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LABORATORY STUDIES OF RESPONSES OF ANURAN AMPHIBIANS TO ROUNDUP EXPOSURES: REFERENCE TOXICANT AND COMPONENTS

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LABORATORY STUDIES OF RESPONSES OF ANURAN AMPHIBIANS TO ROUNDUP EXPOSURES: REFERENCE TOXICANT AND COMPONENTS

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ABSTRACT

Roundup® has been implicated as a possible cause for the declining amphibian populations in North America. Carefully designed laboratory toxicity tests are crucial for accurate risk assessment of the responses of anuran populations to incidental exposures of Roundup® herbicides. The overall objective of these studies was to determine the response of North American anuran species to exposures of Roundup® formulations and components to support or refute the claim that Roundup® is a factor in amphibian decline in North America. Aqueous 96 hour static non-renewal laboratory tests were utilized to (1) evaluate the effectiveness of copper sulfate as a reference toxicant in larval anuran toxicity testing for six species of anurans; (2) compare the toxicity of two formulations of Roundup® containing different salts of glyphosate and surfactant mixtures for three larval ranid species; (3) determine the relative contribution of the two components in the original formulation of Roundup® to the toxicity of the formulation for five species of anurans. Our results indicate that copper sulfate can serve as a suitable reference toxicant in larval amphibian toxicity testing because low concentrations of copper can be used to elicit significant responses in larval anurans which allow for detection of differences in sensitivities between species and accessions of organisms. The results of our study on the comparative toxicity of two formulations of Roundup® herbicides indicate that Roundup WeatherMax® is more toxic to larval anurans than the original formulation of Roundup®. Many Roundup® formulations, including WeatherMax® have proprietary mixtures of surfactants making it difficult to evaluate the source of the toxicity of the formulation, but we can speculate that the difference in surfactant between the two formulations is the cause for the difference in toxicity. Larval amphibian toxicity testing

procedures should be standardized to facilitate spatial and temporal comparisons between species, acquisitions, and laboratories. Our studies also suggest the importance of evaluating whole formulations in risk assessments rather than just the active ingredient to ensure safety for non-target species. The best way to mitigate risk to anuran species could be to control the surfactant portion of Roundup® formulations.

DEDICATION

To my grandmother. Thank you for all of your support and love.

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TABLE OF CONTENTS

Page

Table of Contents (Continued)

Page

LIST OF TABLES

List of Tables (Continued)

Table Page

LIST OF FIGURES

List of Figures (Continued)

Figure Page

CHAPTER I: INTRODUCTION

1.0 Amphibian Decline

The class Amphibia includes frogs, toads, newts, salamanders, and caecilians (Pough *et al*., 2004). Around 90% of all amphibians are anurans or frogs and toads (McDiarmid & Mitchell, 2000).Amphibians play an integral part in ecosystems as predators and prey (Pough *et al*., 2004). They are also important as water quality indicators. Their dependence on water for breeding and larval development, as well as their permeable skin and eggs, which can readily absorb toxic substances, makes them an appropriate sentinel species for aquatic habitats (Berrill *et al.*, 1994; Howe *et al.*, 2004; Mann *et al.*, 2003; McDiarmid & Mitchell, 2000).

Concern has arisen over the global decline of amphibian populations (Edginton *et al.*, 2004; Houlahan *et al.*, 2000; IUCN, 2006; Wojtaszek *et al.*, 2004). Almost one third of all species are considered threatened (IUCN, 2006). It is believed that as many as 120 species of amphibians have become extinct since the 1980's (IUCN, 2006). So far there have been no satisfactory explanations. Many potential factors have been identified. These include habitat loss, chytrid fungi, ultraviolet radiation, pollution, disease, and predation by invasive species (Houlahan *et al.*, 2000; IUCN, 2006).

2.0 Roundup® as a potential factor in amphibian decline

Some limited research has implicated Roundup formulations as a cause of amphibian decline (Mann & Bidwell, 1999; Relyea, 2004, 2005a, 2005b). Other publications and risk assessments (Giesy *et al.*, 2000; Solomon & Thompson, 2003; Thompson *et al.*, 2004; Wojtaszek *et al.*, 2004) have indicated that environmentally

relevant concentrations of Roundup® found in aquatic systems are insufficient to pose risks to native amphibians.

3.0 Reference toxicants for quality assurance

Careful laboratory experiments with sensitive North American anuran species could provide data regarding Roundup® as a contributing factor in amphibian declines. Well-planned experimental design is crucial, and an important part of that experimental design is a positive control or reference toxicant. A reference toxicant measures the response of the test population to a known and essentially unvarying positive control. A reference toxicant allows for assurance of the health of test organisms, as well as comparison between separate acquisitions of organisms, and between laboratories (Dorn *et al.*, 1987; Jop *et al.*, 1986; Lee, 1980). Many substances have been used as reference toxicants including cadmium, chromium, sodium chloride and copper among others (Lee, 1980). Lee (1980) published a list of characteristics for an ideal reference toxicant which includes: universal toxicity, solubility, persistence and stability, toxicity at low concentrations, rapid lethality, and can be readily measured and quantified. Measuring the responses of anuran species to a reference toxicant allows for temporal and spatial transfer of data. Absent the use of a reference toxicant, quality assurance data are limited to untreated control survival, which is necessary but not sufficient for insuring the health and unchanging sensitivity of test organisms. In this study, toxicity tests using the reference toxicant copper sulfate were initiated with toxicity tests of Roundup® formulations and components. Six North American species were tested including *Rana*

pipiens Schreber*, Rana sphenocephala* Cope*, Rana catesbeiana* Shaw*, Rana clamitans* Latreille*, Bufo fowleri* Hinkley*,* and *Hyla chrysoscelis* Laurenti.

4.0 Comparative toxicity of Roundup® formulations

Along with a well planned experimental design including use of a reference toxicant, it is important to consider the fact that there are many different formulations of Roundup®. Each formulation has different forms of glyphosate as well as adjuvants to increase efficacy of the formulation. Roundup® formulations generally consist of two components, glyphosate and a surfactant, which is the critical component in determining toxicity of the formulation. In fact, the majority of the toxicity of Roundup formulations has been attributed to the surfactant (Folmar *et al.*, 1970; Mann & Bidwell, 1999; Perkins *et al.*, 2000; Tsui & Chu, 2003). It is logical that if the toxicity of the formulation is largely attributed to the surfactant, and the surfactant is altered between formulations, then the toxicity of the formulations and the responses of species to those formulations will be altered.

This is an important consideration for anurans because several formulations have been tested to evaluate the role of glyphosate formulations in anuran toxicity (Edginton *et al.*, 2004; Howe *et al.*, 2004; Mann & Bidwell, 1999; Perkins *et al.*, 2000; Relyea, 2004, 2005a, 2005b; Thompson *et al.*, 2004; Wojtaszek *et al.*, 2004). Two formulations are widely used in agriculture and forestry settings, the original formulation of Roundup® and Roundup WeatherMax®. The responses of three North American anurans *R. pipiens, R. sphenocephala,* and *R. clamitans* were measured under similar laboratory conditions to both the original formulation of Roundup® and Roundup WeatherMax®.

5.0 Relative contribution to toxicity of the components of Roundup®

As mentioned previously, the original formulation of Roundup® is a binary mixture containing isopropylamine (IPA) salt of glyphosate and polyethoxylated tallowamine (POEA) surfactant. A large portion of the toxicity observed in Roundup® formulation has been attributed to the surfactant (Folmar *et al.*, 1970; Mann & Bidwell, 1999; Perkins *et al.*, 2000; Tsui & Chu, 2003). The relative contribution to toxicity of the components of Roundup® formulations to anuran species is not well known (Howe *et al.*, 2004; Mann & Bidwell, 1999; Perkins *et al.*, 2000). It is important to understand the relative contributions of glyphosate and surfactant to the toxicity of the mixture as well as any interaction the components have, such as synergism, to ensure safety for amphibians exposed to these formulations. This study involved five species of North American larval anurans, *R. pipiens, R. catesbeiana, R. clamitans, B. fowleri,* and *H. chrysoscelis*. The responses to the binary mixture, and its individual components were measured and the contribution to the toxicity of the formulation was discerned.

6.0 Objectives

The broad goal of this project was to determine the response of North American anuran species to exposures of Roundup® formulations and components to help answer the question: Is Roundup® is a factor in amphibian decline in North America? To accomplish this objective, three experiments were designed and conducted, each with the individual objectives. Aqueous 96h static non-renewal laboratory tests were utilized to (1) evaluate the effectiveness of copper sulfate as a reference toxicant in larval anuran toxicity testing for six species of anurans; (2) compare the toxicity of two formulations of

Roundup® containing different salts of glyphosate and surfactants for three larval ranid species; and (3) determine the relative contribution of the two components in the original formulation of Roundup® to the toxicity of the formulation for five species of anurans.

The objectives of the study using copper sulfate as a reference toxicant in amphibian testing were to: (1) measure the relative sensitivities of six larval anuran species to copper as copper sulfate in aqueous 96 hour acute toxicity tests; (2) measure the relative sensitivity of two separate acquisitions of *R. catesbeiana* and *H. chrysoscelis* and three separate acquisitions of *R. pipiens*; and (3) determine if copper sulfate can be used as a standard reference toxicant in larval amphibian toxicity testing.

The objectives of the study on the comparative toxicity of two formulations of Roundup® were to: (1) measure the response to exposures of the original formulation of Roundup® and Roundup WeatherMax® to three species of larval ranids; and (2) contrast the results of these exposures and compare with existing literature.

The objectives of the study on the relative contribution to toxicity of the components of the original formulation of Roundup® were to: (1) measure the response to exposures of the original formulation of Roundup® and its two components, IPA salt and POEA separately to five species of larval anurans; and (2) determine the relative contribution of the IPA salt and POEA to the toxicity of the mixture.

In the three chapters that follow, data are presented on using copper sulfate as a reference toxicant in larval amphibian testing, the comparative toxicity of two Roundup® formulations to three larval anurans, and the relative toxicity of the components of the original formulation of Roundup® to five larval anurans. The three chapters are presented as independent manuscripts for publication: therefore, some redundancy is necessary.

The three chapters will be submitted to the following journals for publication consideration:

1.) Copper sulfate as a reference toxicant in larval anuran toxicity testing; *Aquatic Toxicology*

2.) Comparative toxicity of two Roundup® brand herbicide formulations to three larval *ranid*s; *Ecotoxicology and Environmental Safety*

3.) Relative toxicity of the components of the original formulation of Roundup® to five North American anurans; *Archives of Environmental Contamination and Toxicology*

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CHAPTER II: COPPER SULFATE AS A REFERENCE TOXICANT IN LARVAL ANURAN TOXICITY TESTING

1.0 Introduction

A reference toxicant measures the response of the test population to a known and essentially unvarying positive control. Reference toxicants are recommended in toxicity studies by the United States Environmental Protection Agency (US EPA) for quality assurance (USEPA, 2002). Sensitivity and health of test organisms can be predicted with the use of reference toxicants. Reference toxicants also allow for comparison of different accessions of test organisms and results from laboratories (Jop *et al.*, 1986). Characteristics of an ideal reference toxicant are listed by Lee (1980) and include universal toxicity, solubility, persistence and stability, toxicity at low concentrations, rapid lethality, and can be readily measured and quantified. Many substances have been used as reference toxicants including chromium, cadmium, chloride and copper (Lee, 1980). We explored copper sulfate as a reference toxicant for amphibian toxicity testing. Copper has been used in toxicity testing for a variety of aquatic organisms including invertebrates, fish, and anurans (Boyd & Williams, 2003; Bridges *et al.*, 2002; Chen *et al.*, 2007; Landé & Guttman, 1973; Lombardi *et al.*, 2002; Murray-Gulde *et al.*, 2002; Suedel *et al.*, 1996; USEPA, 2007). Copper has many of the characteristics of a ideal reference toxicant including: solubility, toxicity at low concentrations, rapid lethality, and can be readily measured and quantified (Lee, 1980). Copper is a trace element and essential micronutrient for plants and animals. At elevated levels copper can become toxic. The bioavailability of copper depends on a number of factors including speciation,

ligand binding, physicochemical properties of water, adsorption to sediments and suspended particles, and the organism of interest (USEPA, 2007).

With increasing concern over amphibian population declines (Houlahan *et al.*, 2000; IUCN, 2006) there is a need for the use of a positive controls in toxicity testing of amphibians so results can be compared between accessions, studies, and laboratories. Not only could copper serve as a suitable reference toxicant in amphibian toxicity testing, but the data from these reference tests can contribute to better water quality standards that insure protection of sensitive amphibian species. Currently, there is only one amphibian species, the Boreal toad (*Bufo boreas*), included in the Aquatic Life Ambient Freshwater Quality Criteria – Copper (USEPA, 2007).

For this research, we chose five anuran species common to South Carolina, the southern leopard frog, *R. sphenocephala,* Fowler's toad, *B. fowleri,* Cope's gray treefrog, *H. chrysoscelis,* American bullfrog, *R. catesbeiana,* and green frog, *R. clamitans* and one species that has been frequently used in toxicity testing, the northern leopard frog, *R. pipiens*. *R. sphenocephala* is native to the eastern United States from New York to the Florida Keys and can be found in any type of freshwater habitat. Breeding can occur year round in some southern habitats and from March to June in northern areas (Conant R, 1998 ; Martsof *et al.*, 1980). *B. fowleri* are native to most habitats in the eastern United States and will breed in many types of water bodies (Conant R, 1998 ; Martsof *et al.*, 1980). Breeding in northern habitats occurs from approximately April to July and in southern habitats breeding occurs from approximately March to May (Conant R, 1998 ; Martsof *et al.*, 1980). *H. chrysoscelis* occurs in eastern North American and is mostly

arboreal (Conant R, 1998 ; Martsof *et al.*, 1980). *H. chrysoscelis* breeds from May to August (Conant R, 1998 ; Martsof *et al.*, 1980). *R. catesbeiana* are widely distributed throughout North America either as natives or introduced species and breeding season can be as long as February to October in its southern ranges (Conant R, 1998 ; Martsof *et al.*, 1980). *R. clamitans* occur in eastern North America and can be found in a variety of habitats including swamps, ponds, lakes, and slow moving rivers and streams (Conant R, 1998 ; Martsof *et al.*, 1980). *R. clamitans* generally breeds from May to June (Conant R, 1998 ; Martsof *et al.*, 1980). *R. pipiens* is common throughout much of northern North America and is found in diverse habitat types (Conant R, 1998 ; Martsof *et al.*, 1980). The breeding season for *R. pipiens* is March to June (Conant R, 1998 ; Martsof *et al.*, 1980).

