed to glyphosate alone. While tail length was significantly affected, tail height and body length were not significantly different from controls.

Gonadal Effects

Abnormal gonads were observed in metamorphs (juveniles) after exposures to both concentrations of POEA, Roundup® Original and Roundup® Transorb. Gonads were categorized as Intersex when maturing primary oocytes were surrounded by somatic and/or medullary tissue or when ovarian tissue was observed at the posterior end of the gonad and testicular tissue at the anterior end of the gonad. Intersex also included enlarged germinal epithelium with a proliferation of oogonia and atretic oocytes observed in juveniles exposed to Roundup® Original and POEA (**Figure 3**). Glyphosate alone had no effect on gonadal development. According to the study authors, although intersex was observed after exposure to POEA, Roundup® Original and Roundup® Transorb, none of the sex ratios from any of the treatment groups was significantly different from controls.

Gene Expression

Expression of thyroid hormone receptor β was roughly 10-fold higher in Stage 42 animals than Stage 25 animals. In stage 25 animals, larvae treated with Roundup® Original (1.8 mg/L) and with Roundup® Transorb (0.6 and 1.8 mg/L) had Tr β levels statistically higher than controls.

Reviewer Comments:

This article reports on a series of studies that were conducted between 1994 and 2001. Frogs eggs used in the study were collected from local ponds and the history of exposure to pesticides is unknown. Additionally, most of the research was conducted using local river water. Although the researchers claim that no organic pollutants have been detected in the river, no data are provided to substantiate this claim.

Loading rate (10 tadpoles/500 mL) in the acute toxicity study seems relatively high. Loading rate for tadpoles in the chronic study (1 tadpole/350 mL) is also relatively high and 96-hr partial water changes may not have provided sufficient water quality conditions to support optimum development and may account for the number of larvae not completing metamorphosis (Gosner Stage 42) by 166 days. This uncertainty is amplified by the relatively high mortality (38%) observed in controls. Although delayed time to metamorphosis appeared to be treatment related, there are insufficient data reported to determine whether water quality may also have been a factor.

It is not clear how the repeat applications of 0.6 and 1.8 mg/L are representative of environmental exposure conditions. Although concentrations are expressed in acid equivalents, all of the data are presented in terms of nominal concentrations.

Review: Toxicity of Glyphosate-based Pesticides to Four North American Frog Species.

PC Code 080803

October 7, 2005

Although sex ratios from field observations of adult phenotypic sex were used to establish background (control) ratios and the current study bases its estimates of sex ratios on juvenile frog gonadal histology, the data indicate that treatment with glyphosate alone did not appear to affect gonadal development relative to controls. However, chronic exposure to POEA alone or glyphosate formulations containing POEA resulted in gonadal developmental effects in the form of intersex (mixed sex). No data were provided to estimate the actual number of frogs exhibiting intersex gonads; however, for POEA alone and Roundup® Original, there appears to be a treatment-related increase in the incidence of intersex. The highest incidence of intersex occurred with Roundup® Transorb at 0.6 mg FAE/L; however, at 1.8 mg FAE/L of Roundup® Transorb, no intersex animals were observed, but the ratio of male to female was 25:75. The lack of statistical significance may be due to high variability in sex ratios from the field observations. At face value though, the data suggest that treatment with POEA and glyphosate formulations containing POEA had a feminizing effect on leopard frogs.

The data indicate that glyphosate alone is practically nontoxic to the amphibian larvae tested on both an acute and chronic exposure basis; however, when the POEA surfactant alone or in combination with glyphosate is toxic to amphibians on both an acute and chronic exposure basis. Gosner stage 20 larvae were less sensitive to acute exposure treatments than larvae at Gosner stage 25. With 96-hr LC_{50} values ranging between 2 and 8 mg FAE/L, glyphosate formulations containing POEA would be classified moderately toxic to aquatic phase amphibians on an acute exposure basis. Acute toxicity testing with glyphosate alone however failed to establish an LC_{50} ($LC_{50} > 17.9$ mg FAE/L). POEA and glyphosate formulations containing POEA appear to affect gonadal development, decreased size at metamorphosis and increased time to metamorphosis. POEA and glyphosate formulations containing POEA exhibited increased incidence of mixed sex. These data suggest that the surfactant and formulations containing the surfactant may impact endocrine-mediated processes.

