Toxicity of herbicide formulations to frogs and the implications for product registration: A case study from Western Australia

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Abstract. Growing concern about the decline of amphibian populations has highlighted the need to assess the potential impact of agricultural chemicals on these animals. Although the relative sensitivity of amphibians to the toxic effects of pesticides and other environmental contaminants has yet to be established, the perceived vulnerability of amphibians to pesticide effects may actually be attributable to their specific habitat requirements. Shallow temporary ponds, essential to the life cycles of many amphibians, are also areas where pollutants may accumulate without substantial dilution. Research in Western Australia has highlighted the potential risk that agricultural chemicals may pose to fauna that inhabit low dilution environments, and indicates that the data currently required for pre-registration assessment of pesticides may be inadequate to effectively protect these environments.

Key words: Amphibian; frog; glyphosate; herbicide; pesticide; surfactant; toxicology.

Introduction

Amphibians are often identified as a group of organisms that are particularly sensitive to environmental pollutants because their dual life cycles and permeable skins provide more opportunities for exposure and more modes of exposure to contaminants than other vertebrates. A limited number of toxicological studies have attempted to evaluate the sensitivity of amphibians relative to other aquatic species (Holcombe et al., 1987; Deyoung et al., 1996; McCrary and Heagler, 1997), although no consistent differences in susceptibility are apparent. This is not surprising because the response of an organism to a chemical contaminant is a function of both biotic and abiotic factors. Indeed, any attempt to identify the most sensitive species or group of organisms, may be unrealistic. The concept of the "most sensitive species" is flawed because species sensitivity can vary markedly depending on factors such as type of contaminant, age and nutritional status, and reproductive state of the organism (Cairns, 1986).

The specific habitat requirements of amphibians, however, may confer upon them a greater vulnerability to the toxic effects of environmental contaminants than other taxonomic groups, even though their relative sensitivities in an acute or chronic toxicity test may be similar. Many amphibians live and breed in ephemerally wet areas that are inundated for only short periods of time. Such environments may be contaminated by spray drift (Lahr, 1997) or direct application of pesticide for the control of aquatic macrophytes (Gardner and Grue, 1996). The lentic nature of such habitats may present pollutants at much higher concentrations, and for longer periods of time than would be experienced by fish and other species in open or lotic water bodies.

About half of the more than 200 species of Australian frogs are dependent on ephemeral or lentic water bodies for the completion of their life cycles (Cogger, 1992). Furthermore, some species, such as members of the genera *Heleioporus*, *Neobatrachus* and *Limnodynastes*, are burrowing species which spend much of their adult lives beneath the soil surface. They live at the margin between wet and dry habitats, and may be adversely affected by the application or accumulation of contaminants along the margins of water bodies.

Agricultural pesticides are a large and chemically diverse group of compounds, which are a widespread source of water contamination. Although successive generations of agricultural pesticides have become less persistent in the environment, and in some instances, more selective for their target species, the toxicological information available for non-target species, including amphibians, is far from adequate (for review see Hall and Henry, 1992; Boyer and Grue, 1995). Furthermore, most pesticides usually incorporate several other chemicals, including organic solvents, adjuvants or surfactants, which do not share the target specificity of the active ingredient. Such chemicals are seldom listed on product labels, and material safety data sheets will often refer to them collectively as “inert” ingredients. However, some of these additives may be more toxic than the active constituent by virtue of their non-selective toxicity. Several studies have noted a large discrepancy between the toxicity of active ingredients and product formulations, indicating that formulation additives significantly increase the toxicity of the formulation (Mayer and Ellersieck, 1986; Linder et al., 1990; Schuytema et al., 1995; Swann et al., 1996).

The potential hazards associated with inert ingredients, was highlighted by research in Western Australia (Bidwell and Gorrie, 1995; Mann and Bidwell, 1999a, 2000, 2001). The toxicity of glyphosate-based herbicides and commercial surfactants (used as wetting and dispersal agents in spray tank mixtures) to Australian frogs was investigated for the first time. This research is summarized here to highlight the potential hazards posed by agricultural products which otherwise have previously been considered as environmentally benign, or for which current application safety margins may be inadequate.