The objectives of this study were to measure the relative sensitivities of six larval anuran species to copper as copper sulfate pentahydrate in 96h aqueous toxicity tests in order to determine if copper sulfate can be used as a reference toxicant in larval anuran toxicity testing. We first measured separately the sensitivities of *R. sphenocephala, B. fowleri, H. chrysoscelis, R. catesbeiana, R. clamitans,* and *R. pipiens* at Gosner stage 25 (Gosner, 1960) to copper sulfate. Second, we measured the relative intraspecific sensitivities of two different acquisitions of *R. catesbeiana* and *H. chrysoscelis* and three separate acquisitions of *R. pipiens* larvae. Third, we compared the relative interspecific sensitivities of the six species from this study and previous literature.

2.0 Materials and Methods

2.1 *Test Substances*

Copper sulfate pentahydrate was used to make all stock solutions for toxicity testing (Table 2.1) (CAS #7758-99-8, Fisher Scientific Inc.).

2.2 *Test Concentration Preparation*

Stock solutions for reference toxicant tests were prepared at a nominal concentration of 1000 mg Cu/L using NANOpure™ water. Dilution water used for test concentrations was moderately hard water (Table 2.2) formulated to simulate general water characteristics of US lakes and streams (Sawyer *et al.*, 1994; Wetzel, 2001).

2.3 *Experimental Design*

Bioassays were performed according to published US EPA methods (USEPA, 2002). Chemical and physical measurements of testing conditions, dilution water, and test solutions were conducted according to published American Society for Testing and Materials(ASTM) methods (ASTM, 2003). Tests were aqueous 96h static non-renewal. Tested exposures were: 25, 50, 75, 85, 95, 100, and 500 µg Cu/L. Testing vessels were 3.8 L glass jars filled to 3 L with test solution. Each of the concentrations and the control were replicated 3 times with 10 animals per replicate. Tadpoles were not fed during tests to avoid compromising water quality. Testing and holding conditions were constant for all species and all tests (Table 2.3) (ASTM, 2003; Edginton *et al.*, 2004; Gosner, 1960; Mann & Bidwell, 1999; Nace, 1974).

2.4 *Animals*

R. sphenocephala, B. fowleri, H. chrysoscelis, and *R. clamitans* egg masses were collected in Pickens and Greenwood Counties, South Carolina (Table 2.5). *R. pipiens* was purchased from Wards Natural Science Rochester NY, Nasco Fort Atkinson WI, and Carolina Biological Supply Co. Burlington NC, and *R. catesbeiana* was purchased from Sullivan Co. Nashville TN and Carolina Biological Supply Co. Burlington NC (Table 2.4). Prior to testing, eggs were allowed to develop into tadpoles which were maintained in ten gallon glass aquaria, other holding conditions remained constant throughout holding and testing (Table 2.5). Tadpoles were tested at Gosner stage 25 (Gosner, 1960). Water used for holding and test concentration dilution was formulated to control for the following parameters: pH, hardness, alkalinity, dissolved oxygen, ammonia, nitrate and nitrite, and chlorine (Table 2.5) (ASTM, 2003; Nace, 1974; USEPA, 2002). While in holding, tadpoles were fed twice daily, *ad libitum*, a mixture of water and ground goldfish flakes(Tetra®) (Nace, 1974). Extra food was removed and tanks were cleaned two times daily and up to 50% water changes were completed every other day to ensure water quality.

2.5 *Endpoints*

Mortality was verified when an organism did not respond to gentle prodding stimuli and did not appear to have any respiratory functions (ASTM, 2003). Mortality was measured every 24 hours for 4 days.

2.6 *Analytical*

Test solution samples were collected for copper concentration verification from every replicate at all concentrations and controls immediately prior to adding animals to test jars. Reference toxicant test samples were acidified with trace metals grade nitric acid (CAS #7697-37-2, Fisher Scientific Inc.) after collection and kept at 3°C prior to analysis. Copper concentrations less than 500 µg Cu/L were determined using a Perkin-Elmer Atomic Absorption spectrophotometer (5100PC model) and US EPA method # 220.2 Atomic Absorption, Furnace Technique (USEPA, 1979). Copper concentrations greater than 500 µg Cu/L were determined using flame atomic absorption and performed according to Method 200.1 (USEPA, 1991).

2.7 *Data Analysis*

Data were analyzed using SAS® Version 9.1 (SAS, 2007). Not all data met the assumptions for parametric analysis. Where appropriate, probit analysis was used to determine the lowest observed effect concentrations (LOEC), no observed effect concentrations (NOEC), LCx values, and 95% confidence intervals (CI). Non-parametric analyses were conducted using two methods. The US EPA MS-DOS application for trimmed Spearman-Karber analysis was used to obtain LC50 values and 95% confidence intervals. Non-parametric rank converted ANOVA's, equivalent to Kruskal-Wallis and Wilcoxon Rank Sum, with Dunnett's test analyses were used to determine LOEC and NOEC values in these cases. LC50 values were defined as significantly different when 95% confidence intervals did not overlap (Thompson *et al.*, 2004). Differences in

concentration-response curves were tested for significance using ANCOVA. Regression analysis (SAS®) was used to generate potency curves for each test.

3.0 Results and Discussion

3.1 *Species sensitivities*

There were both interspecies and intraspecies differences in the relative sensitivities of the six anuran species tested. Control mortality was less than 10% in all tests. *B. fowleri* was the most sensitive species tested with a 96h-LC50 value of 12 µg Cu/L (Table 5). One acquisition of *R. pipiens* was the least sensitive to exposures of copper sulfate with 96h-LC50 value of 116 µg Cu/L (Table 2.6).

3.2 *Sensitivities of multiple acquisitions of a single species:*

The two separate acquisitions of *H. chrysoscelis* had LC50 values of 27 and 35 µg Cu/L and were not significantly different from each other (ANCOVA p = 0.84) (Table 2.6, Figure 2.3). The two acquisitions of *R. catesbeiana* had 96h-LC50 values of 61 and 56 µg Cu/L and were not significantly different from each other (ANCOVA p = 0.53) (Table 2.6, Figure 2.4). Our first acquisition of *H. chrysoscelis* was field collected in July 2007 from Pickens County, South Carolina and our second acquisition was collected in July 2008 from Greenwood County, South Carolina. The first acquisition of *R. catesbeiana* was from Sullivan Co. (Nashville, TN) in July of 2007 and the second acquisition of *R. catesbeiana* was from Carolina Biological Supply Co. (Burlington NC) in June 2008. These acquisitions of *H.* chrysoscelis and *R. catesbeiana* had similar health and sensitivity to the reference toxicant. These results suggest that effective and

consistent rearing and holding conditions is important to quality assurance and perhaps more important than collecting from the same site or vendor. The three acquisitions of *R. pipiens* had a range of sensitivities with 96h-LC50 values from 33 µg Cu/L to 116 µg Cu/L, with acquisition one and two not significantly different from each other (ANCOVA $p = 0.96$) and acquisition three being significantly different from both acquisition one and two (ANCOVA $p < 0.0001$) (Table 2.6, Figure 2.6). Many factors could have affected the sensitivities of these organisms. All three accessions were from vendors. This adds the stress of shipping which could affect the sensitivity of the organisms. Our least sensitive accession of *R. pipiens* was from Carolina Biological which is located nearby in Burlington, NC. The remaining two accessions which were more sensitive (96h-LC50 values of 33 and 58 µg Cu/L) were shipped from vendors located in Wisconsin and New York. Although we expended considerable effort to have the shipments delivered overnight and to keep conditions of acclimation and holding stable once egg masses arrived at the laboratory, the temperature during shipping, distance shipped, and handling of the shipments could affect the sensitivities of the organisms. By collecting egg masses from nearby sources, we were able to supervise the collection methods and conditions, minimize the transport stress, and maintain stable holding conditions.

3.3 *Results of other studies on exposures of copper to amphibians*

Chen *et al.* (2007) reported survival of *R. pipiens* larvae (Gosner 25-42) in a chronic test with copper sulfate at 100 µg Cu/L was significantly decreased compared to

controls. Landé and Guttman (1973) also reported a LC50 value of 150 µg Cu/L for newly hatched *R. pipiens* tadpoles exposed to copper sulfate (Table 2.7). Bridges *et al.* (2002) reported an LC50 value for Gosner stage 25 *R. sphenocephala* tadpoles of 230 µg Cu/L (Table 2.7). These data are somewhat greater than the values obtained in this study and difference could be attributed to differences in age of organisms used in testing as well as water chemistry and testing and holding conditions. Lombardi *et al.* (2002) tested the sensitivity of *R. catesbeiana* tadpoles to copper oxychloride, a fungicide, in acute aqueous 96h toxicity tests (Table 2.7). The reported 96h-LC50 in this study was 2830 µg Cu/L. The author does not specify the stage in development of tadpoles during testing which could account for the relatively high 96h-LC50 reported in this study.

3.4 *Sensitivity of larval anurans compared to other species*

The six larval anuran species that were tested in this research are relatively sensitive to copper. The US EPA's Aquatic Life Ambient Freshwater Quality Criteria – Copper reports the Species Mean Acute Value (SMAV) which is an average LC50 value for each species calculated from published LC50 values which were normalized to a standard set of water chemistry parameters to facilitate comparison (USEPA, 2007). The SMAVs for invertebrates (Table 2.7) *Ceriodaphnia dubia*, *Daphnia magna*, and *Hyalella azteca* indicate these three species are relatively sensitive organisms to copper exposures and they are more sensitive than all anuran species we tested, except *B. fowleri*, the most sensitive species tested (96h-LC50 value of 12 µg Cu/L) (Table 2.6, 2.7)(USEPA, 2007). The six larval anuran species with 96h-LC50 values ranging from 12 to 116 µg Cu/L

(Table 2.6) are similar in sensitivity to fish species such as *Pimephales promelas* and *Oncorhynchus mykiss* which have SMAVs of 69.39 and 22.19 µg Cu/L (Table 2.7) (USEPA, 2007). Only one species of toad, the Boreal toad *Bufo boreas*, is listed in the US EPA water quality criteria mentioned above, SMAV of 47.49 µg Cu/L (Table 2.7) (USEPA, 2007). This value is within the range of 96h-LC50 values of the six anuran species tested in this research.

3.5 *Copper as a reference toxicant*

The results of this study suggest that copper sulfate can serve as a suitable reference toxicant for larval amphibian toxicity testing. Copper has many of the characteristics of a good reference toxicant including solubility in water, toxicity at low concentrations, rapid lethality (96h tests captured the period of action), and is easily measured in water samples with atomic absorption spectrophotometry (Lee, 1980). The relatively high potency of copper sulfate provides the ability to discern interspecies and intraspecies differences in sensitivity. Copper is also toxic to other organisms including invertebrates, fish, and plants which allows for comparison not only within and among species of amphibians but also among different organisms including invertebrates and fish.

4.0 Tables and Figures

Table 2.1: Copper sulfate structure, formula, and CAS No. (Fisher_Scientific, 1999)

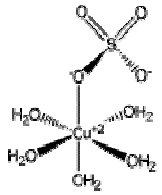
Copper Sulfate	$CuSO_4 \times 5H_2O$
Structure	
CAS No.	7758-99-8

Table 2:2 Substances used to amend reverse-osmosis water to approximate U.S. stream and lake water characteristics (Sawyer *et al.*, 1994; Wetzel, 2001)

Substance	Moderately Hard Dilution Water
CaCO3	2.5 mg / L
NaHCO3	50.9 mg / L
MgSO4 x 7H2O	24 mg / L
CaSO4 x 2H2O	16.5 mg / L
CaCl2 x 2H2O	32.5 mg / L
KCl	1.05 mg / L
KNO3	0.41 mg / L
K2PO4	0.00917 mg / L
Cu Standard (1000ppm)[a]	0.22 mL (110-L)
Se Standard (1000ppm)[b]	0.11 mL (110-L)
Zn Standard (1000ppm)[c]	0.22 mL (110-L)

[a] (Fisher Scientific, 1997a); [b] (Fisher Scientific, 1997b);

[c] (Fisher Scientific, 1997c)

Table 2.3: Holding and testing conditions for larval anurans

Test type	Static non-renewal
Duration	96h
Replicates/treatment	4
Organisms per exposure chamber	10
Endpoint	Mortality
Size of testing chamber	3.8 liters
Volume of dilution in exposure chamber	3 liters
Age of animals[a]	Gosner 25
Simulated site water	Moderately hard
Size of holding vessel	37.9 liter glass aquarium
Volume of dilution in holding	> 1 liter / 50 larvae
Feeding regime[b]	*ad libitum* (Holding) not fed (Testing)
Temperature	20 ± 1 (^{o}C)
Light quality	Cool White
Light intensity	86 ± 8.6 µE/sec
Photoperiod	16-8 light-dark cycle
Aeration[c]	Single-bubble

[a] (Gosner, 1960); (Edginton *et al.*, 2004); (Mann & Bidwell, 1999);

[b] (Nace, 1974); [c] (ASTM, 2003)

Table 2.4: Sources of 6 species of anurans used in copper sulfate reference toxicant tests

Species	Source
Rana sphenocephala	Field collected, Pickens Co. SC, USA
R. clamitans	Field collected, Pickens Co. SC, USA
Bufo fowleri	Field collected, Pickens Co. SC, USA
Hyla chrysoscelis 1	Field collected, Pickens Co. SC, USA
H. chrysoscelis 2	Field collected, Greenwood Co. SC, USA
R. catesbeiana 1	Sullivan Co. Nashville TN, USA
R. catesbeiana 2	Carolina Biological Supply Co. Burlington NC, USA
R. pipiens 1	Nasco Fort Atkinson WI, USA
R. pipiens 2	Wards Natural Science Rochester NY, USA
R. pipiens 3	Carolina Biological Supply Co. Burlington NC, USA

Table 2.5: Water chemistry parameters for testing and holding water (ASTM, 2003; Nace, 1974; USEPA, 2002)

Water Chemistry	Dilution and Test Solution Conditions
pH	6.5 – 8.2
Hardness	150-250 mg/L as $CaCO_3$
Alkalinity	150-250 mg/L as $CaCO_3$
Dissolved Oxygen	≥ 4.0 mg O_2 / L
Ammonia	< 0.2 mg/L
Nitrate & Nitrite	< 0.3 mg/L as Nitrogen
Fluoride	< 1.5 mg/L
Chlorine	< 11 μg/L

Table 2.6: Response of six species of larval anurans to exposures of copper sulfate reference toxicant in aqueous 96h static non-renewal toxicity tests

