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TOXICITY OF GLYPHOSATE AND TRICLOPYR USING THE FROG EMBRYO TERATOGENESIS ASSAY—*XENOPUS*

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Abstract—The effects of glyphosate ([N-phosphonomethyl]glycine) and triclopyr ([[3,5,6-trichloro-2-pyridinyl]oxy]acetic acid) on the embryonic development of *Xenopus laevis* were evaluated using Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX). Rodeo®, the isopropylamine (ipa) salt of glyphosate formulated without a surfactant was found to be the least toxic, with a LC5 and LC50 of 3,779 and 5,407 mg acid equivalent (AE)/L, respectively. The LC5 and LC50 of Roundup®, the ipa salt of glyphosate formulated with a surfactant, was 6.4 and 9.4 mg AE/L, respectively. The surfactant component of Roundup, polyoxyethyleneamine (POEA), had a LC5 and LC50 of 2.2 and 2.7 mg/L, respectively. Garlon® 3A, the triethylamine salt of triclopyr, had a LC5 and LC50 of 119 and 162.5 mg AE/L, respectively. The LC5 and LC50 of Garlon 4®, the butoxyethyl ester of triclopyr, was 6.7 and 9.3 mg AE/L, respectively. Considering a theoretical worst case scenario when the highest rates recommended for glyphosate (12 L of Roundup/ha) or triclopyr (8 L of Garlon/ha) are applied to water 15 cm in depth, the expected environmental concentrations calculated on the basis of AE would be 2.8 and 2.6 mg AE/L, respectively. The margins of safety (LC5/expected environmental concentrations) for frog embryos exposed to these concentrations would be approximately 2, 2, 47, and 1,312 for Roundup, Garlon 4, Garlon 3A, and Rodeo, respectively.

Keywords—Roundup Rodeo Garlon 4 Garlon 3A Frog Embryo Teratogenesis Assay—*Xenopus*

INTRODUCTION

Certain glyphosate and triclopyr formulations are currently registered in some countries to control aquatic weeds. The isopropylamine (ipa) salt of glyphosate formulated with a surfactant (Roundup®, Monsanto Canada, Winnipeg, MB, Canada) and triclopyr formulated as the butoxy-ethyl ester (Garlon 4®, Dow Agro Sciences Canada, Calgary, AB, Canada) are currently used to control weeds in dry-land situations throughout Canada and in many other countries. However, it is the ipa salt of glyphosate formulated without a surfactant (Rodeo®, Monsanto) and triclopyr formulated as the triethylamine salt (Garlon® 3A, Dow Agro Sciences) that are used to control purple loosestrife (*Lythrum salicaria* L.) and other weeds in wetland or aquatic situations in the United States [1].

If herbicides are to be intentionally applied to an aquatic or wetland habit to control aquatic weeds, it is essential to understand the possible effects on nontarget aquatic organisms in those habitats. Laboratory bioassays can be an important part of the risk assessment procedure for chemicals on nontarget organisms. The purpose of this investigation was to examine the effects of triclopyr and glyphosate on the amphibian *Xenopus laevis*. More specifically, the toxicity of two glyphosate formulations and two triclopyr formulations (mentioned above) were examined and compared using the Frog Embryo Teratogenesis Assay—*Xenopus*, commonly known as FETAX.

MATERIALS AND METHODS

Frog Embryo Teratogenesis Assay—*Xenopus* is a 96-h static renewal, whole embryo assay for identifying teratogenic and developmental toxicants. A developmental toxicant is defined as a substance that results in embryo mortality, malformation, or growth inhibition at concentrations far less than those required to affect adult organisms [2]. Frog Embryo Teratogenesis Assay—*Xenopus* was originally developed as an indicator of human developmental hazards, but it is also extremely useful in aquatic toxicology risk assessments [2,3].