Thyroid hormone mRNA expression was highly variable; statistically significant differences were only observed for Gosner stage 25 larvae treated with Roundup® Original at 1.8 mg FAE/L and both concentrations of Roundup® Transorb. However, no statistically significant effect was observed in POEA-treated larvae. It is possible that the high variability associated

The information contained in this article represents a compendium of several studies conducted over roughly seven years. The nonguideline studies provide useful information for gauging the relative toxicity of glyphosate, POEA and formulations of glyphosate containing POEA and/or other surfactants; however, the effects related to chronic exposure may be affected by water quality even though the controls do not suggest such an effect. The relatively high mortality reported for control animals in the chronic toxicity study limits the utility of this study in ecological risk assessment.

October 7, 2005

Table 2. Acute toxicity (median lethal concentration values [LC50] with 95% confidence intervals in parentheses) obtained in 24-h and 96-h exposures of four amphibian species exposed to glyphosate-based herbicides, glyphosate technical material, and polyethoxylated tallowamine surfactant (POEA) at two life stages^a

Species	Gosner stage	Compound	LC50			
			24 h		96 h	
			mg/L	mg FAE/L	mg/L	mg FAE/L
<i>Rana pipiens</i> ^b	25	Roundup Original [®]	11.9 (11.2–12.6)	3.7 (3.5–3.9)	9.2 (NR)	2.9 (NR)
<i>R. pipiens</i> ^c	20	Roundup Original	>25.8	>8	20.9 (19.8–21.9)	6.5 (6.1–6.8)
<i>R. sylvatica</i> ^c	25	Roundup Original	18.1 (16.7–19.6)	5.6 (5.2–6.1)	16.5 (15.7–17.4)	5.1 (4.9–5.4)
<i>R. sylvatica</i> ^c	20	Roundup Original	>25.8	>8	>25.8	>8
<i>Bufo americanus</i> ^c	25	Roundup Original	13.5 (NR)	4.2 (NR)	<12.9	<4
<i>B. americanus</i> ^c	20	Roundup Original	>25.8	>8	25.8 (NR)	8 (NR)
<i>R. clamitans</i> ^d	25	Roundup Original	6.6 (6.1–7.1)	2.0 (1.9–2.2)	6.5 (6.0–7.0)	2.0 (1.9–2.2)
<i>R. clamitans</i> ^c	20	Roundup Original	>25.8	>8	22.8 (21.2–24.5)	7.1 (6.6–7.6)
<i>R. clamitans</i> ^d	25	Glyphosate technical	>38.9	>17.9	>38.9	>17.9
<i>R. clamitans</i> ^d	25	POEA	1.1 (1.1–1.2)	2.4 (2.2–2.5)	1.1 (1.0–1.1)	2.2 (2.1–2.4)
<i>R. clamitans</i> ^d	25	Roundup Biactive [®]	>57.7	>17.9	>57.7	>17.9
<i>R. clamitans</i> ^d	25	Touchdown [®]	>57.7	>17.9	>57.7	>17.9
<i>R. clamitans</i> ^d	25	Glyfos BIO [®]	>57.7	>17.9	>57.7	>17.9
<i>R. clamitans</i> ^d	25	Glyfos AU [®]	29.1 (28.1–30.2)	9.0 (8.7–9.4)	28.6 (27.6–29.6)	8.9 (8.6–9.2)
<i>R. clamitans</i> ^d	25	Roundup Transorb [®]	7.4 (6.9–7.9)	2.3 (2.2–2.4)	7.2 (6.8–7.7)	2.2 (2.1–2.4)

^a Roundup Original, Roundup Biactive, and Roundup Transorb from Monsanto (St. Louis, MO, USA); Touchdown from Syngenta (Wilmington, DE, USA); Glyfos BIO and Glyfos AU from Cheminova (Wayne, NJ, USA). FAE = formulation glyphosate acid equivalents; NR = 95% confidence intervals not reliable.