Species	96h LC50 (95%CI) in µg Cu/L		Potency equation
Bufo fowleri	12	(10, 14)	y = 169.8x + 18.8
Hyla chrysoscelis 2	27	NR	y = 1.8x -4.8
Rana pipiens 1	33	(27, 41)	y = 2.0x - 8.1
H. chrysoscelis 1	35	(32, 38)	y = 1.5x - 2.2
R. catesbeiana 1	56	(50, 63)	y = 0.1x - 47.1
R. pipiens 2	58	(51, 65)	y = 0.1x + 61.2
R. catesbeiana 2	61	(55, 67)	y = 0.2x + 16.2
R. clamitans	70	(63, 78)	y = 1.4x - 41.8
R. sphenocephala	93	(72, 120)	y = 0.1x + 28.1
R. pipiens 3	116	(97, 138)	y = 0.9x - 32.9

Table 2.7: LC50 values for acute aqueous toxicity tests for copper from literature

Species	Acute LC50 in µg Cu/L	Reference
Ceriodaphnia dubia	5.93[a]	(USEPA, 2007)
Daphnia magna	6.00[a]	
Hyalella azteca	12.07[a]	
Oncorhynchus mykiss	22.19[a]	
Pimephales promelas	69.63[a]	
Bufo boreas	47.49 [a]	
Rana pipiens	150	(Landé & Guttman, 1973)
R. sphenocephala	230	(Bridges *et al.*, 2002)
R. catesbeiana	2830	(Lombardi *et al.*, 2002)

[a] Species Mean Acute Value (SMAV) is an average acute LC50 value normalized to a standard set of water chemistry parameters for comparison; (USEPA, 2007)

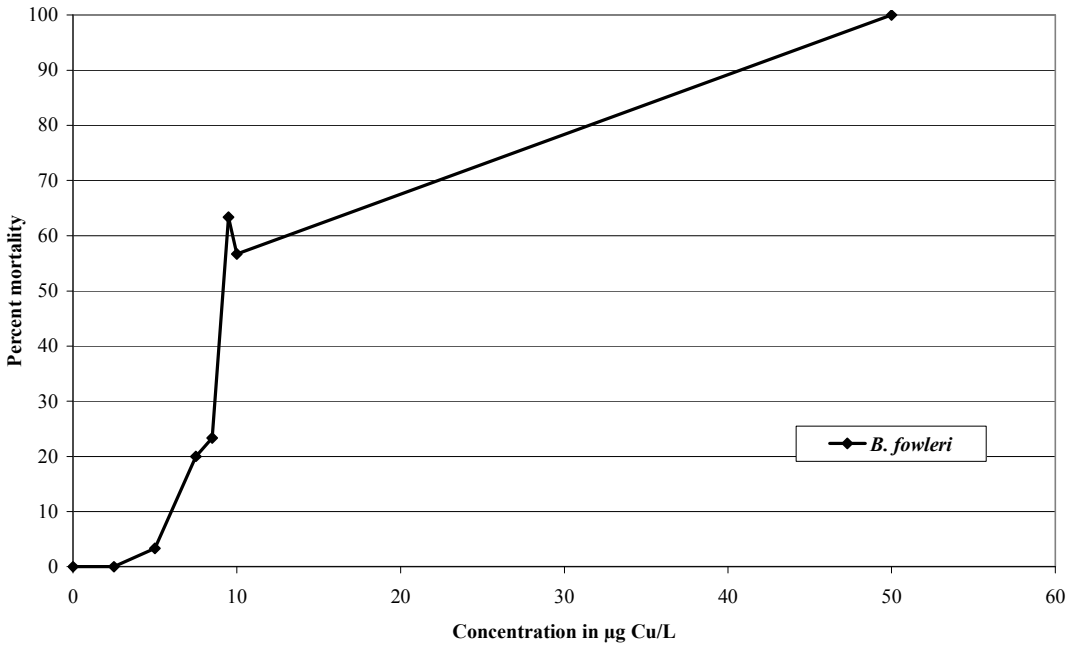


Figure 2.1: Response of *Bufo fowleri* to copper sulfate reference toxicant in 96h acute static non-renewal aqueous toxicity tests

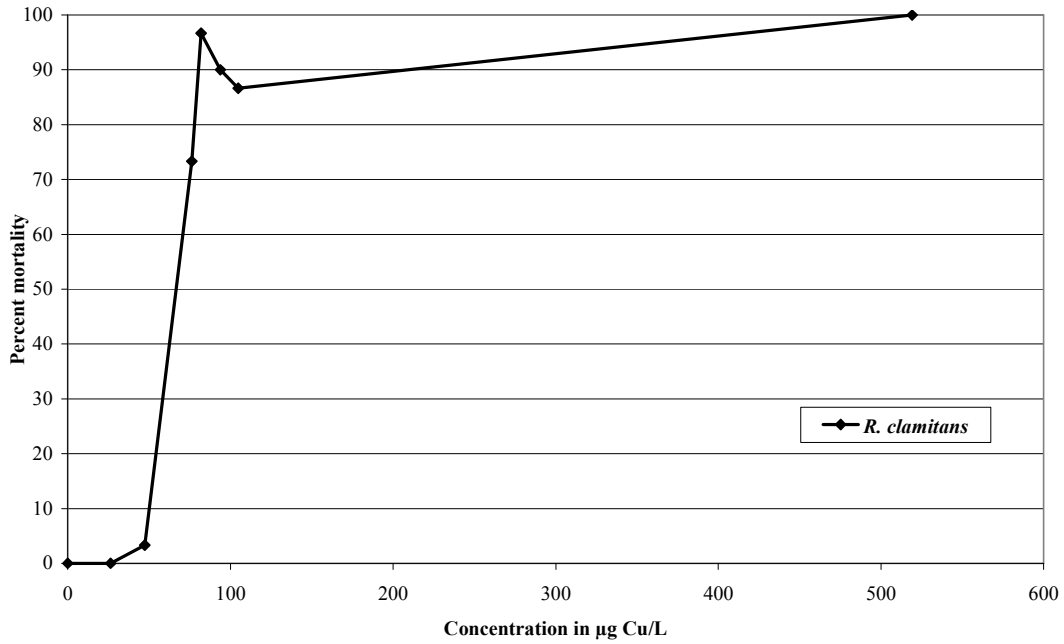


Figure 2.2: Response of *Rana clamitans* to copper sulfate reference toxicant in 96h acute static non-renewal aqueous toxicity tests

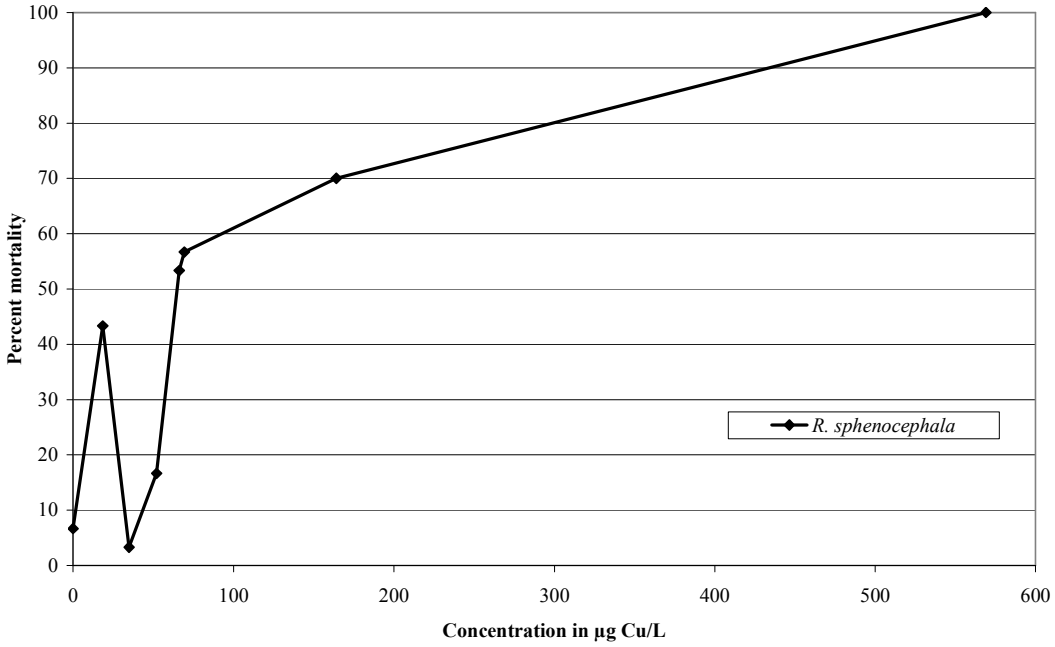


Figure 2.3: Response of *Rana sphenocephala* to copper sulfate reference toxicant in 96h acute static non-renewal aqueous toxicity tests

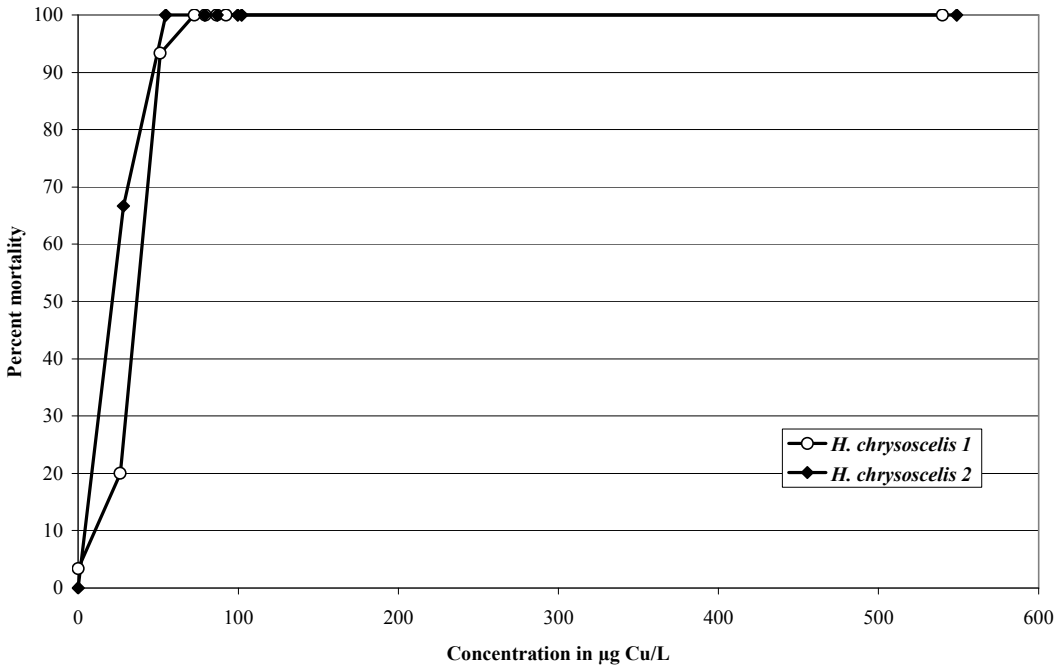


Figure 2.4: Responses of two accessions of *Hyla chrysoscelis* to copper sulfate reference toxicant in 96h acute static non-renewal aqueous toxicity tests

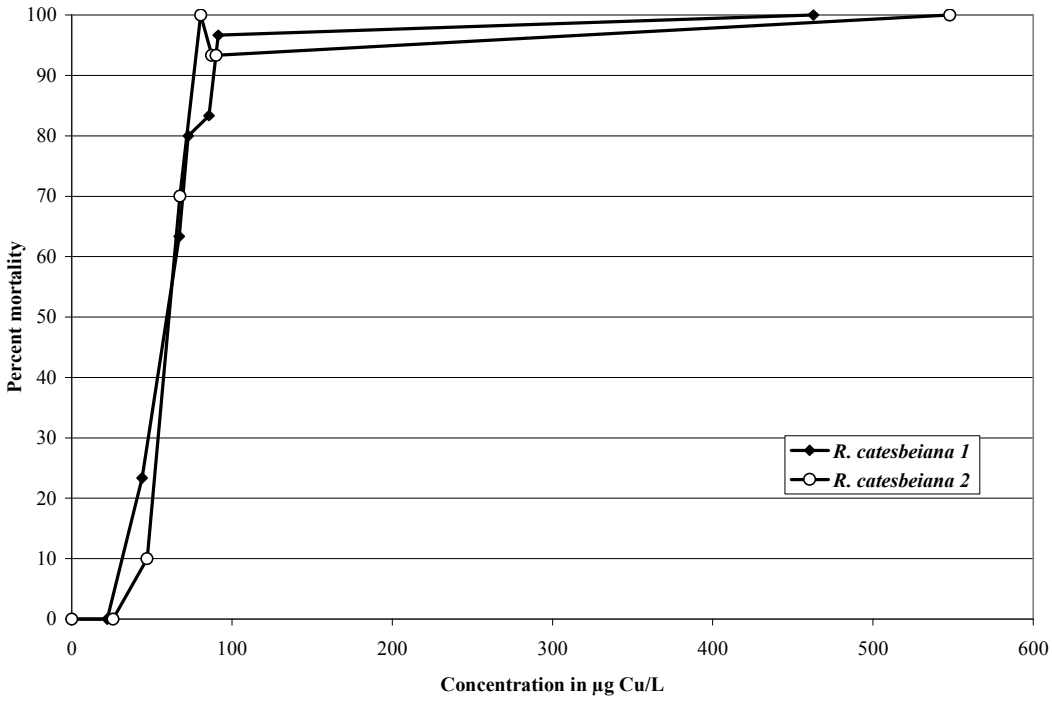


Figure 2.5: Responses of two accessions of *Rana catesbeiana* to copper sulfate reference toxicant in 96h acute static non-renewal aqueous toxicity tests