Chemicals

Two technical formulations of glyphosate were considered in this study, Roundup and Rodeo. Roundup was formulated with a guarantee of 356 g glyphosate acid equivalent(AE) per liter present as the ipa salt. Fifteen percent by volume of the Roundup formulation consisted of the active surfactant polyoxyethyleneamine [4,5]. The Rodeo formulation contained glyphosate as the ipa salt at a concentration of 480 g AE/L water. It did not contain the surfactant polyoxyethyleneamine (POEA). These two formulations of glyphosate were compared on an AE basis.

Two technical formulations of triclopyr were examined in this study, Garlon 4 and Garlon 3A, both with the active ingredient triclopyr (3,5,6-trichloro-2-pyridl-oxyacetic acid). In Garlon 4, triclopyr was formulated as a butoxyethyl ester at a concentration of 480 g AE/L, whereas in Garlon 3A, triclopyr was present as a triethylamine salt at a concentrations of 360 g AE/L. These two formulations of triclopyr were compared on an AE basis.

Fetax solution

Fetax solution was utilized throughout FETAX in both the breeding tank and in the chemical solutions exposed to the *X. laevis* embryos. It was composed of 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$, and 75 mg $MgSO_4$/L distilled water. The pH of the final solution was 7.6 to 7.9.

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Animal care

Xenopus laevis embryos were obtained from a breeding colony at Integrated Explorations, Guelph, Ontario, Canada. The adult *X. laevis* were maintained in an environmentally controlled room at 15°C with a light:dark cycle of 16:8 h. They were housed in glass culture aquaria in dechlorinated tap water. The adult frogs were fed a diet of beef liver.

Breeding Xenopus laevis

One male and one female *X. laevis* were removed from the culture aquariums and placed into fetax solution in a smaller tank used for breeding [2]. The breeding pair were kept at a constant temperature of 24°C, and breeding was initiated by an injection of human chorionic gonadatrophin (Sigma-Aldrich Canada, Oakville, ON, Canada) into the dorsal lymph sac. Both the male and female received two injections 8 h apart, with a total injection of 1,000 IU in a total volume of 1 ml. Eggs were laid and fertilization occurred approximately 10 to 12 h after the final injection. The embryos were removed from the tank within 1 h after laying and dejelled in a fresh 2% w/v L-cysteine solution, adjusting the pH to 8.1 with 1 N NaOH. The embryos were dejelled by pouring the L-cysteine solution over the embryos and swirling the mixture until the jelly coat was removed. The embryos were swirled in the L-cysteine solution for no longer than 3 to 4 min to avoid irreversible damage of the embryos by the L-cysteine solution.

Embryo selection

At the blastula stage, normal embryos were selected for the experiment. The blastula is an embryo composed of a hollow ball of cells just approaching primary organogenesis [3]. Embryos placed under a dissecting microscope were chosen on the basis of cell division, color, size, and stage of development according to established criteria for healthy embryos [6]. In fact, a double selection method was employed. Normally, cleaving embryos were first sorted into dishes containing fresh fetax solution. After a short time, the embryos were sorted again, ensuring that only normally cleaving embryos were selected.

Experimental design

Xenopus laevis embryos were continuously exposed to chemical solutions for the 96-h period of primary organogenesis. During this period, the untreated *X. laevis* embryos developed from the blastula stage of a few hundred cells to a free-swimming larva. Initially, a dose–response experiment was performed to determine the range of concentrations appropriate for each chemical. For each concentration, two 60- $\times$ 15-mm plastic petri dishes were used, each containing 25 embryos and 10 ml of chemical/fetax solution. Four dishes were set up for the negative control containing only fetax solution. Two dishes of 6-aminonicotanamide (2,500 mg/L) (Sigma-Aldrich) with 25 embryos were also set up as a toxic standard or positive control [2]. Data were considered only from those tests that had a low control mortality ($<$2%) and 40 to 60% mortality in the positive control. The petri dishes were then placed in a growth cabinet with a constant temperature of 24°C and continuous light.

In our FETAX procedure, a renewal system was employed. Every 24 h, the chemical solution was removed from the petri dish with a large-mouthed 10-ml pipette to avoid damaging the developing embryos and replaced with fresh solution. Dead embryos were counted and removed daily. Embryos were considered dead if no heart beat was evident.

