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ACUTE TOXICITY OF A HERBICIDE TO SELECTED FROG SPECIES

FINAL REPORT

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PREAMBLE

Glyphosate based herbicides are generally thought of as environmentally friendly because the active ingredient is of relatively low toxicity to animals, although reports in the literature suggest that the formulations may pose a hazard to aquatic animals.

In 1994 a proposal was referred to the Department of Environmental Protection (DEP) for aerially spraying a glyphosate based herbicide over substantial areas of Lake Kunnunura to control the emergent water weed Cumbungi. The Department also received a number of anecdotal reports indicating that frogs had been killed or frog chorus' had been silenced after the application of herbicides (including glyphosate-based herbicide) in or adjacent to aquatic environments.

The DEP therefore commissioned Curtin University's Aquatic Science Research Unit to conduct some toxicity tests on glyphosate herbicide using frog species common to the south-west of Western Australia. An informal steering committee was formed to provide advice and assist with interpretation of the results. The agencies involved were:

- Curtin University;
- Department of Environmental Protection;
- Museum of WA; and
- Water Resources Council of WA.

The results of the studies suggested tadpoles were many times more sensitive to the herbicide formulation tested than adult frogs and that tadpoles were approximately an order of magnitude more sensitive to the formulation than to technical grade glyphosate. These results and the results of other studies reported in the literature tend to suggest that the surfactant used in the formulation is more harmful to aquatic animals than the glyphosate.

Calculations based on recommended application rates for the control emergent aquatic weeds (3 - 4 kg/ha) predict that concentrations of the formulation in shallow water (<5 cm) could be similar to the LC_{50} concentrations for tadpoles. The LC_{50} values for adult frogs also indicate that there may be very little safety margin between predicted concentrations in shallow water and concentrations that could be lethal to frogs. The standard safety buffer is 0.01 times the 96hr LC_{50} concentration. A study by Folmar et al (1979) shows that the toxicity of Roundup to fish changes significantly with temperature and pH, with greatest toxicity at high temperature and pH.

As it is thought to be primarily the surfactant that is responsible for the toxic effect these findings are likely to apply to other formulations too.

These results indicate that the environmental warnings relating to aquatic environments on the labels of herbicide formulations may need to be reviewed in the light of this study and that the recommendations relating to methods of application may also need to be reviewed. However, further study is required to determine the actual concentration and persistence of the formulation in aquatic environments. The DEP is aware that much tighter controls apply to the use of Roundup in aquatic environments in Britain (Guidelines for the Use of Herbicides on Weeds in or near Watercourses and Lakes; Nature Conservancy Council and the Ministry for Agriculture, Fisheries and Food) and understands from correspondence to the WA Government (11 Nov 1991) that it is not registered for use in/over water in the USA.

The findings of this study and other studies reported in the literature suggest that consideration be given to the preparation of a list of low impact surfactants suitable for endorsement for use in aquatic situations. Strategies and/or legislation could then be developed and implemented to ensure that only these surfactants are used in or adjacent to aquatic environments.

This report will be referred to the National Registration Authority for its consideration with a request for it to review label warnings and the relative efficacy and environmental impact (toxicity and persistence) of the various surfactants used in herbicide formulations.

ABSTRACT

The toxicity of technical grade glyphosate and the glyphosate-based herbicide, Roundup®[1] 360, was investigated through acute bioassays with frog species native to Western Australia. In both screening and definitive studies, the formulated herbicide was more toxic to frogs than the glyphosate alone. The 48 h LC_{50} for juvenile (newly emerged) *Crinia insignifera* was 51.8 mg/L Roundup 360, reported as glyphosate active (isopropylamine salt). The 48 h LC_{50} for glyphosate alone was 83.6 mg/L.

Tadpoles were significantly more sensitive to Roundup 360 than juveniles or adults. The 48 h LC_{50} for *Litoria moorei* tadpoles was 11.6 mg/L Roundup 360 as glyphosate, an order of magnitude lower than the 48 h LC_{50} of 121.5 mg/L generated for *L. moorei* tadpoles in exposure to technical grade glyphosate alone. Screening studies with *C. insignifera* tadpoles indicated a similar sensitivity.

C. insignifera adults were significantly more sensitive to Roundup 360 than the larger *L. moorei* adults, demonstrating that species-specific differences in response to the herbicide can be expected.

