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Relative toxicity of the components of the original formulation of Roundup® to five North American anurans

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ABSTRACT

The responses of five North American frog species that were exposed in an aqueous system to the original formulation of Roundup® were compared. Carefully designed and un-confounded laboratory toxicity tests are crucial for accurate assessment of potential risks from the original formulation of Roundup® to North American amphibians in aquatic environments. The formulated mixture of this herbicide as well as its components, isopropylamine (IPA) salt of glyphosate and the surfactant MON 0818 (containing polyethoxylated tallowamine (POEA)) were separately tested in 96h acute toxicity tests with Gosner stage 25 larval anurans. Rana pipiens, R. clamitans, R. catesbeiana, Bufo fowleri, and Hyla chrysoscelis were reared from egg masses and exposed to a series of 11 concentrations of the original formulation of Roundup ® herbicide, nine concentrations of MON 0818 and three concentrations of IPA salt of glyphosate in static (non-renewal) aqueous laboratory tests. LC50 values are expressed as glyphosate acid equivalents (ae) or as mg/L for MON 0818 concentrations for comparison between the formulation and components. R. pipiens was the most sensitive of five species with 96h-LC50 values for formulation tests, for the five species, ranging from 1.80 to 4.22 mg ae/L, and MON 0818 exposures with 96h-LC50 values ranging from 0.68 to 1.32 mg/L. No significant mortality was observed during exposures of 96 h for any of the five species exposed to glyphosate IPA salt at concentrations up to 100 times the predicted environmental concentration (PEC). These results agree with previous studies which have noted that the surfactant MON 0818 containing POEA contributes the majority of the toxicity to the herbicide formulations for fish, aquatic invertebrates, and amphibians. These study results suggest that anurans are among the most sensitive species, and emphasize the importance of testing the herbicide formulation in addition to its separate components to accurately characterize the toxicity and potential risk of the formulation.

1. Introduction

Roundup® brand herbicides contain the active ingredient glyphosate, which is the most extensively used herbicide in the United States (Kiely et al., 2004). The original formulation of Roundup® contains the isopropylamine (IPA) salt of glyphosate and MON 0818 containing a polyethoxylated tallow amine (POEA) surfactant. Glyphosate is a broad spectrum, post-emergent herbicide, which works by inhibiting 5-enolpyruvyl shikimate-3-phosphate synthetase, an enzyme essential for production of aromatic amino acids in plants and some microorganisms (Franz et al., 1997). Animals obtain these aromatic amino acids from their diet and lack this enzyme; therefore, glyphosate is relatively nontoxic to animals (Giesy et al., 2000; Solomon and Thompson, 2003). POEA is a common adjuvant in glyphosate formulations, which enables the aqueous herbicide to stick to the surface of vegetation and aides the herbicide in penetrating the waxy cuticle on plant leaves (Giesy et al., 2000; Solomon and Thompson, 2003). Previous studies have indicated that the toxicity manifested by Roundup® herbicides to aquatic organisms is largely due to the surfactant in the mixture (Folmar et al., 1979; Mann and Bidwell, 1999; Edginton et al., 2004). In the United States, Roundup® branded herbicides are prohibited for direct application to water, however, these herbicides can enter aquatic systems through spray drift, unintended overspray, and to a limited extent through runoff from treated sites, incidentally exposing aquatic and semi-aquatic organisms (Giesy et al., 2000; Solomon and Thompson, 2003).

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Larval anurans have been identified as relatively sensitive organisms to glyphosate based herbicide exposures in laboratory and field studies compared to other aquatic species with acute LC_{50} values ranging from approximately 1–12 mg ae/L (Mann and Bidwell, 1999; Howe et al., 2004; Wojtaszek et al., 2004; Relyea, 2005a, 2005b, 2005c). Since Roundup® herbicides are used for both agricultural and silvicultural applications and relatively sensitive organisms such as larval anurans can be exposed, questions have arisen regarding the toxicity of these exposures (Mann and Bidwell, 1999; Howe et al., 2004; Relyea, 2005a, 2005b, 2005c). Therefore it is important to understand responses of these organisms to exposures of Roundup® formulations as well as the formulation components. Un-confounded laboratory tests using North American anuran species will help to discern the potential risks to these species from incidental exposures as well as the relative contribution of the components of Roundup® to the observed toxicity.