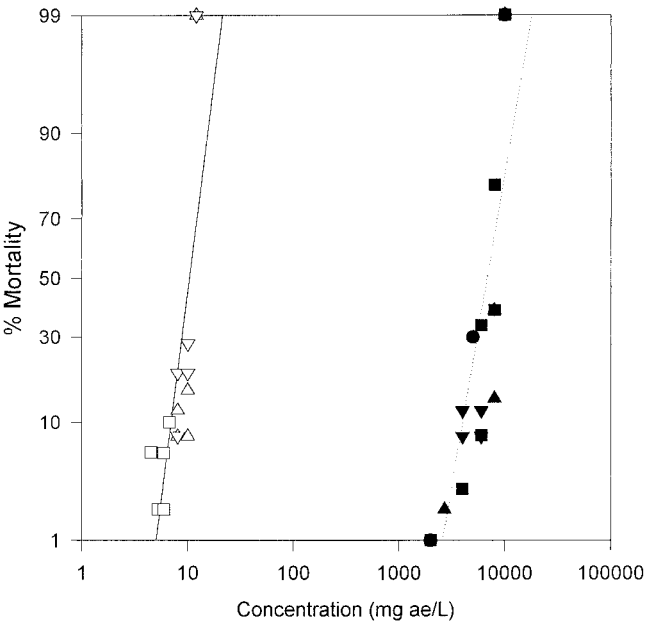

Fig. 1. Comparative toxicity of two formulations of glyphosate, Roundup® and Rodeo®, to *Xenopus laevis* using Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX). Results from different experiments are indicated by differently shaped data points. Each data point is the percent mortality for individual dishes containing 25 embryos. The regression equations in log probit scale are $y = 2.81x + 1.23$ and $y = 3.74x - 9.92$ for Roundup and Rodeo, respectively. Ninety-five percent confidence limits are included. To facilitate plotting of this data in Sigma Plot, data points at 99 and 1 represent 100 and 0% mortality, respectively.

At 96 h, the assay was terminated. Embryo mortality was used to define the 96-h LC50, LC10, and LC5 for each formulation. Total number of dead was recorded and the remaining embryos were preserved in 10% formalin for further microscopic analysis. The length of each embryo was measured and malformations recorded.

Data analysis

Probit analysis was used to generate regression lines in Figures 1, 2, and 3 using Sigma Plot software (SPSS, Chicago, IL, USA). The LC5, LC10, and LC50 values, 95% confidence limits, and the slopes of the probit lines were determined using the U.S. Environmental Protection Agency Probit Analysis Program used for calculating LC/EC values (Version 1.5). Data for growth inhibition of the embryos were subjected to analysis of variance. Corrected mortality and malformation rates were calculated by adjusting for negative control mortality and malformation rates using Abbot's formula [7].

RESULTS

A comparison of LC50 concentrations indicated that the Roundup formulation of glyphosate was 700 times as toxic as the Rodeo formulation (Table 1). However, this difference in toxicity appeared to be due to the toxic effect of the surfactant POEA alone. Even though limited tests were conducted, in each case, POEA was more toxic (LC50 of 6.8 mg/L) than the Roundup formulation (LC50 of 9.3 mg AE/L) (Table 1, Fig. 2). Based on their respective LC50 concentrations, triclopyr formulated as a butoxyethyl ester was 15 times as toxic