The sharp difference in toxicity between technical grade glyphosate and Roundup 360 is probably due to toxicity of the surfactant in the formulated herbicide. While data on environmental concentrations and persistence of the herbicide must be additionally considered to make final conclusions, a potential hazard exists for frogs and tadpoles in shallow water bodies.

[1] Product and Registered Trademark of Monsanto Company

1. Introduction

Evidence from around the world suggests that there has been widespread decline in the populations of many species of frogs in recent years (Young 1990). While a number of factors may have contributed to this decline, concern has been expressed that frogs are particularly affected by habitat loss and by the toxic effect of chemical pesticides (Tyler et al. 1994; Wardell-Johnson and Roberts 1994).

Recent anecdotal reports received by the West Australian Department of Environmental Protection described frog mortalities following the application of chemical herbicides, including those containing glyphosate. The present study was initiated in response to these reports and sought to characterise the toxicity of technical grade glyphosate and the widely available commercial herbicide Roundup 360.

Glyphosate is the active ingredient in a number of commonly used broad spectrum herbicides, with some commercial formulations such as Roundup 360 containing a surfactant component as well. Technical grade glyphosate exhibits low toxicity to fish and low to medium toxicity to aquatic invertebrates (Folmar et al. 1979; Holdway and Dixon 1988; Klemm et al. 1993), although the added surfactant does appear to cause increased toxicity in some formulations (Mitchell et al. 1987; WHO 1994).

Frogs may be exposed to herbicides when the chemicals are applied to waterways for control of emergent weeds, or through runoff or overspray from fields adjacent to permanent or temporary wetlands. The frog species chosen for testing in the present study are among those which breed in shallow waters during winter and spring, a time when herbicide use would be greatest.

2. Materials and methods

2.1 Source of test animals

Screening and/or definitive bioassays were conducted with *Crinia insignifera*, *Litoria adelaidensis* and *Litoria moorei*. Depending on the species, adult, newly emerged or tadpole stages were used. *Crinia* and *Litoria* are representative genera of Leptodactylidae and Hylidae, the two frog families found throughout southern Australia.

Frogs and tadpoles were collected from sites within the Perth metropolitan area and as far south as Mandurah, Western Australia (~ 80 km from Perth). Availability of specimens dictated which collection site was used. All collections were made under permit by personnel from the West Australian Museum.

Bioassays were initiated within two days of receiving the frogs in order to minimise stress associated with being held in the laboratory. Test protocols were reviewed and approved by the Curtin University of Technology Animal Experimentation Ethics Committee.

2.2 Test protocol

Adult *C. insignifera* (15 to 23 mm S-V, snout-vent length) and *L. adelaidensis* (37 to 43 mm S-V) were tested in chambers which facilitated individual exposure to test solutions (Figure 1). The exposure chambers were constructed from short lengths (15 mm for *C. insignifera*, 30 mm for *L. adelaidensis*) of PVC pipe (50 mm diameter) which were covered on one end with 0.5 mm nylon mesh. The opposite side was fitted with the lid of a 55 mm polystyrene petri dish. Bioassays were initiated by placing the chambers into glass beakers which contained the test

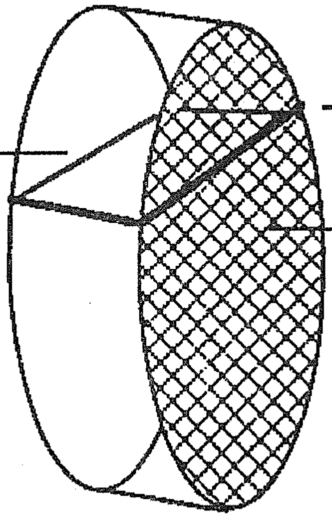

Figure 1. Showing the design of the containers in which frogs were held for exposure to chemicals.

solutions. The chambers were placed in the beakers with the diameter vertical, allowing the meshed side to remain unobstructed and facilitating passage of test solution and air. Bioassays with *C. insignifera* were conducted in 250 ml beakers containing 125 ml of test solution. This provided a 15 mm air space within the exposure chamber. Tests with the larger *L. adelaidensis* were similarly conducted in 250-ml beakers, but with only 100 ml of test solution to allow a 20 mm air space. When undisturbed, the frogs would usually cling to the mesh side of the exposure chamber in the airspace. The volumes of test solution were chosen so that even in this resting position the animals would have some skin contact with the solution. Daily renewal of the bioassay was achieved by pouring off the old test solution and replacing it with new.