Five species of North American anurans, northern leopard frog (*Rana pipiens* Schreber), green frog (*Rana clamitans* Latreille), American bullfrog (*Rana catesbeiana* Shaw), Fowler's toad (*Bufo fowleri* Hinckley), and Cope's gray tree frog (*Hyla chrysoscelis* Laurenti) were chosen to determine the toxicity of the original formulation of Roundup® and its components to larval anurans. This research is intended to contribute to the accurate assessment of potential aquatic risks of the original formulation of Roundup® to North American amphibians. In order to predict responses to potential exposures and partition the toxicity of the components, the formulated mixture of this herbicide as well as its components were separately tested in 96 h acute toxicity tests with sensitive Gosner stage 25 (Gosner, 1960) larval anurans. The results of these acute toxicity tests were used to determine the relative contribution of the components, the IPA salt of glyphosate and the surfactant containing POEA, to the toxicity of the original formulation of Roundup®.

2. Materials and Methods

2.1. Test Substances

The original formulation of Roundup® (MON 2139) and components were supplied by Monsanto Company (St. Louis, MO, USA). The original formulation of Roundup® as supplied was a mixture of the IPA salt of glyphosate at 29.7% acid equivalent (ae) by weight and 15% MON 0818 containing POEA by weight. Separate components of the original formulation of Roundup®, IPA salt of glyphosate (CAS no. 38461-94-0) at 46.0% ae by weight and MON 0818 containing 69.4% POEA by weight (CAS no. 61791-26-2), were also tested individually. Stock solutions of the Roundup® formulation and the components used for toxicity tests were prepared at a nominal concentration of 1000 mg ae/L for the formulation and the glyphosate IPA salt, and 1000 mg/L for MON 0818 using NANOpure™ water.

2.2. Culture water

Water used for holding tanks, controls, and treatments was moderately hard water formulated to simulate general water characteristics of US lakes and streams (Sawyer et al. 1994; Wetzel, 2001). This water was comprised of 2.5 mg $CaCO_3$, 50.9 mg $NaHCO_3$, 24 mg $MgSO_4$, 16.5 mg $CaSO_4$, 32.5 mg $CaCl_2$, 1.05 mg KCl, 0.41 mg KNO_3, 0.009 mg K_2PO_4, 0.22 mL of 1000 ppm Cu reference standard, 0.11 mL of 1000 ppm Se reference standard, 0.22 mL of 1000 ppm Zn reference standard per liter of reverse osmosis filtered water. Water temperature was maintained at 20 ± 1 °C, pH ranged from 6.7 to 7.7, and dissolved oxygen was greater than 4.0 mg O_2/L.

2.3. Experimental design

Bioassays were performed according to the published US EPA method EPA-821-R-02–012 (U.S. Environmental Protection Agency, 2002). Chemical and physical measurements of testing conditions, dilution water, and test solutions were conducted according to the published American Society for Testing and Materials (ASTM) methods (ASTM, 2003). The aqueous tests were 96 h static non-renewal. Concentrations for definitive testing were determined from range-finding tests for the formulation as well as MON 0818. Concentrations tested included 0.3, 0.7, 1.0, 1.4, 1.7, 2.0, 2.4, 2.7, 3.2, 3.8, 5.0, and 7.0 mg ae/L for the Roundup® formulation. The large number and close spacing of testing concentrations in formulation tests enabled the NOEC and LC_{50} values to be calculated precisely. Concentrations for MON 0818 tests included 0.06, 0.18, 0.26, 0.37, 0.44, 0.59, 0.92, 1.25, and 2.00 mg/L. Stock solutions were diluted to definitive test concentrations in 3 L of water for each replicate.