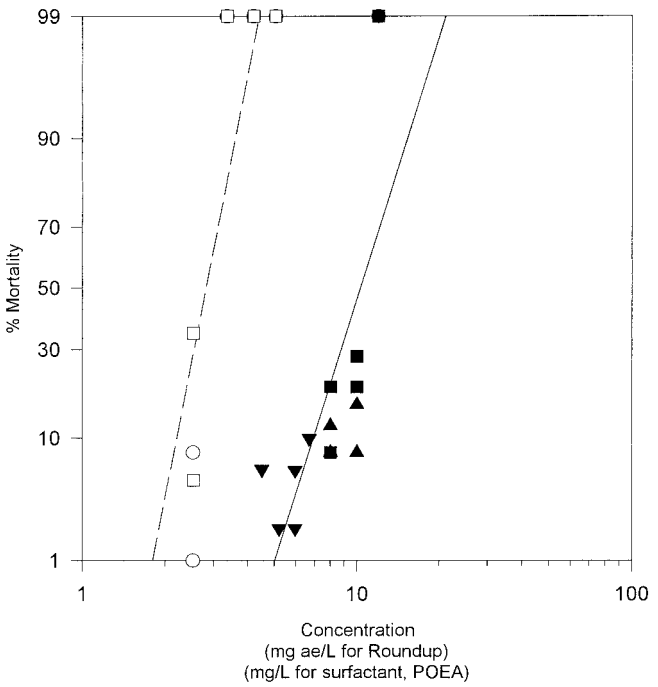

Fig. 2. Comparative toxicity of Roundup® and its surfactant polyoxyethyleneamine (POEA) to *Xenopus laevis* using Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX). Results from different experiments are indicated by differently shaped data points. Each data point is the percent mortality for individual dishes containing 25 embryos. The regression equation for Roundup is $y = 2.8x + 1.2$. Ninety-five percent confidence limits are included. Not enough data were available to determine the regression equation for POEA. To facilitate plotting of this data in Sigma Plot, data points at 99 and 1 represent 100 and 0% mortality, respectively.

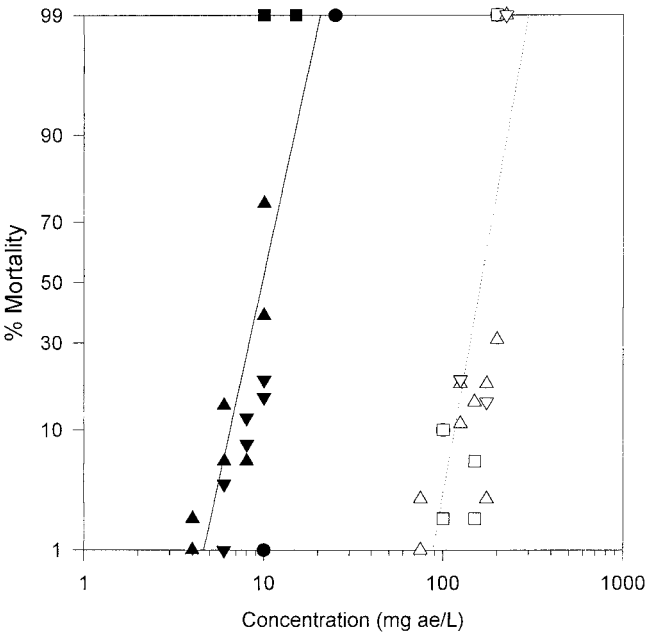

Fig. 3. Comparative toxicity of two formulations of triclopyr, Garlon 4® and Garlon® 3A, to *Xenopus laevis* using Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX). Results from different experiments are indicated by differently shaped data points. Each data point is the percent mortality for individual dishes containing 25 embryos. The regression equations in log probit scale are $y = 4.3x + 0.19$ and $y = 1.7x + 0.024$ for Garlon 4 and Garlon 3A, respectively. Ninety-five percent confidence limits are included. To facilitate plotting of this data in Sigma Plot, data points at 99 and 1 represent 100 and 0% mortality, respectively.

as triclopyr formulated as a triethylamine salt (Table 1, Fig. 3) when compared on an AE basis.

Frog Embryo Teratogenesis Assay—*Xenopus* is a highly sensitive assay for determining the teratogenicity of chemicals. The transparent nature of *X. laevis* embryos allows for examination of internal as well as external development at least up to the 4-d tadpole stage, when this study was terminated. The internal examinations of the *X. laevis* embryos were conducted as described by Bantle et al. [6]. The incidences of malformations such as uncoiling of the gut, edema, blistering, abnormal pigmentation, and axial twisting in control embryos were very low (<5%). Significant increases (analysis of variance, $p \leq 0.05$) in the incidence of malformations were not observed at any concentration of the glyphosate, triclopyr, or surfactant treatments in this study that were not also lethal to the embryos at 96 h.