Glyphosate-Based Formulations and Agricultural Surfactants: A Case Study

Glyphosate (*N*-(phosphonomethyl) glycine), developed in the 1970s, has become one of the most widely used herbicides in the world due to its efficacious weed control properties, low toxicity to wildlife, and negligible environmental persistence (for reviews see Duke, 1988; Malik et al., 1989; WHO, 1994). Glyphosate constitutes the active ingredient in hundreds of different products sold around the world and is usually present as the isopropylamine salt. One of the more widely recognized glyphosate formulations is Roundup® Herbicide, which is manufactured by Monsanto (Monsanto product code MON-2139). Because of its anionic nature glyphosate alone does not easily penetrate plant cuticles. The, herbicide's phytotoxicity is therefore enhanced by the incorporation of a surfactant (Duke, 1988). The surfactant incorporated into most glyphosate formulations is a polyoxyethylene amine derivative (POEA) (NRA, 1996).

Concern over the use of herbicides was raised when the Western Australian Department of Environmental Protection (DEP) received a proposal from the East Kimberly shire council for extensive aerial spraying of Lake Kunnunurra with a glyphosate-based herbicide for the control of the emergent weed cumbungi (*Typha orientalis*). Lake Kunnunurra forms part of the Ord River irrigation system in tropical northwestern Australia. The proposal to spray Lake Kunnunurra had followed numerous anecdotal reports indicating that frogs were dying or disappearing following the application of various herbicides (including glyphosate-based formulations) at various different locations in Western Australia. To address these concerns, the DEP commissioned the Aquatic Science Research Unit of Curtin University of Technology in Perth, Western Australia, to conduct acute toxicity tests using native frogs exposed to glyphosate and the glyphosate-based Roundup® Herbicide.

This study indicated that the glyphosate formulation Roundup® Herbicide was substantially more toxic to tadpoles of the motorbike frog (*Litoria moorei*) and the western froglet (*Crinia insignifera*) than technical grade glyphosate (Bidwell and Gorrie, 1995). In their report to the DEP, Bidwell and Gorrie concluded that the surfactant component of the formulated product was likely to be responsible for its toxicity to tadpoles, and expressed concerns that glyphosate-based formulations, which incorporate similar surfactants may present a toxic hazard to tadpoles if applied over shallow water.

The toxicity to aquatic fauna of the POEA surfactant incorporated into Roundup® Herbicide and most other glyphosate formulations was not a novel finding. Although the DEP report constituted the first such study in amphibians, Folmar et al. (1979) examined the acute toxicity of Roundup®, the POEA surfactant, and technical grade glyphosate acid to four species of aquatic invertebrates and four species of fish. In the Folmar et al. study, the POEA surfactant was of similar toxicity to the formulated product, whereas technical grade glyphosate was an order of magnitude less toxic. These findings were confirmed in subsequent studies (Mitchell et al., 1987; Servizi et al., 1987; Wan et al., 1989).

As a consequence of the Bidwell and Gorrie (1995) report, The Australian National Registration Authority for Agricultural and Veterinary Chemicals (NRA) instigated a special review of glyphosate (NRA, 1996). The scope of this review was restricted to the use of glyphosate formulations in and around aquatic environments with particular reference to the toxicity of formulation surfactants to aquatic organisms. Glyphosate-based herbicides (containing 36% glyphosate and POEA) if applied at the maximum permissible rate of 10.6 kg/ha (NRA, 1996) to a lentic body of water of 5 cm in depth, would leave residues of approximately 21.1 mg/l (whole product). In order to accommodate a fivefold safety margin, the NRA adopted an Australian Commonwealth Environmental Protection Authority (EPA) recommendation that glyphosate formulations for use in aquatic systems should exhibit no toxicity to aquatic organisms at concentrations of at least 100 mg/l (NRA, 1996).