Adult *L. moorei* were too large (40 to 70 mm S-V) for the test chambers described above and so were exposed to the herbicides in replicate 5 L glass beakers containing 200 ml of test solution and holding three frogs each (n=6). The test solution was 1.5 cm deep and largely covered the hindquarters of a frog sitting upright. Each beaker was covered with a Perspex plate through which a number of air holes were drilled.

In contrast to *L. moorei*, newly emerged *C. insignifera* ranged 5 to 11 mm S-V and were too small to be effectively exposed in the test chambers used for full size adults. The bioassay with these frogs was conducted by placing individuals in glass test tubes (30 mm diameter x 120 mm length) with a 0.5 mm nylon mesh across one end. A large swab of glass fiber was inserted through the top of the tube, creating a chamber 10 to 15 mm high. Racks holding 10 tubes were then suspended in two replicate 3 L beakers containing enough test solution to immerse the tubes 5 mm deep. A frog sitting upright in a tube was partly covered in test solution without having to continuously swim. Daily renewal of the bioassay was accomplished by transferring the racks of tubes to alternate beakers containing fresh solution.

Tests with *C. insignifera* and *L. moorei* tadpoles were conducted in 250 ml glass beakers containing 100 ml of test solution. Each beaker held five tadpoles with two or three replicates per concentration.

Daily renewals of the bioassays were achieved by first draining the old test solution over a nylon mesh (in the event a tadpole was accidentally poured out during the change over) and replacing with new.

All bioassays were conducted at 20 ± 2°C under ambient laboratory light.

2.3 Test substance

Technical-grade glyphosate (N-(Phosphonomethyl) Glycine, 96%) was obtained from a local supplier on 23 June 1994. The formulated herbicide "Roundup 360" was purchased from a local retail outlet.

A 1 mg/ml stock solution of glyphosate was prepared by adding 1 g of powdered chemical to 1 L of distilled water. Levels of Roundup 360 were calculated and reported as glyphosate active (isopropylamine salt). Test solutions were prepared by serial dilution of the highest concentration tested. Test concentrations for initial screening studies were based on suggested application levels of the herbicide.

Purity of the technical grade glyphosate and analyses of selected test solutions were conducted by the Chemistry Centre (WA) using high performance liquid chromatography (HPLC).

2.4 Diluent

Aged (three days), aerated laboratory tap water was used as the diluent in the screening tests with *C.insignifera*, *L. adelaidensis* and *L. moorei*. All other bioassays used filtered (30 mm) water collected from a lake on the Curtin University campus.

2.5 Statistical analyses

Mortality data were used to generate LC_{50} values by the Spearman-Karber method (Hamilton et al. 1978). Reported values are based on target (nominal) rather than measured concentrations of glyphosate.

3. Results

Initial screening studies were run between 25 June and 24 July 1994 with adult *C. insignifera* and *L. adelaidensis*. While these studies were conducted primarily to determine if the exposure chambers would work properly, they provided an initial indication that the frogs were significantly more sensitive to Roundup 360 than to technical grade glyphosate. During this test, there was virtually no loss of glyphosate in the test solutions over a 24 h period (Table 1).

A second toxicity screening study with *C. insignifera* was conducted between 11 and 15 August 1994. Again, Roundup 360 was more toxic than technical grade glyphosate. Complete mortality of the frogs was observed at 67.6 and 90 mg/L Roundup 360, while complete mortality in the bioassay with glyphosate was only achieved at a concentration of 135 mg/L (Table 2). Daily LC_{50} values of 39.7 to 52.6 mg/L were generated from the Roundup 360 test, while values of 78.0 to 89.6 mg/L were generated for technical grade glyphosate (Table 3).

Due to difficulties in obtaining sufficient numbers for testing, additional bioassays with *L. adelaidensis* were not conducted.

In a third set of bioassays conducted between 21 and 25 September 1994, adult *L. moorei* and *C. insignifera* tadpoles were exposed to both chemicals. As with the other species tested, Roundup 360 was more toxic to *L. moorei*, although this species was less sensitive to the herbicides than *C. insignifera*. For example, exposure to Roundup 360 at a concentration of 67.6 mg/L resulted in complete mortality of adult *C. insignifera*, but had no apparent effect on *L. moorei* (Tables 2 and 4).