Measurements of embryo length at 96 h indicated that overall embryo growth was a less sensitive indicator of glyphosate

Table 1. Comparative toxicity of two triclopyr formulations, two glyphosate formulations, and the surfactant to *Xenopus laevis* embryos using FETAX[a]

Treatments		Required volume for 100 mg AE/L (ml/L)	Lethal concentration values (mg AE/L)[b]			Slope of probit line
Formulation	Description		LC5	LC10	LC50	
Garlon 4®	Butoxyethyl ester of triclopyr	0.21	8.0 (7.3–8.4)	8.4 (7.9–8.8)	10.0 (9.8–10.3)	16.9
Garlon® 3A	Triethylamine salt of triclopyr	0.28	119.0 (109.4–126.9)	126.9 (117.8–134.3)	159.0 (152.2–165.2)	13.1
Rodeo®	Isopropylamine salt of glyphosate	0.21	5,515.5 (5,086.5–5,842.5)	5,867.2 (5,481.2–6,164.6)	7,296.8 (7,047.8–7,541.6)	13.5
Roundup®	Isopropylamine salt of glyphosate plus surfactant	0.28	7.7 (7.2–8.0)	8.0 (7.6–8.3)	9.3 (9.1–9.6)	18.9
POEA[c]	Surfactant, MONO 0818	0.10	5.8 (5.5–6.0)	6.0 (5.8–6.2)	6.8 (6.6–6.9)	23.8

[a] FETAX = Frog Embryo Teratogenesis Assay—*Xenopus*.
[b] Lethal concentrations were calculated using probit analysis with 95% confidence limits stated. Values for the glyphosate and triclopyr formulations are expressed on an acid equivalent (AE) basis in mg/L.
[c] Polyoxyethyleneamine.

Table 2. Influence of glyphosate and triclopyr formulations on the 96-h growth of *Xenopus laevis* embryos

Treatment[a]	Concentration (mg AE/L)[b]	Mean length (mm)[c] ± SD
Control	0	7.36 ± 0.33
Glyphosate formulations		
Roundup®	5	7.12 ± 0.26
	6	7.44 ± 0.27
	8	7.0 ± 0.24
	10	6.72 ± 0.24*
POEA (surfactant)	6	7.0 ± 0.3
Rodeo®	2,000	7.16 ± 0.28
	4,000	7.16 ± 0.26
	6,000	6.64 ± 0.32*
	8,000	4.6 ± 0.31*
Triclopyr formulations		
Garlon 4®	2.5	7.32 ± 0.29
	6	6.92 ± 0.26*
	8	6.6 ± 0.35*
	10	5.84 ± 0.28*
Garlon® 3A	50	7.56 ± 0.25
	100	7.12 ± 0.30
	125	7.24 ± 0.24
	175	6.2 ± 0.38*
	200	5.68 ± 0.24*

[a] Roundup® contains POEA (a surfactant); Rodeo® does not.
[b] AE = acid equivalent.
[c] Means followed by * are significantly less than the control (analysis of variance $p \leq 0.05$).

or triclopyr toxicity than embryo mortality. Only Garlon 4 reduced embryo growth at a concentration below the LC50 (Table 2).

DISCUSSION

Declining amphibian populations are a major concern of many scientists worldwide. Frogs have been referred to as living barometers for the earth's environmental health [8]. In this study, the effects of two formulations of glyphosate and two formulations of triclopyr on *X. laevis* were evaluated. *Xenopus laevis,* while not indigenous to North America, has been compared with the indigenous species *Rana catesbeiana* (bullfrog) and *Rana pipiens* (leopard frog) in toxicology testing [9]. In these studies, *X. laevis* has proven to be a sensitive indicator organism for embryonic effects and has proven to be a valuable tool for environmental hazard assessments [10,11] (Table 3).