Registrants of all glyphosate-based products available in Australia were asked to provide toxicological information in order to determine which formulations met the new criteria for aquatic use. Eighty-four products were unable to satisfy the new requirements, based on acute toxicity data derived predominantly from tests with non-native fish and invertebrates. The labeling requirements for these products were changed such that the following statements were displayed on the packaging:

> "USE SITUATION — Dry drains and channels, dry margins of dams, lakes and streams.
>
> CRITICAL COMMENTS — Do NOT apply to weeds growing in or over water. Do NOT spray across open water bodies, and do NOT allow spray to enter the water".

Data generated by Bidwell and Gorrie (1995), indicated that tadpoles, and to a lesser extent adults of Australian frogs are also likely to be adversely affected by such formulations under shallow-water conditions (table 1). Subsequent investigations (Mann and Bidwell, 1999a) confirmed that Roundup® Herbicide (a glyphosate isopropyamine formulation containing POEA), was acutely toxic to the tadpoles of four species of Australian frogs between 8.1 and 32.2 mg/l (table 1). Similarly, the glyphosate trimesium formulation, Touchdown® Herbicide (also incorporating POEA) was acutely toxic to tadpoles of the same four species between 27.3 and 48.7 mg/l (table 1). The recently registered Roundup® Biactive, which incorporates different (undisclosed) surfactants, was relatively non-toxic in acute tests (table 1).

At the time of the release of the NRA (1996) review, none of the newer formulations were available in Australia. The alternative was after-market formulation of glyphosate products that contain no surfactant, in combination with a commercially available surfactant. However, this strategy is thwarted by the toxicities of commercial agricultural surfactants, which are essentially similar to the toxicity of POEA (Watkins et al., 1985; Henry et al., 1994).

Subsequent to the NRA review, further research also examined the acute toxicity of two after-market commercial surfactants to the tadpole stages of four species of frogs from southwestern Australia (Mann and Bidwell, 2000, 2001). The after-market surfactants included a nonylphenol ethoxylate (Agral® 600, Crop Care Australasia), and an alcohol alkoxylate (BS1000®, Crop Care Australasia). The

Table 1. Acute toxicity of glyphosate-based herbicides to amphibians as determined at 20°C in static-renewal tests. Unless noted otherwise, all tests were run for 48 h. LC50 denotes a statistically estimated concentration that is expected to be lethal to 50% of tadpoles. All data are for larvae unless noted otherwise (M = metamorph, A = adult). Acute toxicity tests were performed within the context of approval from the Curtin University Animal Ethics Committee. All concentrations are for whole product. Data taken from (1) Mann and Bidwell, 1999a; (2) Bidwell and Gorrie, 1995; (3) Monsanto 1996.

Herbicide	Species	LC50 (mg/l)	Reference
Touchdown® (4LC-E)[a]	*Crinia insignifera*	27.3	1
	Heleioporus eyrei	48.7	1
	Litoria moorei	31.4[d]	1
	Limnodynastes dorsalis	36.2	1
Roundup®	*Crinia insignifera*	10.0	1
(MON 2139)[b]	*Crinia insignifera* (M)	144	1, 2
	Crinia insignifera (A)	137	1, 2
	Heleioporus eyrei	17.5	1
	Litoria moorei	8.1	1
	Litoria moorei	32.2	1, 2
	Limnodynastes dorsalis	8.3	1
Roundup® Biactive	*Crinia insignifera*	>1372	1
(MON77920)[c]	*Heleioporus eyrei*	>1186	1
	Litoria moorei	911	1
	Limnodynastes dorsalis	>1111	1
Roundup® Biactive (MON52276)[c]	*Rana pipiens*	>1040[e]	3

[a] 48% glyphosate trimesium, POEA and alkylpolysaccharide (Crop Care Australasia).
[b] 36% glyphosate and POEA (Monsanto).
[c] 36% glyphosate and undisclosed surfactants (Monsanto).
[d] 24 h exposure.
[e] 96 h exposure.

toxicities of these two classes of surfactants to fish and some invertebrates is well documented (Talmage, 1994). However, very little data is available for amphibians (Mann, 2000; Mann and Bidwell, 2000). Some of the tests performed as part of these studies, were under conditions of high temperature and low dissolved oxygen (DO), both of which are factors that might affect surfactant toxicity in amphibian habitat. For these tests, a different nonylphenol ethoxylate was used (Teric GN8, Huntsman Corporation Australia Ltd.).