The difference in toxicity between technical grade glyphosate and Roundup 360 was dramatically illustrated by data from the screening bioassay with *C. insignifera* tadpoles. Here, complete mortality of the tadpoles occurred at 22.5 mg/L Roundup 360, while glyphosate levels as high as 180 mg/L had no effect (Table 5). *C. insignifera* tadpoles were also significantly more sensitive to the formulated herbicide than were adults.

The definitive test of Roundup 360 using *C. insignifera* adults was initiated on 14 October 1994. At this time, it was difficult to obtain adults of the size used for testing in August, so the bioassay was run with newly emerged individuals with lengths ranging from 5 to 10 mm S-V. Unfortunately, by Day three of the 96 h test, the control frogs were showing signs of stress, possibly due to their containment, so the test was considered valid only up to 48 h (Table 6). The 48 h LC_{50} for this bioassay was 51.8 mg/L as glyphosate (95% CI 42.1 - 63.8 mg/L). This value was not significantly different from the 48 h LC_{50} of 49.4 mg/L as glyphosate generated for larger adult *C. insignifera* in August 1994.

Definitive bioassays with tadpoles were conducted between 18 and 22 January 1995. In this test, *L. moorei* was used in place of *C. insignifera* since the later were not readily available. Side-by-side testing of both glyphosate and Roundup 360 again illustrated greater toxicity of the formulated herbicide (Table 7). Daily LC_{50} values ranged between 110.8 and 127.0 mg/L for glyphosate alone and 7.7 to 12.7 for Roundup 360 reported as glyphosate. The sensitivity of *L. moorei* tadpoles was comparable to *C. insignifera* tadpoles (Tables 5 and 7).

Water quality parameters were within acceptable limits for the definitive tests with adult frogs and tadpoles. Dissolved oxygen levels ranged between 6.4 and 8.1 mg/L, while pH ranged between 6.9 and 7.4 across all treatments.

Table 1. Measured concentrations of fresh and 24h old solutions of technical grade glyphosate.

Target Concentration (mg/L)	Measured Concentration (mg/L)
12.5, freshly prepared	12.9
12.5, 24 h old	12.8
100, freshly prepared	110
100, 24 h old	110

Table 2. Toxicity of technical grade glyphosate and Roundup 360 (expressed as glyphosate active) to adult *C. insignifera* in a screening study conducted between 11 and 15 August 1994.

Toxicant (mg/L)	# Exposed	Cumulative % Mortality			
		24 h	48 h	72 h	96 h
0	5	0	0	20	20
Roundup 360 11.1	5	0	0	0	0
Glyphosate 22.3	5	0	0	0	0
Roundup 360 22.3	5	0	0	0	0
Glyphosate 45	5	0	0	0	0
Roundup 360 45	5	20	20	40	60
Glyphosate 67.6	5	20	40	40	40
Roundup 360 67.6	5	100	----	----	----
Glyphosate 90	5	40	40	60	60
Roundup 360 90	5	80	100	----	----
Glyphosate 135	5	100	----	----	----

Table 3. Preliminary LC_{50} values (95% confidence interval) for adult *C. insignifera* in screening bioassays with glyphosate or Roundup 360 (expressed as glyphosate active).

Time	LC_{50} (mg/L)	
	Glyphosate	Roundup 360
24 h	89.6 (73.6 - 108.9)	52.6 (39.3 - 70.5)
48 h	83.6 (67.4 - 103.6)	49.4 (40.5 - 60.2)
72 h	72.0 (62.9 - 96.6)	44.2 (34.7 - 56.3)
96 h	78.0 (62.9 - 96.6)	39.7 (31.1 - 50.5)

Table 4. Toxicity of technical grade glyphosate and Roundup 360 (expressed as glyphosate active) to adult *L. moorei* in a screening test conducted between 21 and 25 September 1994.

Toxicant (mg/L)	# Exposed	Cumulative % Mortality 24 h	48 h	72 h	96 h
0	6	0	0	0	0
Glyphosate 22.5	6	0	0	0	0
Roundup 360 22.5	6	0	0	0	0
Glyphosate 45	6	0	0	0	0
Roundup 360 45	6	0	0	0	0
Glyphosate 67.6	6	0	0	0	0
Roundup 360 67.6	6	0	0	0	0
Glyphosate 90	6	0	0	0	0
Roundup 360 90	6	0	0	17	67
Glyphosate 180	6	0	0	0	0
Roundup 360 180	6	50	67	100	----

Table 5. Toxicity of technical grade glyphosate and Roundup 360 (expressed as glyphosate active) in a screening test with *C. insignifera* tadpoles.