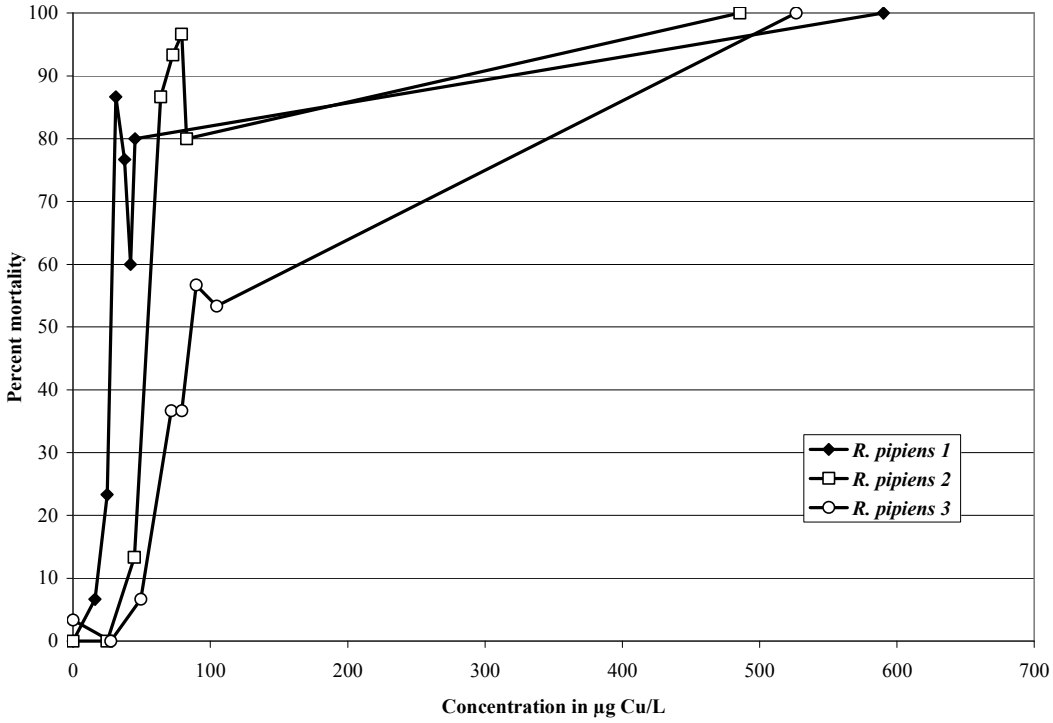


Figure 2.6: Responses of three accessions of *Rana pipiens* to copper sulfate reference toxicant in 96h acute static non-renewal aqueous toxicity tests

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CHAPTER III: COMPARATIVE TOXICITY OF TWO ROUNDUP® BRAND HERBICIDE FORMULATIONS TO THREE LARVAL RANIDS

1.0 Introduction

Glyphosate is the most applied agricultural pesticide in the United States (Kiely *et al.*, 2004) and is the active ingredient in Roundup® brand herbicide formulations. Two major uses of Roundup® formulations are for control of unwanted vegetation in agriculture and silviculture settings. In agricultural settings, Roundup® is used for Roundup Ready® crops, preparation of fields for crop planting and no-till or conservation farming (Monsanto, 2004). In silviculture, Roundup® formulations are used for the removal of competing vegetation, site preparation, and weed control (Cantrell, 1985). Roundup® formulations containing glyphosate can enter aquatic systems through spray drift, overspray, and runoff from treated sites (Giesy *et al.*, 2000; Solomon & Thompson, 2003). During both agriculture and silviculture applications, adjacent aquatic systems and ephemeral pools, as well as indigenous fauna such as amphibians, may be incidentally exposed.

There has been concern over declining amphibian populations (Houlahan *et al.*, 2000; IUCN, 2006). Amphibians can be exposed to water contaminants such as pesticides at multiple stages in their life cycle. Aquatic embryos, larval stages and adults are susceptible to exposure to water contamination due to their dependence on water for breeding and early life stages and their highly permeable skin (Berrill *et al.*, 1994; Howe *et al.*, 2004; Mann *et al.*, 2003; McDiarmid & Mitchell, 2000). Recently, questions have arisen regarding the toxicity of exposures of Roundup® formulations to amphibians

(Mann & Bidwell, 1999; Relyea, 2005a, 2005b, 2005c). Other publications and risk assessments (Giesy *et al.*, 2000; Solomon & Thompson, 2003; Thompson *et al.*, 2004; Wojtaszek *et al.*, 2004) have indicated that environmentally relevant concentrations of Roundup® found in aquatic systems as result of overspray, drift, and runoff are insufficient to pose risks to amphibians.

The two formulations of Roundup® used in this research, the original formulation of Roundup® and Roundup WeatherMax®, are binary mixtures containing a salt of glyphosate and a surfactant. The original formulation of Roundup® contains the isopropylamine (IPA) salt of glyphosate and a surfactant, polyethoxylated tallow amine (POEA). In Roundup WeatherMax®, the potassium salt of glyphosate is used along with a surfactant that is a proprietary mixture. Glyphosate's herbicidal activity involves inhibition of the enzyme, 5-enolpyruvyl shikimate-3-P synthetase, which is essential in aromatic amino acid synthesis (Franz *et al.*, 1997). Animals lack this synthesis pathway therefore glyphosate is relatively nontoxic to animals (Giesy *et al.*, 2000; Solomon & Thompson, 2003). Surfactants are a common adjuvant in herbicide formulations. They allow the liquid herbicide to stick to vegetation and penetrate the surface of the plant leaves (Giesy *et al.*, 2000; Solomon & Thompson, 2003). Previous studies have noted that the toxicity of the original formulation of Roundup® to non-target species is primarily from the surfactant component (Folmar *et al.*, 1970; Giesy *et al.*, 2000; Howe *et al.*, 2004; Mann & Bidwell, 1999; Perkins *et al.*, 2000; Solomon & Thompson, 2003; Thompson *et al.*, 2004; Tsui & Chu, 2003). Roundup Weather Max® has a proprietary surfactant and data regarding its toxicity to aquatic species have not been published.

Aqueous 96h copper sulfate reference toxicant tests were initiated with tests of the Roundup® formulations to monitor health and sensitivity of test organisms.

Unconfounded laboratory tests using North American anuran species can discern the potential risks to these species from incidental exposures to Roundup® formulations. By testing two formulations of Roundup® with different surfactant components we can determine the relative risk of these formulations for amphibians. Testing three species in the same genus can help determine if closely related species respond similarly to these formulations. The objectives of this research were (1) to measure the toxicity of the original formulation of Roundup® containing POEA to northern leopard frog (*Rana pipiens* Schreber), southern leopard frog (*Rana sphenocephala Cope*) and green frog (*Rana clamitans* Latreille); (2) to measure the toxicity of Roundup WeatherMax® containing a proprietary surfactant to *R .pipiens, R. sphenocephala, and R. clamitans;* and (3) to contrast the results from these exposures and compare with existing literature.

2.0 Materials and Methods

2.1 Chemicals

Both Roundup® formulations were provided by Monsanto Co. (St. Louis MO). The original formulation of Roundup® is a binary mixture of the isopropylamine (IPA) salt of glyphosate (29.7% acid equivalents (AE)) (Table 3.1) and polyethoxylated tallow amine (POEA) surfactant (15%). Roundup WeatherMax® formulation is composed of the potassium salt of glyphosate (39.9 AE %) and a proprietary mixture of surfactants.

Copper sulfate pentahydrate was used to make all copper reference toxicant stock solutions (CAS #7758-99-8, Fisher Scientific Inc.).

Stock solutions for Roundup® formulations used for toxicity testing were prepared at a nominal concentration of 1000 mg AE/L. Stock solutions for reference toxicant tests were prepared at a nominal concentration of 1000 mg Cu/L using NANOpure™ water. Dilution water used for test concentrations was moderately hard water (Table 3.2) formulated to simulate general water characteristics of US lakes and streams (Sawyer *et al.*, 1994; Wetzel, 2001) and this water was also used for acclimating and holding animals prior to testing.

2.2 Animals

Egg masses were field collected (*R. sphenocephala* and *R. clamitans*) in Pickens County, South Carolina, or purchased from vendors (*R. pipiens* from Wards Natural Science, Rochester NY and Carolina Biological Supply Co., Burlington NC). Egg masses were quarantined and acclimated to water and laboratory conditions and the health of tadpoles was closely monitored (ASTM, 2003). During holding, tadpoles were fed twice daily, *ad libitum*, a mixture of ground goldfish fish flakes (Tetra®) in water (Nace, 1974). Holding tanks were cleaned twice daily and up to 50% water changes were completed every other day to ensure water quality. Tadpoles were reared to Gosner stage 25 (Gosner, 1960) prior to testing.

2.3 Experimental design

Bioassays were performed according to published US EPA methods (USEPA, 2002). Chemical and physical measurements of testing conditions, dilution water, and test solutions were conducted according to published American Society for Testing and Materials(ASTM) methods (ASTM, 2003). Aqueous tests were 96 hour static non-renewal. Concentrations for definitive testing were determined by range finding tests. Formulation 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 both Roundup® formulations. Reference toxicant test exposures were: 25, 50, 75, 85, 95, 100, and 500 µg Cu/L. Testing vessels were 3.8 L glass jars filled with three L of test solution. There were four replicates per concentration and untreated control with 10 tadpoles per replicate in formulation tests and there were three replicates per concentrations and control with 10 tadpoles per replicate in reference toxicity tests. Tadpoles were not fed for the duration of the test to preserve water quality. Test jars were gently aerated with single bubble aeration (ASTM, 2003), similar to holding tanks (Table 3.3). Water used for holding and test concentration dilution was controlled for the following parameters: pH, hardness, alkalinity, dissolved oxygen, ammonia, nitrates and nitrites, and chlorine (Table 3.4) (ASTM, 2003; Nace, 1974). Holding and testing conditions were consistent for all species (Table 3.3).

2.4 Endpoints

The primary endpoint 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 (ASTM, 2003). Endpoints were measured and dead animals removed daily for 4 days.

2.5 Analytical

Test solution samples were collected for concentration verification from all replicates, concentrations, and controls immediately prior to adding animals to test jars. Samples were stored in silanized glass vials at 3°C prior to analysis. Glyphosate concentrations were determined using Dionex Ultra-Mate-3000 High Performance Liquid Chromatography (HPLC) with autosampler and Variable Wavelength Detector system with Dionex Chromeleon software (Dionex Corp., Sunnyvale CA). Method used for derivatization and analysis of glyphosate in water samples was supplied by Monsanto Co. (St. Louis, MO) (Powell *et al.*, 1990). Copper concentrations below 500 µg Cu/L were determined using a Perkin- Elmer Atomic Absorption spectrophotometer (5100PC model) and EPA method # 220.2 Atomic Absorption, Furnace Technique (USEPA, 1979). Copper concentrations at or above 500 µg Cu/L were determined using flame atomic absorption and performed according to Method 200.1 (USEPA, 1991).

2.5 Data Analysis

Data were analyzed using SAS® Version 9.1 (SAS, 2007). Not all data met the assumptions for parametric analysis. Where appropriate, probit analysis was used to determine lowest observed effect concentrations (LOEC), no observed effect concentrations (NOEC), LCx values, and 95% confidence intervals (CI). Non-parametric

analysis was conducted using two programs. The US EPA MS-DOS application for trimmed Spearman-Karber analysis was used to obtain 96h-LC50 values and 95% confidence intervals. Non-parametric rank converted ANOVA's, equivalent to Kruskal-Wallis and Wilcoxon Rank Sum with Dunnett's test analyses were used to determine LOEC and NOEC values in these cases. Differences in concentration-response curves were tested for significance using ANCOVA. Regression analysis (SAS®) was used to generate potency slopes for each test.

3.0 Results

In tests with copper sulfate reference toxicant, all frog species had similar sensitivities with LC50's ranging from 0.06 to 0.12 mg Cu/L (Table 7). All exposures of both formulations and reference toxicant tests were verified analytically. HPLC analysis for glyphosate concentration was performed on both formulation tests for all species tested. Recovery of glyphosate was between 85 and 115% for all tests. Copper concentrations were determined by atomic absorption spectrophotometry. The analytically verified replicates for each concentration were added and the mathematical mean of those values was calculated. The statistical analysis and results were based on these analytically verified mean values. Control mortality was less than 10% in all tests.

The linear portion of the potency curve, from the LOEC to the concentration eliciting 100% mortality was used to calculate the potency slope. The linear equation calculated from this portion of the potency curve contains a key piece of information: the degree of response exhibited by a population of organisms to increasing concentrations of

a toxicant, which can be shown by the slope of the concentration-response line (Perkins *et al.*, 2000). Another piece of information important in evaluating differences among species is the threshold level which can be estimated by averaging the NOEC and LOEC (Suter, 1990). We did not log-transform these data so we were able to more accurately pinpoint LC50 values and estimate the best fitting potency slope for the range of action.

In the toxicity tests with the original formulation of Roundup®, *R. pipiens* was the most sensitive species tested, followed by *R. sphenocephala* and *R. clamitans* with 96h-LC50 values ranging from 1.80 to 4.55 mg AE/L (Table 3.5). *R. clamitans* was 2.5 times less sensitive than *R. pipiens*. Potency slopes for *R. pipiens*, *R. sphenocephala*, and *R. clamitans* were all significantly different from zero (p-values of 0.0002, < 0.0001, and < 0.0001 respectively) (Figures 3.1-3.3). The potency slopes for exposures to the original formulation of Roundup® were significantly different from each other ($p < 0.0001$).

R. sphenocephala was the most sensitive species tested to exposures of Roundup WeatherMax® followed by *R. pipiens*, and *R. clamitans* with 96h-LC50 values ranging from 1.37 to 2.77 mg AE/L (Table 3.6). Potency slopes for Roundup WeatherMax® exposures for all three species were significantly different from zero ($p < 0.0001$) and significantly different from each other ($p < 0.0001$) (Figures 3.1-3.3).

Roundup WeatherMax® was more toxic than the original formulation of Roundup® for two of the species tested, *R. sphenocephala* and *R. clamitans*. *R. sphenocephala* was 1.5 times more sensitive to WeatherMax® and *R. clamitans* was 1.6 times more sensitive to WeatherMax®. Potency slopes for Roundup WeatherMax® for *R. sphenocephala* and *R. clamitans* also had steeper slopes and lower thresholds than for the

original formulation of Roundup®. *R. pipiens* was more sensitive to the original formulation of Roundup® with a 96h-LC50 of 1.80 mg AE/L compared a 96h-LC50 for WeatherMax® of 2.27 mg AE/L. Potency slopes for the original formulation of Roundup® were significantly different from potency slopes for Roundup WeatherMax® for all three species ($p < 0.0001$).

4.0 Discussion

Differences in the LC50 values for closely related species such as *R. pipiens* and *R. sphenocephala* suggest that evolutionary relatedness cannot be used to accurately estimate responses of two related species. Howe *et al.* (2004) noted that in tests of the original formulation of Roundup® that three species in the same genus, *R. pipiens, R. sylvatica* and *R. clamitans* did not respond similarly to exposures with 96h-LC50 values of 2.9, 5.1 and 2.0 mg AE/L respectively (Table 3.7). While *R. pipiens* was the most sensitive species tested under these conditions and with these chemicals, the wide range in LC50 values among these closely related species indicates the importance of acquiring data from multiple species and the risk of extrapolation from one species to another even within the same genus.