The toxicity of the two surfactants was manifested as a dose dependent, non-specific narcosis. Narcosis is a common means by which organic chemicals elicit effects, and is the result of chemicals accumulating within cell membranes in a non-specific manner, and resulting in decreased activity and reduced reaction to external stimuli (van Wezel and Opperhuizen, 1995). The sensitivity displayed by Australian tadpoles was similar to that displayed by the commonly used amphibian test species

Table 2. Acute toxicity data for amphibian larvae exposed to agricultural surfactants. EC50 denotes a statistically estimated concentration that is expected to cause immobilisation in 50% of tadpoles. All tests were 48 h static renewal tests run at 20°C and full oxygen saturation unless noted otherwise. Acute toxicity tests were performed within the context of approval from the Curtin University Animal Ethics Committee. All concentrations are for active ingredient. Data taken from Mann and Bidwell (2001).

Surfactant product	Species	EC50 (immobilisation) (mg/l)
BS 1000® [a]	*Crinia insignifera*	6.0
	Heleioporus eyrei	25.4
	Litoria moorei	<11.0
	Limnodynastes dorsalis	4.3
Agral® 600[b]	*Crinia insignifera*	3.5
	Heleioporus eyrei	12.1
	Litoria moorei	4.6
	Limnodynastes dorsalis	4.1
	Xenopus laevis	2.3
	Bufo marinus	5.4
Teric GN8[c]	*Bufo marinus*	4.1[d]
	Bufo marinus	2.2[e]
	Bufo marinus	1.8[f]
	Bufo marinus	1.4[g]

[a] 100% alcohol alkoxylate (Crop Care Australasia).
[b] 60% nonylphenol ethoxylate (Crop Care Australasia).
[c] 100% nonylphenol ethoxylate (Huntsman Corporation, Australia Ltd.).
[d] 30°C, flow through, 12 h, >5.7 mg/l dissolved oxygen.
[e] 30°C, flow through, 12 h, 1.7-2.3 mg/l dissolved oxygen.
[f] 30°C, flow through, 12 h, 1.2-1.7 mg/l dissolved oxygen.
[g] 30°C, flow through, 12 h, 0.8-1.3 mg/l dissolved oxygen.

Xenopus laevis (Mann and Bidwell, 2000, 2001, table 2) and some species of fish and invertebrates (Talmage, 1994). Agral® 600 was acutely toxic to the tadpoles of four species of Australian frogs between 3.5 mg/l and 12.1 mg (active ingredient)/l and BS1000® was acutely toxic to the same four species between 6.0 and 25.4 mg/l (table 2). Some species differences in sensitivity were observed, although this may have been related to animal size. High temperature-low DO conditions resulted in a two to threefold increase in toxicity (table 2).

The Current State of Pesticide Registration in Australia

In Australia, registration of a pesticide formulation is contingent on submission by the registrant, of adequate toxicity data for birds, mammals and other vertebrates, aquatic organisms and non-target invertebrates, and native vegetation. Various types of data are stipulated, including acute, short-term and long term toxicity studies, reproduction studies, developmental studies, genotoxicity studies, and studies on the toxicity of metabolites and impurities, other adverse effects and toxicology of mixtures (http://www.nra.gov.au/index.html).

The stringent registration requirements in Australia are, however, relatively recent, and formulations registered several years ago, may not comply with them. A pre-existing formulation will only be encompassed by the tighter requirements if the registrant applies for a change to the formulation's label, or a change to the formulation's specifications, or, if a special review of a class of products is instigated, as was the case for glyphosate formulations.

