Toxicity of a garden herbicide to the larvae of two amphibians: a cautionary tail

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AS populations of many amphibian species continue to decline on a global scale, habitat alteration has become recognized as a major threat (see Skelly *et al*., 2003; Stuart *et al*., 2004). However, the past 10 or so years also has seen a surge of evidence that implicates chemical contamination as an additional factor that may contribute to populations declines, especially with regard to agricultural chemicals that are toxic to amphibians (see Berrill *et al*., 1997).

It was more than 25 years ago that Beebee (1979) stressed the importance of suburban parks and gardens as refuges and breeding sites for British amphibians; interest among British homeowners in maintaining existing ponds or creating new ones is increasing (Beebee & Griffiths, 2000; see also Beebee, 1996). Of concern in the context of garden ponds as effective amphibian refuges should be the ready availability of insecticides and herbicides for residential use. Chemicals used to manage pests (often the same or similar compounds to those employed in agriculture) are easily purchased by homeowners and, even if not applied directly to ponds used as breeding sites by amphibians, may enter the water incidentally via aerial drift and run-off. Here we describe a test for toxic effects of such a pesticide in two species of amphibians.

Ortho® GroundClear® Triox Vegetation Killer (hereafter GROUNDCLEAR) is a terrestrial, broad-spectrum herbicide that is freely available commercially in the United States, where it is recommended for use by landscapers and gardeners to control unwanted weeds. Its primary active ingredient is isopropylamine salt of glyphosate at 5% by mass. Imazapyr is the secondary active ingredient, also as isopropylamine salt and at 0.08% by mass. Remaining ingredients are listed as 'inert' and include surfactant to facilitate penetration into plant tissues. Pure glyphosate has a mean half-life of 47 days in soil and a half-life ranging from 12 days to 10 weeks in aquatic habitats (Extoxnet, 2006).

Residential use of glyphosate-containing formulations has increased in the United States as former fields and wildlands have been converted into housing developments. Indeed, the overall use of glyphosate as a herbicide in the United States is second only to atrazine (EPA, 2006), the latter notorious for its action as an endocrine disruptor (see Hayes, 2005). Even though many glyphosate-containing formulations (including GROUNDCLEAR) are not intended for aquatic application, and are so labeled on their containers, with use by homeowners comes the potential for careless application in the vicinity of habitats used as breeding sites by amphibians. Thus, it is important to determine whether formulations such as GROUNDCLEAR might impact non-target species, especially those life stages the survival of which is crucial for population persistence. We exposed larvae of the African clawed frog, *Xenopus laevis* (a model amphibian species in ecotoxicological studies), and of the North American long-toed salamander, *Ambystoma macrodactylum*, to a range of concentrations of GROUNDCLEAR, and determined mortality as an end-point after 96 hrs of exposure.

We obtained fertilized *Xenopus* eggs from Xenopus 1, Inc (Dexter, Michigan), a commercial supplier, approximately 48 hrs after fertilization. They were maintained in aged tap water at 15°C through hatching until they had progressed to larval developmental stages 48–50. We captured gravid female *Ambystoma* from a pond near Pullman, Washington, in February and March 2003. They were placed in plastic boxes containing aged tap water and rocks, and allowed to lay eggs. These eggs then were transferred into larger aquaria containing aerated and aged tap water at approximately 15°C. Hatched larvae were removed and placed into similar aquaria until they had progressed to developmental stages 6–9.

Our testing protocol was adapted from FETAX (see Hoke & Ankley, 2005). The manufacturer's recommended concentration of GROUNDCLEAR for use (one part formulation to four parts water) was set as '1'; all other concentrations were serial dilutions of this. Concentrations of 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 were used. All solutions were made using aged, double-distilled tap water, and all studies included appropriate controls consisting of aged, double-distilled tap water only. In the *Xenopus* study, we included two replicates for each pesticide concentration and the water control, with 7 larvae per replicate. In the *Ambystoma* study, we included two replicates for each pesticide concentration and the water control, with 5 larvae per replicate. These are minimum numbers stipulated for FETAX.

Larvae of appropriate developmental stages were allocated randomly to treatments and placed into plastic boxes that each contained 1 L of the respective solutions. They were then left at approximately 15°C for 96 hrs of static exposure, during which time they were not fed and their media were not aerated. Survivors at 96 hrs were counted and then euthanized in a saturated solution of chloretone (1,1,1-tricholor-2-methyl-propanolol).