The variance in responses of anuran species and their sensitivities relative to other commonly tested animal species suggests that anurans should be evaluated for ecological risks from application of extensively used herbicides and other high volume high use chemicals (Table 3.7). Currently, amphibians are not typically included in toxicity

testing of chemicals for their safety of application in the environment. An accurate environmental risk assessment needs to include these relatively sensitive species.

As mentioned before, previous studies have found that the majority of the toxicity of Roundup® formulations comes from the surfactant component (Folmar *et al.*, 1970; Giesy *et al.*, 2000; Howe *et al.*, 2004; Mann & Bidwell, 1999; Perkins *et al.*, 2000; Solomon & Thompson, 2003; Thompson *et al.*, 2004; Tsui & Chu, 2003). Of the two formulations tested in this study, Roundup WeatherMax® appears to be the more toxic formulation for two of the three species tested. In a concurrent study, three additional species were tested for their sensitivities to Roundup WeatherMax® and the original formulation of Roundup®, Cope's gray treefrog *Hyla chrysoscelis,* Fowler's toad *Bufo fowleri, and* American bullfrog *Rana catesbeiana*. Of those three species, *B. fowleri* and *R. catesbeiana* were more sensitive to Roundup WeatherMax® while *H. chrysoscelis* was more sensitive to the original formulation of Roundup® (Table 3.7) (Fuentes, 2008). The results of the Fuentes (2008) study as well as this research indicate that four of six species of larval anurans were more sensitive to exposures of Roundup WeatherMax® than exposures of the original formulation of Roundup®. Our lack of knowledge about the proprietary surfactant mixture in Roundup WeatherMax® makes it difficult to confirm the source of toxicity in the formulation.

Since surfactants in formulations of Roundup® are the major contributors to toxicity, it is necessary to investigate these surfactants for their safety to non-target organisms at environmentally relevant concentrations. Although the surfactants are only listed as "other ingredients" on glyphosate formulation labels, they are an

environmentally relevant component which needs to be evaluated in environmental risk assessments.

5.0 Tables and Figures

Table 3.1: Formula and Environmental Properties for Glyphosate:

Property	**Value**
Molecular formula	$C_3H_8NO_5P$
CAS No.	1071-83-6
Water solubility[a] (mg/L)	10,000-15,7000 at 25°C
Log Kow[a]	–4.59 to –1.70
H (Pa-m3/mol)[a]	1.41×10^{-5}
Koc (L/kg)[b]	9-60,000; geometric mean (n=28), 2,072
Kd [b]	3-1,188; geometric mean (n=28), 64
BCF[c]	Low
Photolysis half-life (d)[d]	Stable
Hydrolysis half-life (d) [d]	Stable
Biodegradation half-life (d)[e]	60

[a] (Mackay *et al.*, 1997); [b] (Giesy *et al.*, 2000); [c] (Brandt, 1983);(Brandt, 1984); (Veith *et al.*, 1979) [d] (WSSA, 1983); [e] (Brandt, 1983); (WSSA, 1983)

Table 3.2: Substances used to amend reverse-osmosis water to approximate U.S. stream and lake water characteristics (Sawyer *et al.*, 1994; Wetzel, 2001)

Substance	Moderately Hard Dilution Water
CaCO3	2.5 mg / L
NaHCO3	50.9 mg / L
MgSO4 x 7H2O	24 mg / L
CaSO4 x 2H2O	16.5 mg / L
CaCl2 x 2H2O	32.5 mg / L
KCl	1.05 mg / L
KNO3	0.41 mg / L
K2PO4	0.00917 mg / L
Cu Standard (1000ppm)[a]	0.22 mL (110-L)
Se Standard (1000ppm)[b]	0.11 mL (110-L)
Zn Standard (1000ppm)[c]	0.22 mL (110-L)

[a] ((Fisher Scientific, 1997a)); [b] ((Fisher Scientific, 1997b));

[c] ((Fisher Scientific, 1997c))

Table 3.3: Holding and testing conditions for larval anurans

Test type	Static non-renewal
Duration	96h
Replicates/treatment	4
Organisms per exposure chamber	10
Endpoint	Mortality
Size of testing chamber	3.8 liters
Volume of dilution in exposure chamber	3 liters
Age of animals[a]	Gosner 25
Simulated site water	Moderately hard
Size of holding vessel	37.9 liter glass aquarium
Volume of dilution in holding	> 1 liter / 50 larvae
Feeding regime[b]	*ad libitum* (Holding) not fed (Testing)
Temperature	20 ± 1 (^{o}C)
Light quality	Cool White
Light intensity	86 ± 8.6 µE/sec
Photoperiod	16-8 light-dark cycle
Aeration[c]	Single-bubble

[a] (Gosner, 1960);(Edginton *et al.*, 2004); (Mann & Bidwell, 1999);

[b] (Nace, 1974); [c] (ASTM, 2003)

Table 3.4: Water chemistry parameters for testing and holding water (ASTM, 2003; Nace, 1974; USEPA, 2002)

Water Chemistry	Dilution and Test Solution Conditions
pH	6.5 – 8.2
Hardness	150-250 mg/L as $CaCO_3$
Alkalinity	150-250 mg/L as $CaCO_3$
Dissolved Oxygen	$\geq$ 4.0 mg O_2 / L
Ammonia	< 0.2 mg/L
Nitrate & Nitrite	< 0.3 mg/L as Nitrogen
Fluoride	< 1.5 mg/L
Chlorine	< 11 µg/L

Table 3.5: Responses to the original formulation of Roundup® of three species of Gosner stage 25 larval ranids in aqueous static non-renewal acute toxicity tests measured in mg AE/L

Species	NOEC	LOEC	96h LC50 (95%CI)		Potency equation	Threshold
Rana pipiens	1.29	1.32	1.80	(1.73, 1.88)	y = 92.5x -116.1	1.3
R. sphenocephala	1.52	1.81	2.05	(1.90, 2.20)	y = 47.9x - 54.2	1.7
R. clamitans	3.42	3.89	4.55	(4.34, 4.78)	y = 26.6x - 80.5	3.7

Table 3.6: Responses to Roundup WeatherMax® of three species of Gosner stage 25 larval ranids in aqueous static non-renewal acute toxicity tests measured in mg AE/L

Species	NOEC	LOEC	96h LC50 (95%CI)		Potency equation	Threshold
Rana pipiens	1.65	1.68	2.27	(2.18, 2.36)	y = 65.5x - 98.4	1.7
R. sphenocephala	0.68	0.98	1.33	(1.22, 1.45)	y = 54.7x - 26.2	0.8
R. clamitans	1.91	2.37	2.77	(2.67, 2.87)	y = 62.2x - 123.0	2.1

Table 3.7: Responses of organisms to two formulations of Roundup® in acute aqueous toxicity tests [a]

Species	Original formulation	WeatherMax®	Citation
Ceriodaphnia dubia	4.2[b]		
Hyalella azteca	1.1[b]		Tsui and Chu 2004
Salmo gairdneri	6.1		
Pimephales promelas	1.7		Folmar 1979
Xenopus laevis	9.3		Perkins *et al.* 2000
Lymnodynastes dorsalis	3.0[b]		
Litoria moorei	2.9-11.6[b]		Mann and Bidwell 1999
Heleioporus eyrei	6.3[b]		
Crinia insignifera	3.6[b]		
Rana pipiens	2.9		
Rana clamitans	2		Howe *et al.* 2004
Rana sylvatica	5.1		
Bufo americanus	< 4.0		
Hyla chrysoscelis	2.5	3.3	
Bufo fowleri	4.2	2.0	Fuentes 2008
Rana catesbeiana	2.8	2.0	
Rana pipiens	1.8	2.3	
Rana sphenocephala	2.1	1.4	Summary results from this research
Rana clamitans	4.6	2.8	

[a] 96h-LC50 values originally published in mg/L converted to mg AE/L for comparison; [b] 48h LC50

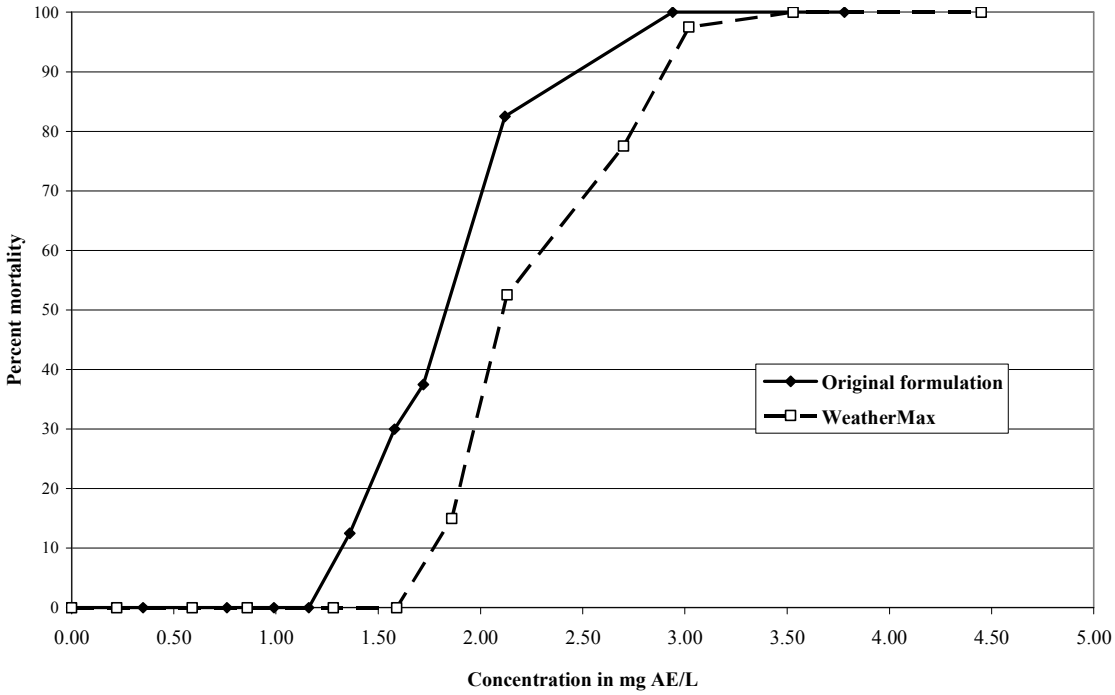

Figure 3.1: Response of *Rana pipiens* to the original formulation of Roundup® and Roundup WeatherMax® in aqueous 96h static non-renewal toxicity tests

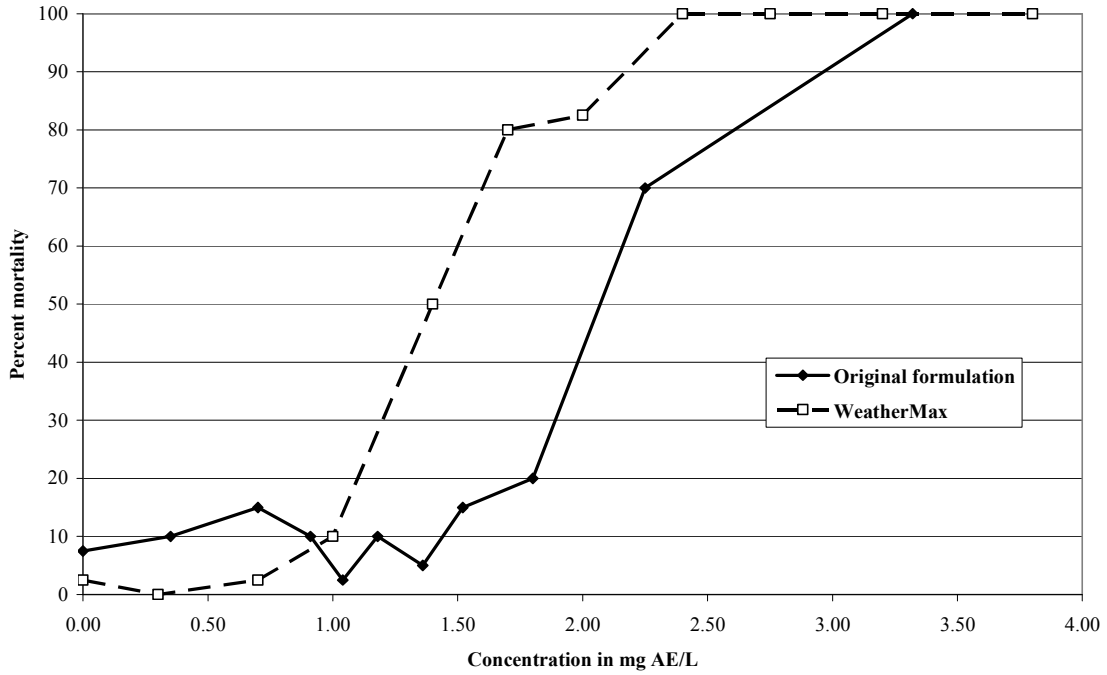

Figure 3.2: Response of *Rana sphenocephala* to the original formulation of Roundup® and Roundup WeatherMax® in aqueous 96h static non-renewal toxicity tests

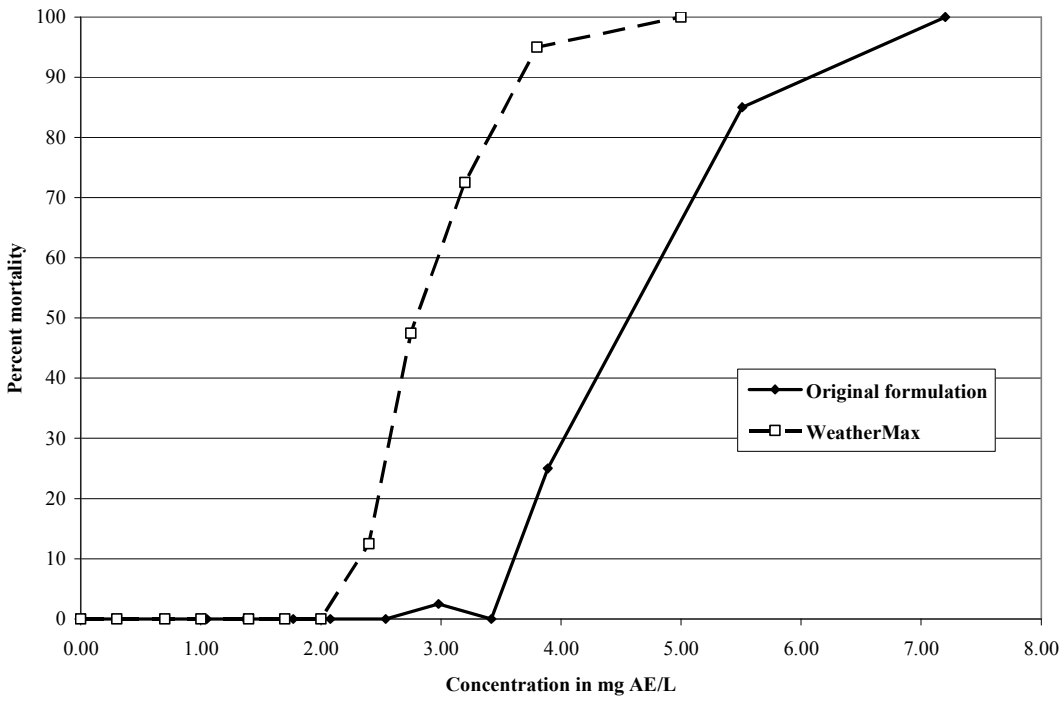

Figure 3.3: Response of *Rana clamitans* to the original formulation of Roundup® and Roundup WeatherMax® in aqueous 96h static non-renewal toxicity tests