Glyphosate IPA salt concentrations were based on the predicted environmental concentration (PEC) immediately following an application of herbicide at a recommended label application rate 0.84 kg ae/ha (0.75 lb ae/acre) (personal communication, Joy Honegger 2008) into a body of water with a depth of 20.3 cm. This depth corresponds to the depth proposed for a field study to be completed after the laboratory portion of this study, which would create ponds 20.3 cm deep including the worst case scenario depth of 15.2 cm from Solomon and Thompson, (2003) and 5.1 cm to account for evaporation calculated according to the evaporation rates for South Carolina and time of year corresponding to herbicide treatment to the ponds. Concentrations for glyphosate IPA salt tests included 0.42, 4.15, and 41.48 mg ae/L, which correspond to the PEC, ten times the PEC and 100 times the PEC.

Test vessels were 3.8 L glass jars filled with 3 L of test solution. In tests with the original formulation of Roundup®, there were four replicates per concentration and untreated control with 10 tadpoles per replicate. In MON 0818 and IPA salt of glyphosate tests, there were three replicates of each concentration and untreated control with 10 tadpoles per replicate. Tadpoles were not fed for the duration of the test to preserve water quality. Jars were gently aerated with single bubble aeration (ASTM, 2003). Frog eggs were field collected as available and tests were completed as eggs became available. All standard operating procedures were approved by Clemson University's Institutional Animal Care and Use Committee.

2.4. Animals

Egg masses were collected (*B. fowleri*, *R. catesbeiana*, *H. chrysoscelis*, *R. clamitans*) in Pickens and Greenwood Counties, South Carolina, or purchased from vendors (*R. pipiens* from Wards Natural Science, Rochester NY, Nasco, Fort Atkinson WI, and Carolina Biological Supply Co., Burlington NC, *R. catesbeiana* from Sullivan Co., Nashville TN and Carolina Biological Supply Co., Burlington NC). During holding, tadpoles were fed twice daily *ad libitum* a mixture of ground goldfish fish flakes (Tetra™) in water (Nace, 1974). Eggs and tadpoles were maintained in glass aquaria with a 16:8 light:dark cycle and single bubble aeration. Holding tanks were cleaned twice daily and up to 50% water changes were completed every other day to maintain good water quality. Tadpoles were reared to Gosner stage 25 prior to testing. Previous research has shown that this stage in amphibian development is more sensitive to exposures of contaminants than either embryo, earlier larval stages, later larval stages or adults (Berrill et al., 1994; Mann and Bidwell, 1999; Edginton et al., 2004; Howe et al., 2004).

2.5. Observed endpoint

The endpoint observed was mortality. Mortality was determined when an organism did not appear to have any respiratory functions or movement and did not respond to gentle prodding stimuli using a glass stir rod or removal from water (ASTM, 2003). Mortality was assessed and dead animals removed daily for four days.

2.6. Analytical

Analytical concentration verification was completed at Clemson University, Clemson South Carolina. Test solution samples were collected for glyphosate verification from dilution water, stock solution, and every replicate at all concentrations and controls immediately prior to adding animals to test chambers. Samples were stored in silanized glass vials at 3 °C prior to analysis. Glyphosate concentrations (reported as glyphosate acid equivalents, ae) were determined using Dionex Ultra-Mate-3000 High Performance Liquid Chromatography with autosampler and Variable Wavelength Detector system with Dionex Chromeleon software (Dionex Corp., Sunnyvale CA), and a YMC-Pack ODS-AM column with a 40 uL injection volume and 500 nm as the primary wavelength. Methods used for derivatization and analysis of glyphosate in water samples were supplied by Monsanto Company (Powell et al., 1990). A glyphosate analytical standard (99.8% purity) was used to create calibration standards. The range of recovery was 85–115% according to external standards.

2.7. Data analysis

Data were analyzed using SAS® Version 9.1 (SAS, 2007). 96-h LC_{50} values were considered significantly different from each other when their 95% confidence intervals did not overlap. Where appropriate, probit analysis was used to determine the no observed effect concentration (NOEC), LC_{50} values, and 95%