^b 2000 Chronic study.

^c 1994 Study.

^d 2001 Study.

(Source: Howe, C. M., M. Berrill, B. D. Pauli, C. C. Helbing, K. Werry and N. Veldhoen. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environmental Toxicology and Chemistry 23(8): 1928–1938.)

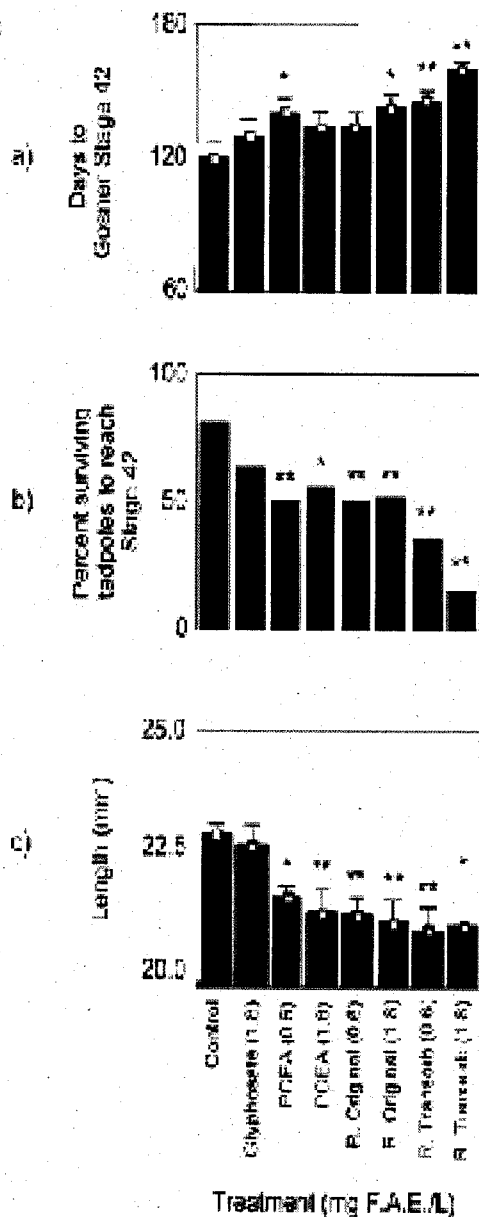


Fig. 1. Metamorphosis data for four species tadpoles exposed to glyphosate technical, the polyethoxylated tallowamine surfactant (POEA), Roundup Original® (R. Original; Monsanto, St. Louis, MO, USA) and Roundup Transorb® (R. Transorb; Monsanto). a. Days to metamorphic climax. b. Percentage of surviving tadpoles to reach metamorphic climax. c. Snout-vent length (mm). Compound concentrations in mg formulation acid equivalents (FAE; see text) follow compound names. Bar height represents the mean, and error bars represent the standard error. * $p < 0.05$, ** $p < 0.01$ compared to control.

Source: Howe, C. M., M. Berrill, B. D. Pauli, C. C. Helbing, K. Werry and N. Veldhoen. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. *Environmental Toxicology and Chemistry* 23(8): 1928 - 1938.