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CHAPTER IV: RELATIVE TOXICITY OF THE COMPONENTS OF THE ORIGINAL FORMULATION OF ROUNDUP® TO FIVE NORTH AMERICAN ANURANS

1.0 Introduction

Roundup® brand herbicides contain the active ingredient glyphosate, which is the most extensively used herbicide in the United States (Kiely *et al.*, 2004). Roundup® formulations can enter aquatic systems, incidentally exposing aquatic and semi-aquatic organisms through spray drift, overspray, and runoff from treated sites (Giesy *et al.*, 2000; Solomon & Thompson, 2003). The original formulation of Roundup® is a binary mixture of the isopropylamine (IPA) salt of glyphosate and polyethoxylated tallow amine (POEA) surfactant. Glyphosate is a broad spectrum, post-emergent herbicide (Franz *et al.*, 1997). Glyphosate works by inhibiting the enzyme 5-enolpyruvyl shikimate-3-P synthetase which is 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 & Thompson, 2003). POEA is a common adjuvant in glyphosate formulations (Giesy *et al.*, 2000; Solomon & Thompson, 2003). It enables the liquid herbicide to stick to the surface of vegetation and aids the herbicide in penetrating the waxy cuticle on plant leaves (Giesy *et al.*, 2000; Solomon & Thompson, 2003).

Since Roundup® is used for both agriculture and silviculture applications and relatively sensitive organisms such as larval anurans can be exposed, questions have arisen regarding the toxicity of these exposures (Howe *et al.*, 2004; Mann & Bidwell, 1999; Relyea, 2005a, 2005b, 2005c). Previous studies have indicated that the toxicity

manifested by Roundup® is largely due to the surfactant in the binary mixture (Folmar *et al.*, 1970; Mann & Bidwell, 1999; M. Tsui & Chu, 2003). Given that the specific mode of action of glyphosate is a pathway found only in plants and microorganisms, it is likely that the surfactant would be the more potent component in Roundup®. Larval anurans have been identified as relatively sensitive organisms to Roundup® exposures in laboratory and field studies (Howe *et al.*, 2004; Mann & Bidwell, 1999; Relyea, 2005a, 2005b, 2005c; Wojtaszek *et al.*, 2004) so it is important to understand responses of these organisms to exposures of Roundup® as well as its components. Unconfounded laboratory tests using North American anuran species can 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 Treefrog (*Hyla chrysoscelis* Laurenti), were chosen to determine the toxicity of the original formulation of Roundup® to larval anurans in this research. *R. pipiens* is common throughout much of northern North America and is found in diverse habitat types (Conant & Collins, 1998 ; Martsof *et al.*, 1980). The breeding season of *R. pipiens* is March to June (Conant & Collins, 1998 ; Martsof *et al.*, 1980). *R. clamitans* occur in eastern North America and can be found in a variety of habitats including swamps, ponds, lakes, and slow moving rivers and streams (Conant & Collins, 1998 ; Martsof *et al.*, 1980). *R. clamitans* generally breeds from May to June (Conant & Collins, 1998 ; Martsof *et al.*, 1980). *R.*

catesbeiana are widely distributed throughout North America either as natives or introduced species and breeding season can be as long as February to October in its southern ranges (Conant & Collins, 1998 ; Martsof *et al.*, 1980). *B. fowleri* are native to most habitats in the eastern United States and will breed in many types of water bodies (Conant & Collins, 1998 ; Martsof *et al.*, 1980). Breeding in northern habitats occurs from approximately April to July and in southern habitats breeding occurs from approximately March to May (Conant & Collins, 1998 ; Martsof *et al.*, 1980). *H. chrysoscelis* occurs in eastern North American and is mostly arboreal (Conant & Collins, 1998 ; Martsof *et al.*, 1980). *H. chrysoscelis* generally breeds from May to August (Conant & Collins, 1998 ; Martsof *et al.*, 1980).

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, we separately tested the formulated mixture of this herbicide as well as its components in 96 hour acute toxicity tests with sensitive Gosner stage 25 (Gosner, 1960)larval anurans, specifically northern leopard frogs, green frogs, American bullfrogs, Fowler's Toads, and Cope's gray treefrog. The results of these toxicity tests were used to determine the relative contribution of the components, the IPA salt of glyphosate and POEA surfactant, to the toxicity of the original formulation of Roundup®.

2.0 Materials and Methods

2.1 Chemicals

The original formulation of Roundup® and components were supplied by Monsanto Co. (St. Louis, MO). The original formulation of Roundup® is a binary mixture composed of the IPA salt of glyphosate (Table 4.1) at 29.7% acid equivalent (AE) and POEA surfactant at 15%. Separate components of the IPA salt of glyphosate at 46.0% AE and POEA surfactant at 69-73% 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 IPA salt, and 1000 mg/L for POEA using NANOpure™ water. Dilution water used for test concentrations was moderately hard water (Table 4.2) formulated to simulate general water characteristics of US lakes and streams (Sawyer *et al.*, 1994; Wetzel, 2001)and was the same water used for acclimating and holding animals prior to testing.

2.2 Experimental design

Bioassays were performed according to published US EPA methods (USEPA, 2002). Chemical and physical measurements of testing conditions, dilution water, and test solutions were conducted according to published American Society for Testing and Materials(ASTM) methods (ASTM, 2003). The aqueous tests were 96 hour static non-renewal. Concentrations for definitive testing were determined from range finding tests for the formulation as well as POEA. 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, concentrations for IPA salt included 0.42, 4.15, and 41.48 mg AE/L and concentrations for POEA included 0.06, 0.18, 0.26, 0.37, 0.44, 0.59, 0.92, 1.25, and 2.00 mg/L. IPA salt concentrations were based on the predicted environmental concentration (PEC) immediately following an application of herbicide at a recommended label application rate of 2.3 liters (L)/hectare(ha) (Monsanto Co. 2008) into a body of water with a depth of 17.6 cm. Three concentrations of IPA salt were tested: PEC, 10 times the PEC, and 100 times the PEC. A copper sulfate (CAS #7758-99-8, Fisher Scientific Inc.) reference toxicant was used to ensure health of test organisms and to compare the sensitivities across species. A 96h aqueous static non-renewal test was initiated with formulation and component tests each time availability of organisms permitted. Seven concentrations: 25, 50, 75, 85, 95, 100, and 500 µg Cu/L were tested.

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 four replicates of an untreated control with 10 tadpoles per replicate. In POEA and reference toxicant tests there were three replicates of each concentration and 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), as in holding tanks (Table 4.3). Water used for holding and test concentration dilution was controlled for the following parameters: pH, hardness, alkalinity, dissolved oxygen, ammonia, nitrates and nitrites, and chlorine (Table 4.4) (ASTM, 2003; Nace, 1974). Holding and testing conditions were consistent for all species (Table 4.3).

2.3 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. catebeiana* from Sullivan Co. Nashville TN and Carolina Biological Supply Co. Burlington NC). Water used for holding and test concentration dilution was formulated with the following parameters: pH, hardness, alkalinity, dissolved oxygen, ammonia, nitrate and nitrite, and chlorine (Table 4) (ASTM, 2003; Nace, 1974; USEPA, 2002). Holding and testing conditions were consistent for all species (Table 4.3). During holding, tadpoles were fed twice daily *ad libitum* a mixture of ground goldfish fish flakes (Tetra™) in water (Nace, 1974). Holding tanks were cleaned twice daily and up to 50% water changes were completed every other day to ensure water quality. Tadpoles were reared to Gosner stage 25 (Gosner, 1960) prior to testing. Previous research has shown that this stage in amphibian development is more sensitive to exposures of contaminants than either embryo and earlier larval stages or later larval stages and adults (Berrill *et al.*, 1994; Berrill *et al.*, 1993; Edginton *et al.*, 2004; Howe *et al.*, 2004; Mann & Bidwell, 1999).

2.4 Endpoints

The primary 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). Endpoints were measured and dead animals removed daily for 4 days.

2.5 Analytical

Test solution samples were collected for glyphosate or copper concentration verification from every replicate at all concentrations and controls immediately prior to adding animals to test jars. Formulation and IPA salt samples were stored in silanized glass vials at 3°C prior to analysis. Glyphosate concentrations were determined using Dionex Ultra-Mate-3000 High Performance Liquid Chromatography (HPLC) with autosampler and Variable Wavelength Detector system with Dionex Chromeleon software (Dionex Corp., Sunnyvale CA). Methods used for derivatization and analysis of glyphosate in water samples were supplied by Monsanto Co. (St. Louis, MO)(Powell *et al.*, 1990). Reference toxicant test samples were acidified with trace metals grade nitric acid (CAS #7697-37-2, Fisher Scientific Inc.) after collection and kept at 3°C prior to analysis. Copper concentrations below 500µg/L were determined using a Perkin- Elmer Atomic Absorption spectrophotometer (5100PC model) and EPA method # 220.2 Atomic Absorption, Furnace Technique (USEPA, 1979). Copper concentrations at or above 500 µg/L were determined using flame atomic absorption and performed according to the Analytical Method 200.1 (USEPA, 1991).

2.5 Data Analysis

Data were analyzed using SAS® Version 9.1 (SAS, 2007). Not all data met the assumptions for parametric analysis. Where appropriate, probit analysis was used to

determine the lowest observed effect concentration (LOEC), no observed effect concentration (NOEC), LCx values, and 95% confidence intervals (CI). Non-parametric analyses were conducted using two programs. The USEPA MS-DOS application for trimmed Spearman-Karber analysis was used to obtain LC50 values and 95% confidence intervals. Non-parametric rank converted ANOVA's, equivalent to Kruskal-Wallis and Wilcoxon Rank Sum with Dunnett's test analyses were used to determine LOEC and NOEC values in these cases. Differences in concentration-response curves were tested for significance using ANCOVA. Regression analysis (SAS®) was used to generate potency curves for each test.

3.0 Results

In tests with copper sulfate reference toxicant, all frog species had similar sensitivities with 96h- LC50's ranging from 11.72 to 69.93 µg Cu/L (Table 4.7). All exposures of formulation, the IPA salt component, and reference toxicant tests were verified analytically. HPLC analysis for glyphosate concentration was performed on all formulation tests and IPA salt tests for all species tested. Recovery of glyphosate was between 85 and 115% for all tests. Copper concentrations were determined by atomic absorption spectrophotometry. The analytically verified replicates for each concentration were added and the mathematical mean of those values was calculated. The statistical analysis and results were based on these analytically verified mean values. Control mortality was less than 10% in all tests.

R. pipiens was the most sensitive species to exposures of the original formulation of Roundup® followed by *H. chrysoscelis, R. catesbeiana* and *B. fowleri*, and *R. clamitans* with 96h-LC50 values ranging from 1.80 to 4.55 mg AE/L (Table 5). *R. pipiens*, the most sensitive species tested, was 2.5 times more sensitive than *R. clamitans* to the original formulation of Roundup®. Potency slopes for all five species exposed to the original formulation of Roundup® were significantly different from zero (ANCOVA) with p-values of <0.0001 for *R. clamitans*, *H. chrysoscelis*, *R. catesbeiana* and *B. fowleri,* and p = 0.0002 for *R. pipiens* (Figures 4.1-4.5). Potency slopes for the original formulation of Roundup® for all five species were also all significantly different from each other (ANCOVA p-values of <0.0001).

For POEA exposures, *R. pipiens* was the most sensitive species tested and *R. clamitans* was the least sensitive species tested with 96h-LC50 values ranging from 0.68 to 1.32 mg/L (Table 4.6). *R. clamitans* was two times less sensitive to POEA than *R. pipiens*. Potency slopes for all five species exposed to POEA were significantly different from zero with ANCOVA p-values of 0.0003 for *R. clamitans*, 0.0045 for *H. chrysoscelis*, and <0.0001 for *B. fowleri, R. catesbeiana,* and *R. pipiens*. Potency slopes for POEA for all five species were also all significantly different from each other with ANCOVA p-values ranging from 0.0003 to <0.0001 except potency slopes for *B. fowleri* and *R. catesbeiana* which were not significantly different from each other (p = 0.26) (Figures 4.1-4.5). No significant mortality was observed during exposures of 96h for any of the five species exposed to IPA salt at the three concentrations tested which represent a

predicted environmental concentration (PEC) based on a label application rate of 2.3 L/ha, 10 times the PEC, and 100 times the PEC (0.42 to 41.5 mg AE/L) (Figures 4.1-4.5).

The relative contribution (RC) of the components, POEA and IPA salt, to the toxicity of the formulation was calculated according to the method of Tsui and Chu (2003). In order to calculate RC values, the toxic units are needed and 96h-LC50 values for the formulation and both components must be obtained. In this study, 96h-LC50 values were not obtained for the IPA salt component because it was nontoxic to tadpoles at all concentrations tested. At tested concentrations of 100 times the PEC, we saw no significant mortality. Mann and Bidwell (1999) also tested the IPA salt on Australian anuran species at up to approximately 400 mg AE/L and saw no mortality, which is about 1000 times our PEC of 0.42 mg AE/L. Perkins *et al.* (2000) published a 96h-LC50 value of 7296.8 mg AE/L for *Xenopus laevis* (Table 8). This 96h-LC50 value was used to estimate toxic units. For *R. pipiens*, *R. catesbeiana*, *B. fowleri*, and *R. clamitans*, POEA contributed 100% of the toxicity to the formulation. Only 30% mortality was observed after 96h in the highest concentration of POEA tested for *H. chrysoscelis* and we were unable to calculate 96h-LC50 values or the RC for this species due to lack of mortality.