Toxicant (mg/L)	# Exposed	Cumulative % Mortality 24 h	48 h	72 h	96 h
0	10	0	0	0	0
Glyphosate 22.5	10	0	0	0	0
Roundup 360 22.5	10	20	100	---	---
Glyphosate 45	10	0	0	0	0
Roundup 360 45	10	100	---	---	---
Glyphosate 67.6	10	0	0	0	0
Roundup 360 67.6	10	100	---	---	---
Glyphosate 90	10	0	0	0	0
Roundup 360 90	10	100	---	---	---
Glyphosate 180	10	0	0	0	0
Roundup 360 180	10	100	---	---	---

Table 6. Toxicity of Roundup 360 (expressed as glyphosate active) to newly emerged *C. insignifera*.

Roundup 360 (mg/L)	# Exposed	Cumulative % Mortality 24 h	48 h	72 h
0	20	5	10	20
11.25	20	0	5	15
22.5	20	0	5	10
45	20	30	40	65
90	20	35	85	100
180	20	90	100	----
LC_{50} (95 % confidence interval)		88.7 (68.6 - 114.6)	51.8 (42.1 - 63.8)	*

* - no LC_{50} calculated due to control mortality

Table 7. Toxicity of technical grade glyphosate and Roundup 360 (expressed as glyphosate active) to *L. moorei* tadpoles.

Toxicant (mg/L)	# Exposed	Cumulative % Mortality 24 h	48 h	72 h	96 h
0	15	0	0	0	0
Roundup 360 1.12	15	0	0	0	0
Roundup 360 2.25	15	0	0	0	0
Roundup 360 4.5	15	0	0	0	20
Roundup 360 9.0	15	0	13	40	53
Glyphosate 11.25	15	0	0	0	0
Roundup 360 18.0	15	100	---	---	---
Glyphosate 22.5	15	0	0	7	13
Roundup 360 36	15	100	---	---	---
Glyphosate 45	15	0	0	0	0
Glyphosate 90	15	0	7	13	27
Glyphosate 180	15	100	---	---	---

Table 8. LC_{50} values (95% confidence intervals) for *L. moorei* tadpoles exposed to technical grade glyphosate or Roundup 360 (expressed as glyphosate active).

Time	LC_{50} (mg/L)	
	Glyphosate	Roundup 360
24 h	127.0 (90.0 - 180.0)	12.7 (9.0 - 18.0)
48 h	121.5 (111.2 - 132.9)	11.6 (10.3 - 13.1)
72 h	116.0 (102.2 - 131.8)	10.6 (9.0 - 12.4)
96 h	110.8 (95.2 - 128.4)	7.66 (6.1 - 9.6)

4. Summary

The results of this study indicate that technical grade glyphosate and Roundup 360 can be acutely toxic to adult frogs and tadpoles in laboratory bioassays. The LC_{50} values generated are within the range of values reported for a number of aquatic organisms in a summary of toxicity data for glyphosate and Roundup compiled by the World Health Organisation (WHO 1994).

Of the two chemicals, Roundup 360 was more toxic to frogs than technical grade glyphosate alone. Most glyphosate formulations contain a surfactant which can have greater toxicity to non-target species than the active agent itself (Servizi et al. 1987; Klemm et al. 1993). Certain classes of surfactants exert an effect on aquatic organisms by causing damage to the gill membrane (Able 1974). The presence of a surfactant in the formulation that was tested may then explain why tadpoles, which respire with gills, were significantly more sensitive to Roundup 360 than adult frogs. Glyphosate formulations which do not contain surfactants have been identified elsewhere as preferable for use as herbicides in wetlands (Klemm et al. 1993).

Formulations which include glyphosate and surfactant are commonly used to control emergent water weeds in a number of Australian localities. The recommended application rates of Roundup 360 can range between 1.8 and 5.4 kg/ha. At these levels, concentrations of herbicide resulting from direct application or overspray may pose a risk to frogs and tadpoles in shallow wetland areas (< 5 cm in depth). While data concerning actual environmental concentrations and persistence of these herbicides are necessary to fully assess the potential impact on frog populations, labelling amendments which advise of a potential hazard in wetlands are necessary.

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