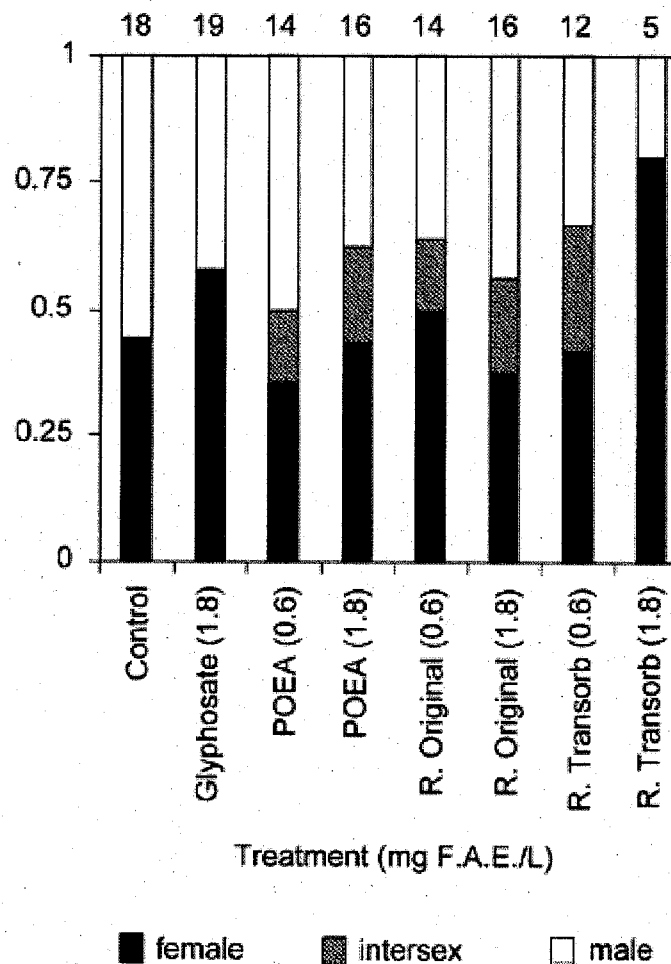


Fig. 3. Sex ratio of control and treated metamorphs based on observations of gonadal histology at metamorphic climax (Gosner stage 42). Tadpoles were exposed to glyphosate technical, the polyethoxylated tallowamine surfactant (POEA), Roundup Original® (R. Original; Monsanto, St. Louis, MO, USA), and Roundup Transorb® (R. Transorb; Monsanto). Compound concentrations in mg formulation acid equivalents (FAE; see text) follow compound names. Numbers above bars = n .

Source: Howe, C. M., M. Berrill, B. D. Pauli, C. C. Helbing, K. Werry, and N. Veldhoen. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. *Environmental Toxicology and Chemistry* 23(8): 1928 - 1938.

REQUEST FOR 6(a)(2) SCREEN

DATE: 10/5/2005

Week of 9/30/2005

SRRD/S. Howard-Miller for R.
McNally

FROM: Kathleen O'Malley
Information Services Branch/IRSD

TO: ___ George Ghali (HED/**Tox, **Onco, Residue, Product Chem)

___ Nancy McCarroll (HED/Genotoxicity)

___ HED Product Chemistry (Chemistry Science Advisory Council)

___ Kathleen Raffaele (HED/Developmental Neurotoxicity)

___ Jerry Blondell (Human Health/HED)

___ Kit Farwell (Animal Safety/HED)

___ John Redden (RD/Products - Acute Tox)

___ Bill Schneider (BPPD)

___ Hardip Singh/Cara Dzubow (EFED)

___XX___ ShaRon Carlisle (AD)

___ Mark Perry (SRRD/Products - Acute Tox)

___ Pesticide Resistance Team (Sharlene Matten/BPPD, Phil Hutton/BPPD,
Kathy Davis/FEAD)

Attached are copies of transmittal letters and/or study conclusions of recently received 6(a)(2) data submissions. Please screen this information and provide me with the results by hand/mail/fax delivery of this screen cover sheet with your comments to me in Room 216 CM2 (7502C), Fax: 305-7670.

IDENTIFICATION NUMBER: Screen Summary of Study MRID #46650501

PRODUCT NUMBER: 524-445

CHEMICAL NAME: Glyphosate, isopropylamine salts, Roundup

DESCRIPTION: Toxicity of Glyphosate Pesticides to 4 North American Frog Species
(Journal Article)

Briefly describe the adverse effect:

Does this submission warrant an expedited review? ___Yes ___No

If yes, explain:

Expedited review is appropriate if the results change our regulatory position on this chemical or product.
Examples are:

Acute Tox - label change or change in tox category
If end point is more sensitive than current basis for regulatory position

Antimicrobial Product Efficacy Failure - label change: reduced claims or dosage increased

158.134 Flagging Statement is positive

If expedited, recommended timeframe for review:

Other Comments:

Please respond by: **ASAP**

Signature

Date