The higher toxicity of the butoxy-ethyl ester formulation of triclopyr to *X. laevis* in this study is likely due to greater uptake and greater accumulation of this more lipophilic molecule [12,13] by the embryo compared with the triethylamine salt of triclopyr. However, it could also be due to other formulation differences. This increased toxicity associated with the triclopyr ester formulation has been seen for several other aquatic species (Table 4).

The slope of the dose–response curve in probit analysis defines the degree of toxic response exhibited by an organism. The slope associated with the dose–response line of Garlon 4 was steeper than the slope associated with the dose–response line of Garlon 3A (Fig. 3). This indicates that the mortality of *X. laevis* was rapidly affected with a minimal increase in exposure concentration (Fig. 3). This phenomenon has also been documented by Kreutzweiser et al. for Garlon 4 toxicity to rainbow trout and chinook salmon [14]. The sharp lethal threshold in these toxicity tests for the butoxyethyl ester form of triclopyr suggests that, at a distinct level of exposure, accumulation in the embryos exceeds the elimination rate and thus becomes highly toxic [14]. This implies that, as lethal concentrations are approached, small increases in concentration will result in substantial increases in mortality.

Surfactants can enhance the biological uptake of pesticides and are often recommended for use by the manufacturer to supplement a pesticide. The higher toxicity of Roundup (with surfactant POEA) compared with Rodeo (no surfactant) to *X. laevis* in this study can be attributed to the toxicity of the surfactant itself, which had a LC50 of 6.8 mg/L compared with an LC50 of 7,296.8 mg/L for Rodeo (glyphosate alone) (Table 1). Even though a limited number of tests were performed to evaluate the effects of the surfactant POEA on *X. laevis*, each test showed a lower LC50 value for POEA alone than for either Roundup or Rodeo (Table 1). More studies are needed, but it seems likely that the surfactant itself is responsible for the greater toxicity displayed by the Roundup formulation of glyphosate. It seems less likely that the greater toxicity is due to enhanced uptake of glyphosate by the embryos. The manufacturer recommends that a surfactant be added to Rodeo sprays and that additional surfactant be added to sprays of Roundup to enhance the efficacy of these herbicides. The results in our study indicate that adding more of the POEA surfactant would likely increase toxic effects of these glyphosate formulations on *X. laevis* embryos. More research is needed to determine the safest surfactants to include in glyphosate formulations intended for use in or near aquatic environments.

In a possible worse case scenario, if the highest recommended rates (12 L/ha for Roundup [G. Paquette, personal communication] and 8 L/ha for Garlon 4 [T. Haagsma, personal communication]) were applied to water 15 cm in depth, the expected environmental concentration would be 2.88 mg AE/L and 2.56 mg AE/L, respectively. The resulting margins of safety (LC5/expected environmental concentrations) would be ap-

Table 3. Effects of various pesticides on *Xenopus laevis*

Pesticide tested	Experimental conditions	LC50	Reference
Naphthalene	3-week larval assay	2.1 mg/L	[10]
Malathion	FETAX[a] (96 h)	10.9 mg/L	[21]
Parathion	FETAX (96 h)	14.7 mg/L	[21]
Paraquat technical	FETAX (96 h)	8.1 mg/L	[11]
Paraquat formulation	FETAX (96 h)	−8.1 mg/L	[11]
Dieldrin	96 hour	40.4–49.5 μg/L	[9]
Gluthion	FETAX (96 h)	6.1–6.3 mg/L	[21]

[a] FETAX = Frog Embryo Teratogenesis Assay—*Xenopus.*

Table 4. Increased toxicity associated with Garlon 4® (ester formulation of triclopyr) compared with Garlon 3A® (amine formulation of triclopyr) on several aquatic species

