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Toxicity of Herbicide Mixtures: Atrazine and Roundup® Toxicity to Daphnia Magna

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TOXICITY OF HERBICIDE MIXTURES: ATRAZINE AND ROUNDUP ® TOXICITY TO *DAPHNIA MAGNA*

Jennifer A. Stiles

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May 29, 2013

Herbicide use in agricultural systems often causes runoff to contain chemical mixtures that may have greater deleterious effects on non-target organisms than the same chemicals would have when found alone. Little information is known about how some of these chemicals act in mixtures, though many of their individual effects have been described. It is important to understand what effects these chemical mixtures have to identify protective application levels. In this experiment, a 21-day chronic *Daphnia magna* toxicity test was conducted using an atrazine and Roundup® mixture. Five concentrations of atrazine were tested (0.4, 0.8, 1.2, 1.6, 2.0mg/L). The mixture treatments contained the same concentrations of atrazine with a constant concentration of Roundup® (450mg/L, 2% glyphosate). A spike in reproduction was observed at the lowest concentration of atrazine (0.4mg/L), with a subsequent decrease in reproduction with increasing concentration of atrazine. In the mixture the Roundup® control caused significantly reduced reproduction in the *D. magna*, though the combination of atrazine and Roundup® resulted in increased reproduction. Log linear regression analysis was used to predict overall trends in the treatments.

Introduction

The increasing use of pesticides in industrial agriculture raises concern about increasing chemical content in run-off from farms. According to the U.S. EPA, the herbicide known as atrazine, is one of the most widely used pesticides in the United States (EPA, 2013). Monitoring of drinking water and freshwater atrazine levels is being extensively conducted in the Midwest where there is abundant corn production and heavy atrazine use (EPA, 2013). Roundup® is another herbicide that is widely used to control weeds in both agricultural and non-agricultural settings. The active ingredient in Roundup® is glyphosate and according to the U.S. EPA in 2009, approximately 135 million pounds of glyphosate equivalents are applied each year to agricultural crops (EPA 2009). Because of the extensive use of pesticides like atrazine and Roundup®, it is important to examine the effects to non-target organisms. Atrazine has been determined to be a potential neuroendocrine toxicant, (Cooper et al. 2007)(Palma et al. 2009), and because of its heavy use in agricultural settings, there is the potential for agricultural runoff of this herbicide and other herbicides such as Roundup®. Deener (2000) found that 90% of over 200 mixtures of common pesticides in aquatic systems are toxic according to "concentration addition". While the specific mixture tested here was not evaluated by Deener, additive effect of the toxicity of mixtures of atrazine and other organo-phosphate inhibitors was observed. Little is known about the toxicity of mixtures, this research seeks to shed light on the effects to *Daphnia magna* reproduction in an atrazine and Roundup® mixture, as the two may be found together in the environment as a result of agricultural runoff. *D. magna* are an important freshwater indicator species and were examined in this study because of the large data sets that we've begun to collect on individual herbicidal effects to their growth, behavior and reproduction.

While it is known that atrazine is a neuroendocrine disruptor, its specific mode of action is still being studied. Its chlorohydrocarbon structure (Figure 1A) is likely an important factor in understanding its toxicity. Glyphosate, the active ingredient in Roundup®, is an organophosphate (Figure 1B), a chemical class known for its endocrine disruption (Curtis, 2001)(Glusczak *et al.* 2006)

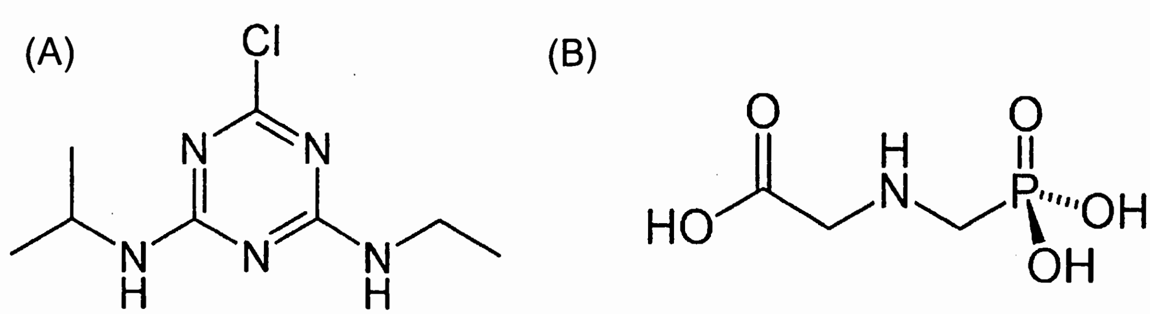

Figure 1. (A) Structure of atrazine. (B) Structure of glyphosate, the active ingredient in Roundup®.