We obtained 100% mortality for *Xenopus* larvae in all concentrations of GROUNDCLEAR greater than and inclusive of 0.001 of that recommended for application. In the three highest concentrations, we observed that the death of all larvae occurred within minutes of initial exposure. At a concentration of 0.001, all larvae were dead within 4 hrs. Frog larvae at concentrations of 0.0001 and 0.00001 exhibited no mortality; neither did larvae in the water control (Table 1).

Larval mortality was complete for *Ambystoma* in all concentrations of GROUNDCLEAR greater than and inclusive of 0.001. Observations of larvae exposed to the highest concentrations indicated that they ceased to move, and likely died, within minutes of initial exposure. At lower concentrations, death occurred within hours to days. Larval mortality was 70% at a concentration of 0.0001, and zero both at 0.00001 and in the water control (Table 1).

These data indicate that, under controlled conditions in the laboratory, acute exposure to GROUNDCLEAR results in rapid death for larvae of *X. laevis* and *A. macrodactylum* even at very low concentrations (discussed further below). Since we used a formulation that is a chemical 'cocktail,' we cannot attribute mortality to any particular component. However, surfactants added to formulations such as GROUNDCLEAR may be as or more toxic than glyphosate alone (Howe *et al*., 2004; Mann & Bidwell, 1999; Perkins *et al*., 2000). Of course, it is to all components of formulations that organisms will be exposed simultaneously. In addition, all of our solutions were made up in aged, double-distilled water. The physicochemical properties of natural pond water are both complex and diverse, and it is possible that the relative toxicities of the components of herbicide formulations may be affected.

Percent mortality:

Solution	*X. laevis*	*A. macrodactylum*
1.0	100	100
0.1	100	100
0.01	100	100
0.001	100	100
0.0001	0	70
0.00001	0	0
Water control	0	0

Table 1. Percent mortality for larvae of the frog *Xenopus laevis* and the salamander *Ambystoma macrodactylum* exposed to solutions of six concentrations of GROUNDCLEAR, plus water controls, for 96 hrs.

GROUNDCLEAR is a formulation that is not intended for aquatic use (and is so labeled), and thus should not be applied directly to habitats in which amphibians breed (we note that there are available other glyphosate formulations that are designed for use in water to control weeds). However, aquatic habitats could be contaminated by aerial drift and/or run-off of terrestrial pesticides such as GROUNDCLEAR, especially in the confines of a residential garden; we stress that such habitats are increasingly important as amphibian breeding sites.

In addition, formulations such as GROUNDCLEAR are most likely to be applied in the spring and early summer, times when embryos and larvae of many amphibians may be in adjacent bodies of water. Thus, at least on a local scale, application of formulations such as GROUNDCLEAR in the vicinity of breeding ponds in principle could compromise the survivorship of young life-history stages. We note that studies of community effects in semi-natural aquatic mesocosms of glyphosate formulations intended for terrestrial use demonstrated considerable to complete mortality for the larvae of five anuran species (Relyea, 2005).

What are the magnitudes of the risks imposed by residential pesticides to amphibians in garden ponds in reality? These are difficult to assess, but (very?) rough estimates can be obtained by some simple calculation: we provide an example. GROUNDCLEAR is recommended for use with one part formulation mixed in four parts water. Imagine that 5 L of such a mixture is applied in the vicinity of a small garden pond containing a volume of 100 L of water. Assume that 0.1 L of the mixture enters the water by drift/run-off. That would give a concentration of 0.001 GROUNDCLEAR in the pond, which would appear to be sufficient to kill both species used in our study (see Table 1). Of course, the risk would decline as the volume of drift/run-off decreases and/or the volume of water in the pond increases.

We certainly acknowledge the artificiality of aspects of our laboratory study. Nevertheless, our results lead us to advocate use of the precautionary principle concerning application of pesticides in the vicinity of ponds in gardens used by breeding amphibians: do not do it unless you know it is safe. At present, this is tantamount to saying “don’t do it at all;” we stress the crucial need for additional research to determine the extent to which weed-free patios may result in frog-free ponds.

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