Species	Chemical formulation	Toxicology studies		Reference
		Type of study	Concentration	
Bluegill sunfish	Garlon 4®	96-h LC50	0.87 mg/L	[21]
Bluegill sunfish	Garlon® 3A	96-h LC50	891 mg/L	[21]
Rainbow trout	Garlon 4®	96-h LC50	0.74 mg/L	[21]
Rainbow trout	Garlon® 3A	96-h LC50	552 mg/L	[21]
Daphnia	Garlon 4®	48-h LC50	2.2 mg/L	[21]
Daphnia	Garlon® 3A	48-h LC50	775–1,170 mg/L	[21]

proximately 2.7 for Roundup and 3.1 for Garlon 4 (Table 5). If Rodeo and Garlon 3A were applied at these same rates on an acid equivalency basis, the margins of safety would be approximately 1,915 and 47, respectively, as long as additional surfactants were not added to the spray mixtures (Table 5). Thus, the highest recommended rates of either glyphosate or triclopyr applied during *X. laevis* 96-h embryo development did not elicit a toxic response.

When assessing the risk associated with a herbicide application on an aquatic habitat, both the toxicity of the chemical as well as the expected exposure must be considered. In actual practice, triclopyr and glyphosate are applied during mid to late summer, well after the early stages of amphibian development. Since both glyphosate and triclopyr dissipate rapidly in the environment and residues are rarely detectable the following spring [15–18], the actual margins of safety for amphibian embryos may be significantly higher.

Several studies have confirmed that glyphosate dissipates quickly from the surface waters of lentic systems and that sediment adsorption or biodegradation are the initial means of glyphosate loss from the water column [16,19]. Glyphosate has been found to dissipate rapidly from surface waters, leaf litter residues, and soil with a DT50 of 3.5 to 11.2, <14, and 45 to 60 d, respectively.

Triclopyr also dissipates quickly in water, primarily due to rapid photodecomposition, with a half-life of 2.1 to 10 h [20,21]. Solomon et al. [15] evaluated the dissipation of 0.25 kg/ha and 2.5 kg/ha Garlon 4 in limnocorrals. The dissipation of Garlon 4 from water was rapid, with less than 5% of the applied herbicide detected on day 15 and no detectable levels present at day 42.

This study demonstrates that the toxic effects of pesticides are very dependent on formulation type (i.e., amine vs ester) as well as on other components in the formulations, such as surfactants. Before one can assess the risks associated with the use of these chemicals in the natural environment, further testing must be performed. Even though the embryos considered in FETAX represent a sensitive life stage of *X. laevis* development, other studies could be conducted to compare the sensitivity of other growth stages. For example, larvae 1 to 2 weeks of age may be more sensitive than embryos due to the presence of gills and a higher surface-to-volume ratio.

In this study with small volume solutions in petri dishes, the solutions were changed every 24 h to minimize the effects of loading into the embryos. However, it is possible that somewhat lower concentrations could be toxic in larger volume studies or studies in the natural environment where the supply of herbicide to the embryo could be virtually unlimited. Longer exposures in a natural surface water environment, recognizing the potential influence of loading densities at different stages of development of native amphibian life cycles, must be studied to fully determine the risk associated with glyphosate and triclopyr use in aquatic environments.

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Table 5. Margin of safety calculations for glyphosate and triclopyr applied at the highest recommended application rates to water 15 cm in depth

Treatments	Highest recommended application rate (L/ha)[a]	EEC in water 15 cm in depth[b]	Margin of safety (LC5/EEC)[c]
Triclopyr			
Garlon 4®	8	2.56 mg AE/L	3.1
Garlon® 3A	10.72	2.56 mg AE/L	46.5
Glyphosate			
Roundup®	12	2.88 mg AE/L	2.7
Rodeo®	8.9	2.88 mg AE/L	1,915.1

[a] Highest recommended application rate for Garlon 4® and Roundup® were in accordance with personal communications from Tip Haagsma (Dow Elanco) and Guy Paquette (Monsanto), respectively.
[b] EEC = expected environmental concentration that would result when the chemical is applied to water 15 cm in depth; AE = acid equivalent.
[c] Margin of safety is calculated by dividing the LC5 concentration (required for 5% mortality) by the EEC.

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