In the experiment described here, we tested the effects of an atrazine and Roundup® mixture on *D. magna* reproduction. The same test performed in our lab in March 2012 observed *D. magna* with enhanced reproduction in both the atrazine alone and in the mixture with Roundup®. Because of the unexpected nature of this data, we sought to repeat the test to see if the herbicides again caused an increase in reproduction.

Hypothesis

Based on the results of a similar experiment conducted in March 2012, it was hypothesized that compared to the negative control, there would be an increase in reproduction of *D. magna* exposed to both atrazine and Roundup® alone, with an even greater increase in reproduction observed in the mixtures.

Methods

One 21-day chronic toxicity test was conducted on *D. magna* according to ASTM E1193-97 (2011a). The general procedures were conducted according to Markiewicz and Sofield (2011). The concentrations of atrazine used were based on the results from an acute test in February 2012 and a chronic test in March 2012. *D. magna* reproduction was tested at five concentrations of atrazine alone and then in a mixture with a constant concentration of Roundup®. The *D. magna* originally came from Aquatic Bio System Inc., and were cultured in the lab for approximately two months prior to the start of the experiment. Offspring from this culture were less than 24 hours old at the start of the test. Each *D. magna* was placed into a separate 250mL beaker with 100mL of test solution (Table 1). There were ten replicates for each treatment. The positive control consisted of 1.4mg/L of sodium lauryl sulfate. The Roundup® formulation used had 2% glyphosate. Solutions were renewed on a weekly basis and offspring counted and removed daily.

Table 1. Treatment concentrations of atrazine and Roundup® both alone and in mixture for exposure of *D. magna*.

Treatment	[atrazine] (mg/L)	[Roundup®] (mg/L)
Negative Control	0.0	0.0
Atrazine 1	0.4	0.0
Atrazine 2	0.8	0.0
Atrazine 3	1.2	0.0
Atrazine 4	1.6	0.0
Atrazine 5	2.0	0.0
Roundup® Control	0.0	450
Mixture 1	0.4	450
Mixture 2	0.8	450
Mixture 3	1.2	450
Mixture 4	1.6	450
Mixture 5	2.0	450

Reconstituted hard water was made according to ASTM E729-96 (2011b). Water quality parameters including hardness, alkalinity, pH and dissolved oxygen were tested on a weekly basis and found to be within the acceptable parameters

(2011b). During the experiment, *D. magna* were kept in an environmental chamber and were exposed to a 16:8 photoperiod and the temperature held constant at 20±2°C. They were fed *Pseudokirchneriella subcapitata* every two days. Statistical analysis was conducted and graphs created using the R packages: agricolae and drc.

While the presence of sediment in the environment may help sequester some organic chemicals, the presence of sediment and additional dissolved oxygen has not been shown to decrease toxicity to *Ceriodaphnia dubia*, a closely related species to *D. magna* (Phyu *et al.* 2013). Therefore, laboratory analysis with *D. magna* and atrazine can be more readily applied to environmental conditions.

Results

Daphnia magna exposed only to hard water, produced an average of 30 offspring over the 21 day period. According to ASTM, the data quality objective for the negative control is an average of atleast 60 offspring per *D. magna*, which was not met in this experiment (ASTM 2011a). Data quality objectives for the positive control were met.

D. magna treated with the five concentrations of Atrazine had reproduction trending downward (Figure 1). A previous study conducted in this lab found acute mortality at 2.5 mg/L. With this downward trend from 0.4 to 2.0 mg/L in this chronic study, it appears that Atrazine at the upper concentrations is inhibiting their reproduction (Figure 1). For reproductive effects, 2.0mg/L seems to be close to the limit for non-lethal effects, as in a previous trial in the same laboratory, concentrations of 2.5mg/L and greater resulted in mortality.

In the mixture treatment with five concentrations of atrazine and 450 mg/L Roundup® (9mg/L glyphosate), an average increase in reproduction was observed compared with atrazine-only treatments. However, Roundup® alone significantly inhibited the reproduction of *D. magna* (Figure 2). Other studies have found acute mortality in *Daphnia magna* at 30 mg/L (World Health Organization, 1994 and Alberdi *et al.* 1996). These studies support our findings of inhibition at the concentrations tested. Statistical significance of inhibition is discussed below.