The linear portion of the potency curve, from the NOEC to the concentration eliciting 100% mortality was used to calculate the potency slope. The linear equation calculated from this portion of the potency curve contains a key piece of information, namely the degree of response exhibited by a population of organisms to increasing concentrations of a toxicant, which can be shown by the slope of the concentration-

response line (Perkins *et al.*, 2000). A linear equation was chosen to represent the potency curve. By not log-transforming our data we are able to more accurately pinpoint LC50 values and estimate the best fitting potency slope for the range of action. The threshold level is the range between the NOEC and LOEC and can be estimated by taking the average of the LOEC and NOEC values (Suter, 1990). In Roundup® formulation exposures, *H. chrysoscelis* and *B. fowleri* have similar slopes, 44.7 and 47.2, but the threshold level for *B. fowleri* is 2.2 times greater than for *H. chrysoscelis.* From our data, it is obvious that both the slope and threshold are important for determining the most sensitive species and potential risks. For both the original formulation of Roundup® and POEA, *R. pipiens* has the steepest slope and the lowest threshold confirming that it is the most sensitive species tested (Table 4.5 and 4.6).

4.0 Discussion

Previous research has shown that invertebrates, fish, and anurans have 48 to 96h-LC50 values ranging from 1.1 to 11.6 mg ae/L for exposures to the original formulation of Roundup® (Table 4.8). By comparing our data with previous research, *R. pipiens* is one of the most sensitive species tested among invertebrates, fish, and anurans. Only *Hyalella azteca* and *Pimephales promelas* had lower 48 and 96h-LC50 values, respectively (Folmar *et al.*, 1979; M. T. K. Tsui & Chu, 2004). Less information is available regarding the effects of exposures of POEA on animals (Giesy *et al.*, 2000; Solomon & Thompson, 2003) (Table 4.8). Our results agree with previous studies which have noted that POEA contributes the majority of the toxicity to the herbicide

formulations for fish, invertebrates, and amphibians and again our study results suggest that anurans are among the most sensitive species (Folmar *et al.*, 1979; Howe *et al.*, 2004; Mitchell *et al.*, 1987; Perkins *et al.*, 2000; Wan *et al.*, 1989). Howe *et al.* (2004) reported a 96h-LC50 values of 2.9 mg AE/L and 2.0 mg AE/L for *R. pipiens* and *R. clamitans*, respectively, for exposures to the original formulation of Roundup® (Table 4.8). In comparing these reported values to our data, *R. pipiens* 96h-LC50 value of 1.80 mg AE/L is 1.6 times lower and *R. clamitans* 96h-LC50 value of 4.60 mg AE/L is 2.3 times higher than the values reported in Howe *et al.* (2004). These differences in 96h-LC50 values could be due to different methods of collection, holding, and/or testing.

NOEC values ranged from 1.29 to 3.42 mg AE/L for the original formulation of Roundup®, with *R. pipiens* the most sensitive and *R. clamitans* the least sensitive (Table 4.5). By comparing the NOEC values and the PEC values, the margin of safety (NOEC/PEC) can be determined. For the original formulation of Roundup®, three recommended one-time application rates are 2.3 L/ha, 4.7 L/ha, and 11.7 L/ha, with the latter being the maximum applied amount allowed per year on crops (personal communication, Monsanto Co. St. Louis MO). The PEC for these label rates immediately after application into a 13.2 cm deep water body would be 0.55, 1.11, and 2.77 mg AE/L respectively. Using these estimates and our calculated NOEC values the margins of safety for the lowest PEC of 0.55 mg AE/L would range from 2.4 to 6.2 for the five species tested. For a PEC of 1.11 mg AE/L the range in margins of safety is 1.2 to 3.1. For the highest PEC of 2.77 mg AE/L, the range in margins of safety is 0.5 to 1.2 for the five species tested. A margin of safety value less than one signifies a NOEC value

above the PEC, indicating that at this application rate toxic effects on larval amphibians could be possible. With this in mind, it is important to remember that the NOEC values calculated in this research are based on conservative aqueous laboratory tests and do not take into account the strong affinity both glyphosate and POEA have for binding with soil and sediment (Giesy *et al.*, 2000; Solomon & Thompson, 2003). This would likely increase the NOEC values and increase the margins of safety.

While POEA contributed essentially 100% of the toxicity of the original formulation of Roundup®, there appeared to be synergy between the POEA and IPA salt components in the formulation tests. When comparing the formulation tests and POEA component tests, 96h-LC50 values for the POEA component tests were higher than would be expected if the toxicity was simply additive and POEA was contributing 100% of the toxicity. For example, in the formulation test with *R. pipiens* the 96h-LC50 value was 1.80 mg AE/L. Since POEA is 15% of the total formulation, the expected 96h-LC50 value for a POEA test, with this species, would be 0.27 mg/L. The actual 96h-LC50 value for the *R. pipiens* POEA component test was 0.68 mg/L. In tests with POEA alone, 96h-LC50 values were higher than expected based on formulation tests. This could imply slight synergism between the two components, as would be expected for a herbicide and adjuvant components on target species. This slight synergism makes the formulation more toxic than either of the components separately to non-target species. These results show the importance of testing the herbicide formulation as well as its separate components to accurately characterize the toxicity and potential risk of the formulation.

These results indicate that species of North American anurans including *R. pipiens* are among the most sensitive organisms tested to date to exposures of the original formulation of Roundup®. Our results also indicate that the surfactant in the formulation contributes the majority of the toxicity. Wan *et al.* (1989) showed that toxicity of Roundup® formulations could be reduced by decreasing the percentage of POEA in the formulation.

5.0 Tables and Figures

Table 4.1: Formula and Environmental Properties for Glyphosate:

Property	**Value**
Molecular formula	$C_3H_8NO_5P$
CAS No.	1071-83-6
Water solubility[a] (mg/L)	10,000-15,7000 at 25°C
Log Kow[a]	–4.59 to –1.70
H (Pa-m3/mol)[a]	1.41×10^{-5}
Koc (L/kg)[b]	9-60,000; geometric mean (n=28), 2,072
Kd [b]	3-1,188; geometric mean (n=28), 64
BCF[c]	Low
Photolysis half-life (d)[d]	Stable
Hydrolysis half-life (d) [d]	Stable
Biodegradation half-life (d)[e]	60

[a](Mackay *et al.*, 1997); [b](Giesy *et al.*, 2000); [c](Brandt, 1983);(Brandt, 1984); (Veith *et al.*, 1979) [d](WSSA, 1983); [e](Brandt, 1983); (WSSA, 1983)

Table 4.2: Substances used to amend reverse-osmosis water to approximate U.S. stream and lake water characteristics (Sawyer *et al.*, 1994; Wetzel, 2001)

Substance	Moderately Hard Dilution Water
CaCO3	2.5 mg / L
NaHCO3	50.9 mg / L
MgSO4 x 7H2O	24 mg / L
CaSO4 x 2H2O	16.5 mg / L
CaCl2 x 2H2O	32.5 mg / L
KCl	1.05 mg / L
KNO3	0.41 mg / L
K2PO4	0.00917 mg / L
Cu Standard (1000ppm)[a]	0.22 mL (110-L)
Se Standard (1000ppm)[b]	0.11 mL (110-L)
Zn Standard (1000ppm)[c]	0.22 mL (110-L)

[a] (Fisher Scientific, 1997a); [b] (Fisher Scientific, 1997b); [c] (Fisher Scientific, 1997c)

Table 4.3: Holding and testing conditions for larval anurans

Test type	Static non-renewal
Duration	96h
Replicates/treatment	4
Organisms per exposure chamber	10
Endpoint	Mortality
Size of testing chamber	3.8 liters
Volume of dilution in exposure chamber	3 liters
Age of animals[a]	Gosner 25
Simulated site water	Moderately hard
Size of holding vessel	37.9 liter glass aquarium
Volume of dilution in holding	> 1 liter / 50 larvae
Feeding regime[b]	*ad libitum* (Holding) not fed (Testing)
Temperature	20 ± 1 (^{o}C)
Light quality	Cool White
Light intensity	86 ± 8.6 µE/sec
Photoperiod	16-8 light-dark cycle
Aeration[c]	Single-bubble

[a] (Gosner, 1960);(Edginton *et al.*, 2004); (Mann & Bidwell, 1999);

[b] (Nace, 1974); [c] (ASTM, 2003)

Table 4.4: Water chemistry parameters for testing and holding water (ASTM, 2003; Nace, 1974; USEPA, 2002)

Water Chemistry	Dilution and Test Solution Conditions
pH	6.5 – 8.2
Hardness	150-250 mg/L as $CaCO_3$
Alkalinity	150-250 mg/L as $CaCO_3$
Dissolved Oxygen	$\geq$ 4.0 mg O_2 / L
Ammonia	< 0.2 mg/L
Nitrate & Nitrite	< 0.3 mg/L as Nitrogen
Fluoride	< 1.5 mg/L
Chlorine	< 11 µg/L

Table 4.5: Response of five species of Gosner stage 25 larval anurans to exposures of the original formulation of Roundup® measured in mg AE/L

Species	NOEC	LOEC	96h-LC50 (95%CI)		Potency equation	Threshold
R. pipiens	1.29	1.32	1.80	(1.73, 1.88)	y = 92.5x -116.1	1.3
H. chrysoscelis	1.74	2.10	2.50	(2.38, 2.63)	y = 44.7x - 62.2	1.9
R. catesbeiana	2.02	2.52	2.77	(2.66, 2.89)	y = 66.9x - 145.5	2.3
B. fowleri	3.40	3.95	4.21	(4.08, 4.33)	y = 47.2x -147.0	3.7
R. clamitans	3.42	3.89	4.55	(4.34, 4.78)	y = 26.6x - 80.5	3.7

Table 4.6: Response of five species of Gosner stage 25 larval anurans to exposures of POEA measured in mg/L

Species	NOEC	LOEC	96h LC50 (95%CI)		Potency equation	Threshold
R.pipiens	0.38	0.40	0.68	(0.63, 0.74)	y = 158.2x - 57.4	0.4
B.fowleri	0.59	0.92	0.80	(0.75, 0.85)	y = 135.6x - 64.0	0.8
R.catesbeiana	0.59	0.92	0.83	(0.77, 0.90)	y = 97.4x - 46.4	0.8
H. chrysoscelis	0.59	0.92	[a]			0.8
R.clamitans	0.92	1.25	1.32	(1.23, 1.41)	y = 66.5x - 55.6	1.1

[a] insufficient mortality to calculate LC50

Table 4.7: Response of five Gosner stage 25 larval anurans to copper sulfate reference toxicant measured in μg Cu/L

Species	**96h-LC50 (95%CI)**		**Potency equation**	**Threshold**
B. fowleri	11.72	(9.99, 13.75)	y = 169.8x + 18.8	9.0
R. pipiens	32.86	(26.51, 40.73)	y = 2.0x - 8.1	27.9
H. chrysoscelis	35.09	(32.48, 37.91)	y = 1.5x - 2.2	13.2
R. catesbeiana	61.07	(55.35, 67.39)	y = 0.2x + 16.2	36.4
R. clamitans	69.93	(62.87, 77.79)	y = 1.4x - 41.8	61.8

Table 4.8: Responses of organisms to Roundup® and POEA in acute aqueous toxicity tests with 96h-LC50 values originally published in mg/L converted to mg AE/L for comparison ([b] 48h LC50)

Species	Roundup® mg AE/L	POEA mg/L	Citation
Ceriodaphnia dubia	4.2[b]		
Hyalella azteca	1.1[b]		Tsui and Chu 2004
Salmo gairdneri	6.1	2.0	Folmar 1979
Pimephales promelas	1.7	1.0	
Xenopus laevis	9.3	6.8	Perkins *et al.* 2000
Lymnodynastes dorsalis	3.0[b]		
Litoria moorei	2.9-11.6[b]		Mann and Bidwell 1999
Heleioporus eyrei	6.3[b]		
Crinia insignifera	3.6[b]		
Rana pipiens	2.9		
Rana clamitans	2		Howe *et al.* 2004
Rana sylvatica	5.1		
Bufo americanus	< 4.0		
Rana pipiens	1.8	0.7	
Hyla chrysoscelis	2.5		Summary results from this research
Rana catesbeiana	2.8	0.8	
Bufo fowleri	4.2	0.8	
Rana clamitans	4.6	1.3	

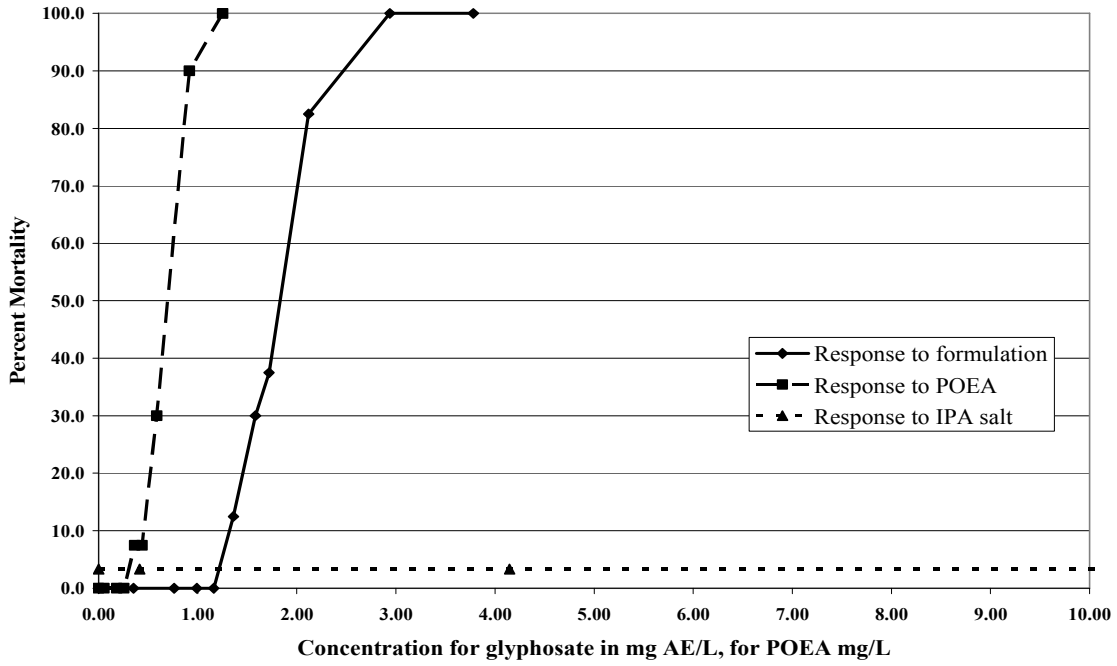

Figure 4.1: Response of *Hyla chrysoscelis* to the original formulation of Roundup®, POEA and IPA salt in 96h aqueous static non-renewal toxicity tests