Pesticide-registrants are not obliged to provide amphibian toxicity data. This situation belittles the importance of amphibians in many ecosystems. Amphibians are often the main vertebrate group at risk of exposure to contaminants in ephemeral systems (Lahr, 1997). Although amphibians have not been demonstrated to be universally more sensitive to environmental contaminants than other taxonomic groups, only a very narrow suite of amphibian species have been used for toxicity testing. The majority of toxicity tests that utilize amphibians have used species of the genera *Rana*, and *Bufo*, or *Xenopus laevis* (Mann and Bidwell, 1999b). Australian frog species in particular are severely under-represented in toxicity tests (Mann and Bidwell, 1999b).

Moreover, ephemeral systems display large fluctuations in temperature, dissolved oxygen, pH, and light intensity, which can substantially alter the toxicological profile of environmental contaminants (Lahr, 1997). The current reliance by Australian regulatory authorities upon toxicological data derived from studies at 20°C with fully oxygenated test-water, is likely to misrepresent the physical and chemical environment in which amphibians live. Many of our tropical species live and breed in waters, which commonly exceed 40°C, and are deficient in oxygen (Tyler, 1994).

The case study presented here highlights the important role that standard acute toxicity tests can have in frog conservation. However, it is also important to recognize that issues related to chemical effects upon amphibians and regulation of chemicals may require more complex test systems than single species laboratory bioassays. A number of authors have criticized reliance on data generated solely from laboratory tests (Kimball and Levin, 1985; Rowe and Dunson, 1994; Cairns et al., 1996) and have cited the need for multi-phase micro- or mesocosms and field validation in order to understand the risks posed by chemical contaminants. Unfortunately, the cost of running these tests can often be prohibitive.

Summary

The NRA mandated changes with regard to glyphosate can be considered, to some extent, as a landmark conservation success-story. Certainly the findings of Bidwell and Gorrie (1995) had a profound impact on the aquatic usage of glyphosate-based formulations by precipitating the NRA special review of glyphosate. However, despite these positive aspects, reports of frog mortalities or cessation of frog chorus following herbicide application in Australia have continued following the prescribed changes in glyphosate herbicide labeling. Very few of these reports are ever substantiated, and if reported in the scientific literature, the authors are often reliant on the accuracy of information from landowners or pest control agencies with regard to the nature of the chemical application. For example, Tyler and Williams (1996) documented mortality of large numbers of adult burrowing frogs (*Neobatrachus pictus*) following seasonal rainfall several months after the alleged application of a glyphosate-based herbicide. Establishing a true cause and effect relationship in such a situation is not possible, especially because we can not be certain what chemicals have been applied and in what combinations or concentrations.

The after-market formulation of pesticide cocktails is a practice fraught with potential hazards. There are no controls on inappropriate tank mixing of pesticides, surfactants and other additives apart from guidelines set down by individual agricultural industries and chemical manufacturers. Apart from the risk of overdosing a tank with one or more ingredients, the mixing of different pesticides and additives is likely to result in various additive, antagonistic or synergistic interactions amongst chemicals. Predicting the hazards associated with these practices is difficult if not impossible.

Furthermore, the continued availability of nonylphenol ethoxylate- and alcohol alkoxylate-based surfactants is somewhat of an aberration given their apparent toxicity. The continued use of nonylphenol ethoxylates is currently the subject of considerable debate in Europe and the USA because of concerns regarding the persistence, toxicity, and potential estrogenic properties of their degradation products (Renner, 1997). However, calls to phase them out are being resisted by some regulatory authorities (Renner, 1997). Nevertheless, the existence of surfactant species with superior toxicological properties negates the need for the sale of older style surfactants for either pre- or post-market pesticide formulation, irrespective of the application situation. This is particularly important if considered within the context of the risk to terrestrial or semi-aquatic fauna, which live and breed in low dilution habitats.

Further expansion of the toxicity database for amphibians will facilitate their inclusion in future risk assessments of pesticides. However, it is ultimately the end user who will decide whether or not to follow the label guidelines derived from this toxicity data. Unfortunately, the “more is better” approach, and the mixing of “pesticide cocktails” is a continuing practice associated with the use of agricultural chemicals. The future of amphibians at risk from agricultural chemicals will not only depend on good science to document the potential for deleterious effects, but an increased public awareness of the vulnerability and importance of these organisms in aquatic systems.

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