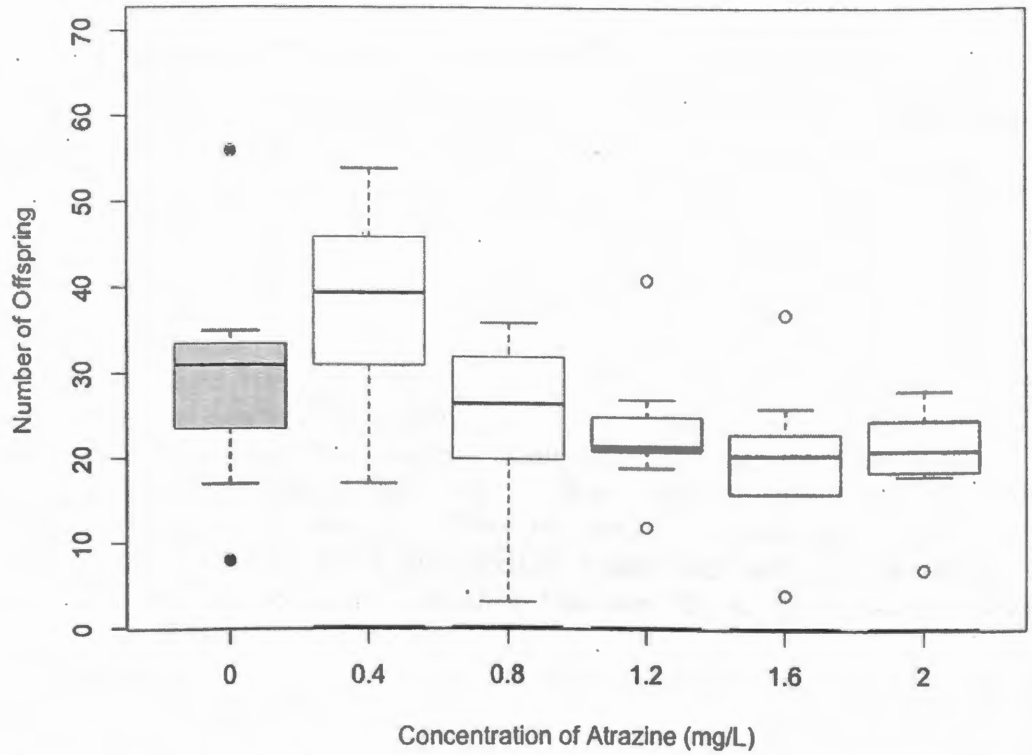

Figure 1. Boxplot of atrazine-only data.. Grey box represents the negative control.

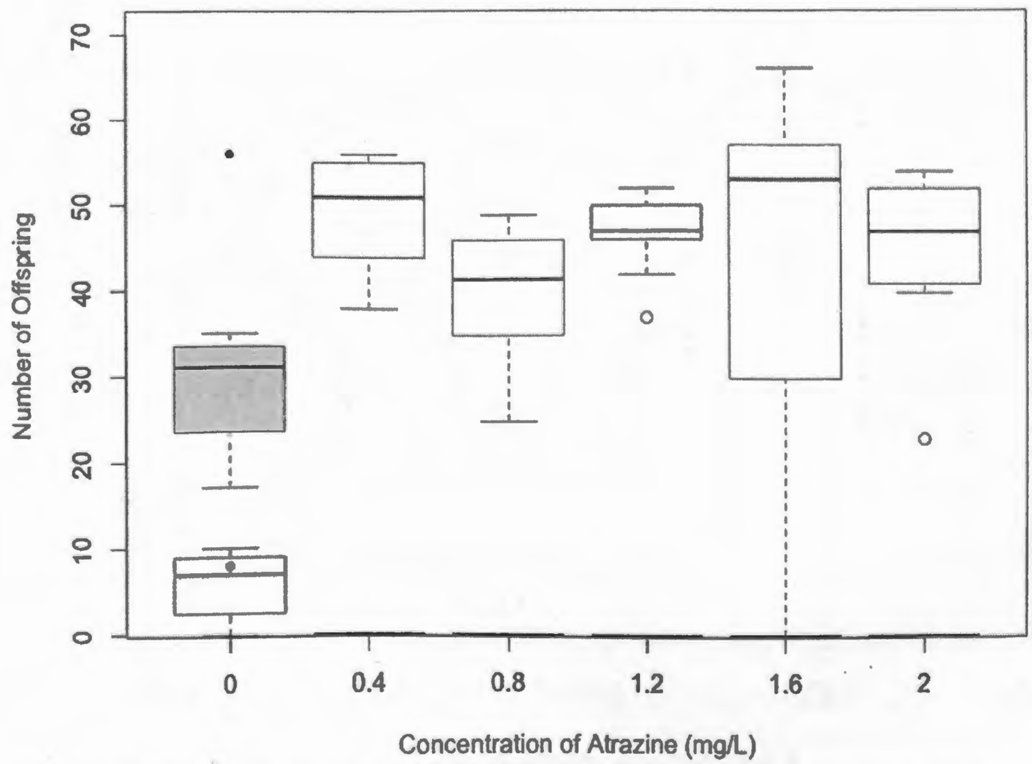

Figure 2. Boxplot of *D. magna* reproduction in the atrazine and Roundup® mixture. The grey box represents the negative control.