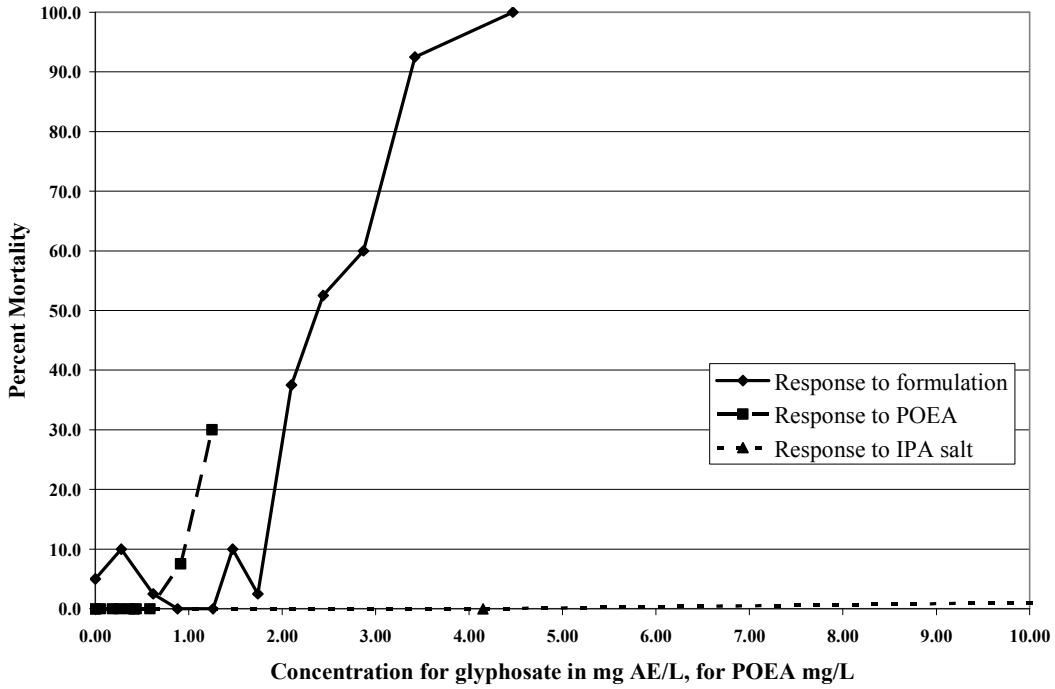

Figure 4.2: Response of *Hyla chrysoscelis* to the original formulation of Roundup®, POEA and IPA salt in 96h aqueous static non-renewal toxicity tests

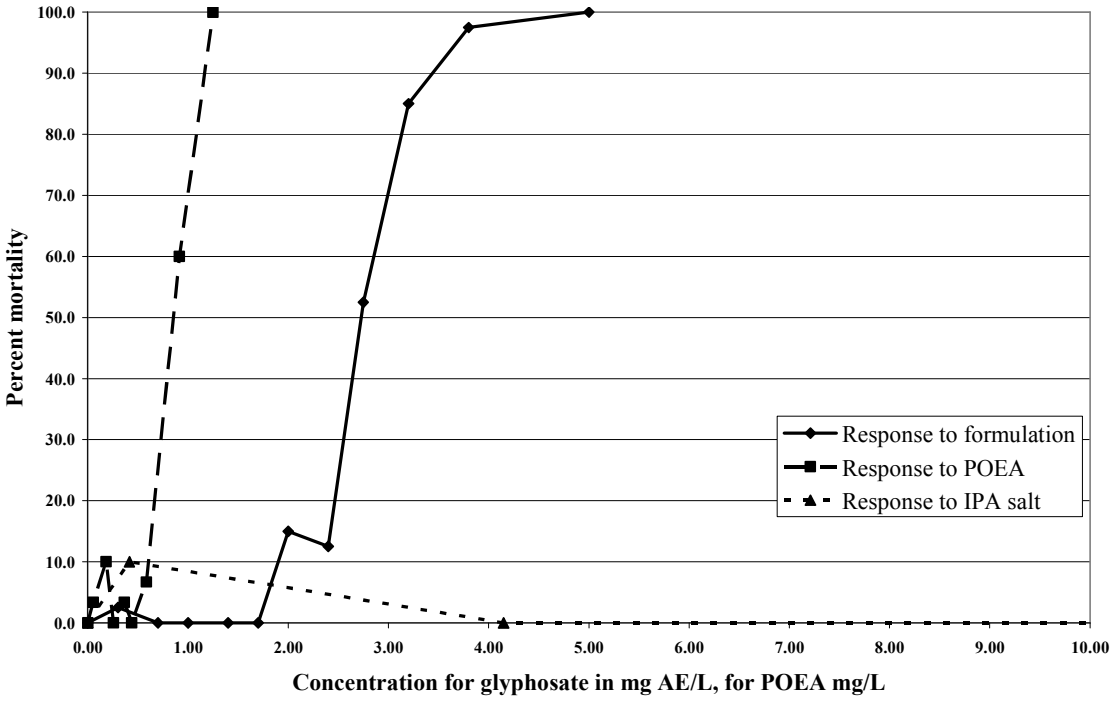

Figure 4.3: Response of *Rana catesbeiana* to the original formulation of Roundup®, POEA and IPA salt in 96h aqueous static non-renewal toxicity tests

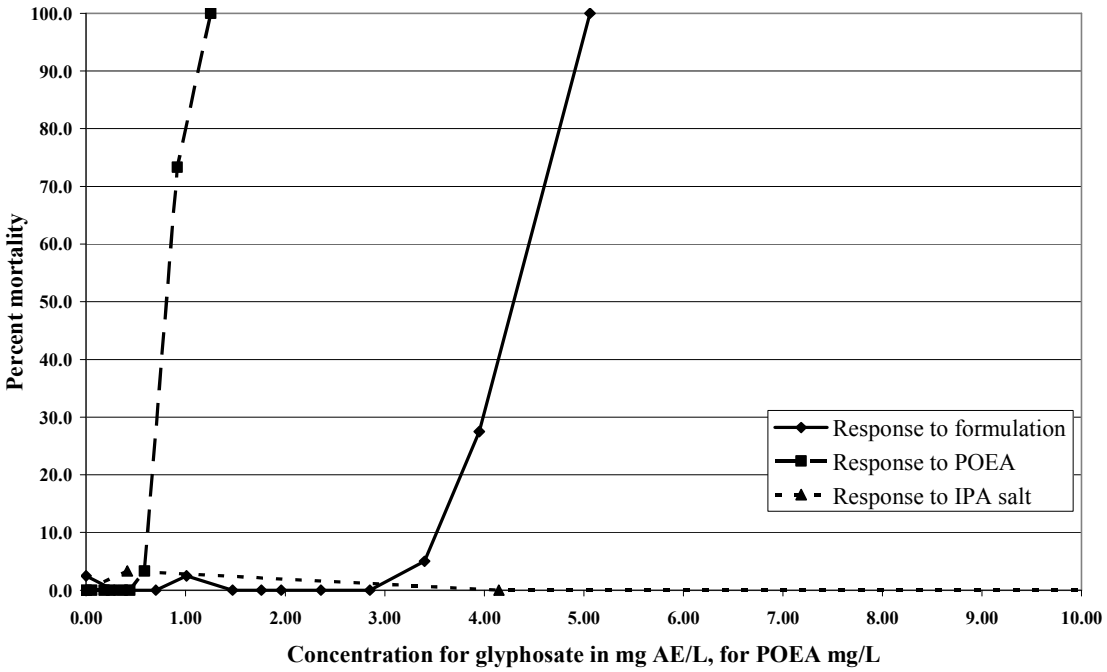

Figure 4.4: Response of *Bufo fowleri* to the original formulation of Roundup®, POEA and IPA salt in 96h aqueous static non-renewal toxicity tests

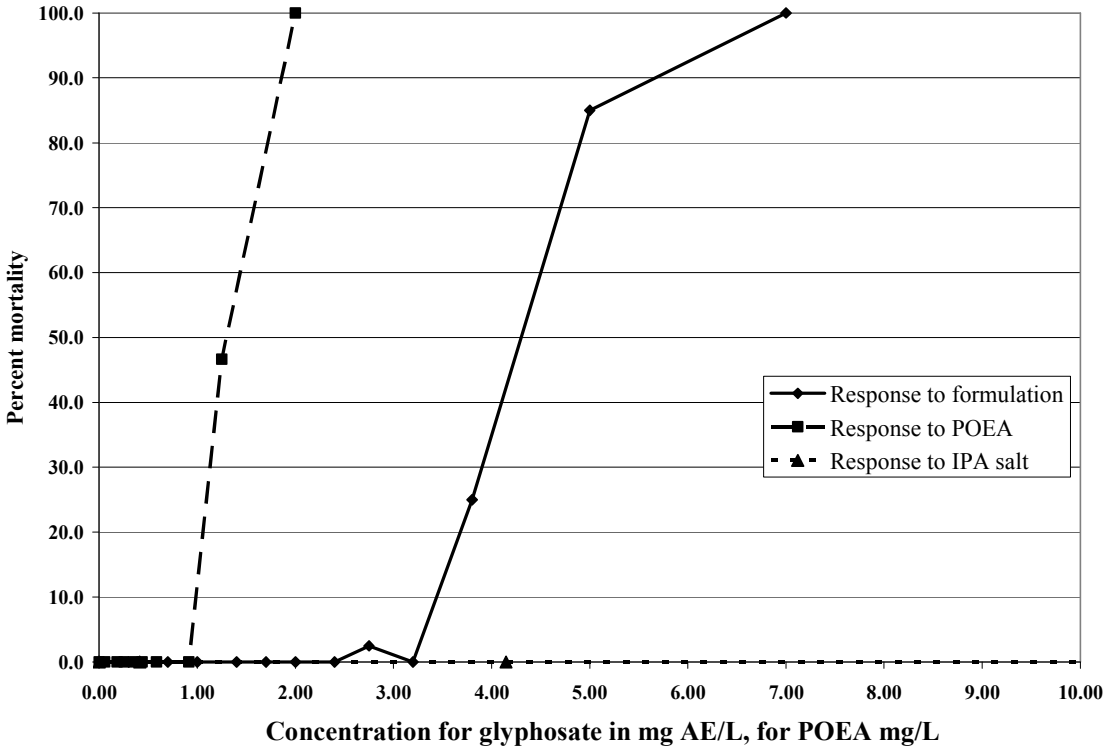

Figure 4.5: Response of *Rana clamitans* to the original formulation of Roundup®, POEA and IPA salt in 96h aqueous static non-renewal toxicity tests

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CHAPTER V: CONCLUSIONS

There has been concern over declining amphibian populations all over the world (Houlahan *et al.*, 2000; IUCN, 2006). A few recent studies have implicated Roundup® herbicide products as a factor in this amphibian decline (Mann & Bidwell, 1999; Relyea, 2005a, 2005b, 2005c), while other studies have refuted this idea (Giesy *et al.*, 2000; Solomon & Thompson, 2003; Thompson *et al.*, 2004; Wojtaszek *et al.*, 2004). Careful designed and implemented laboratory experiments with sensitive North American anuran species could provide data to help answer the question: Is Roundup® is a factor in amphibian decline in North America?

There were three separate studies included in this research; the objectives of each study are listed below. The objectives of the study using copper sulfate as a reference toxicant in amphibian testing were to: (1) measure the relative sensitivities of six larval anuran species to copper as copper sulfate in aqueous 96 hour acute toxicity tests, (2) measure the relative sensitivity of two separate acquisitions of *R. catesbeiana* and *H. chrysoscelis* and three separate acquisitions of *R. pipiens* and (3) determine if copper sulfate can be used as a reference toxicant in larval anuran toxicity testing.

The objectives of the study on the comparative toxicity of two formulations of Roundup® were to: (1) measure the responses to exposures of the original formulation of Roundup® and Roundup WeatherMax® of three species of larval ranids and (2) contrast the results of these exposures and compare with existing literature.

The objectives of the study on the relative contribution to toxicity of the components of the original formulation of Roundup® were to: (1) measure the response

to exposures of the original formulation of Roundup® and its two components, IPA salt and POEA separately of five species of larval anurans and (2) determine the relative contribution of the IPA salt and POEA to the toxicity of the mixture.

The results of our first study suggest that copper sulfate can serve as a reference toxicant in larval amphibian toxicity testing. Copper has many of the characteristics of a good reference toxicant including solubility in water, toxicity at low concentrations, rapid lethality, and is easily measured in water samples with atomic absorption spectrophotometry (Lee, 1980). Copper is also toxic to other organisms including invertebrates, fish, and plants which allows for comparison not only within and between species of amphibians but also between different organisms such as invertebrates and fish. Low concentrations of copper elicit responses in larval anurans which allows for easy detection differences in sensitivity between species and between accessions of organisms.

The results of our study on the comparative toxicity of two formulations of Roundup® brand herbicides, the original formulation of Roundup® and Roundup WeatherMax® indicate that Roundup WeatherMax® is the more toxic to larval anurans at lower concentrations that the original formulation of Roundup®. Previous studies have concluded that the majority of the toxicity of Roundup® formulations comes from the surfactant (Folmar *et al.*, 1970; Giesy *et al.*, 2000; Howe *et al.*, 2004; Mann & Bidwell, 1999; Perkins *et al.*, 2000; Solomon & Thompson, 2003; Tsui & Chu, 2003). Since surfactants in formulations of Roundup® are the major contributors to toxicity it is

necessary to investigate these surfactants, not just the active ingredients, for their safety to non-target organisms at environmentally relevant concentrations.

The results of our third study on the toxicity of the components of the original formulation of Roundup® indicate that the major contributor to toxicity is the surfactant, POEA. Although POEA contributes the majority of the toxicity to the formulation, there appears to be slight synergism between the components, POEA and the IPA salt of glyphosate. This slight synergism makes the formulation more toxic than either of the components separately to non-target species. These results show the importance of testing the herbicide formulation as well as its separate components to accurately characterize the toxicity of the formulation.

The toxicity of Roundup® formulations is controlled by the surfactant. Use rates of formulations in the field suggest a small margin of safety for larval anurans when using our conservative unconfounded aqueous toxicity tests to determine no observed effect levels. Laboratory tests including sediment and field tests which simulate more realistic exposure situations will likely increase the margin of safety. The exposure can be controlled by regulating the amount of surfactant in the herbicide formulation. The biggest return for mitigation of the risk to anuran species is likely to control the surfactant.

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