Log Linear Regression and LSD Analysis

Using the statistical software, R, a three parameter log linear regression analysis was performed on the atrazine only and the mixture treatments with both the negative control and the Roundup® control. *D. magna* exposed to atrazine showed an overall downward trend in reproduction (Figure 3), though there was an observed initial spike in reproduction at the lowest concentration tested (0.4mg/L) which was confirmed using a Least Significant Difference (LSD) analysis (Table 1).

Log linear analysis was attempted on the mixture treatments using two, three, four and five parameters, but the P values were such that there was no observed significant difference. LSD analysis showed some significant differences between the negative control and mixture treatments at 0.4, 1.2 and 2.0mg/L of atrazine (Table 1). The mixture treatments enhanced the reproduction of *D. magna*. The Roundup® control was also found to be significantly different from the mixture treatments with an overall reduction in offspring in the Roundup® control followed by a reproduction spike in the presence of atrazine (Table 1).

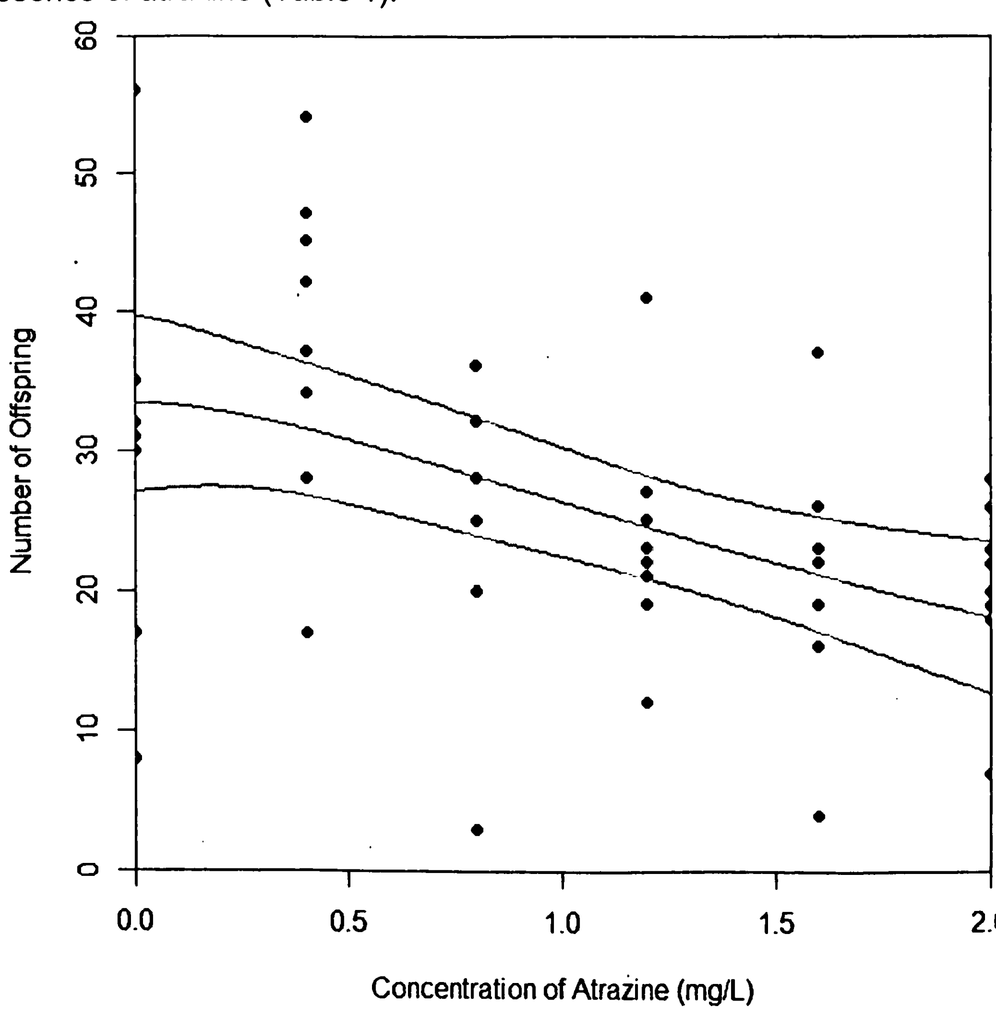

Figure 3. A three parameter log linear regression of *D. magna* reproduction with five concentrations of atrazine. The blue curve represents the prediction of reproduction and the red curves indicate the upper and lower confidence intervals.

Table 1. Least Significant Difference analysis. Different letters represent a significant difference.

Concentration of Atrazine (mg/L)	Atrazine only treatments	Mixture Treatments
0 (negative control)	ab	a
0 (Roundup® control)	not applicable	c
0.4	a	b
0.8	b	ab
1.2	b	b
1.6	b	ab
2.0	b	b

Discussion

The relatively low average reproduction observed in the negative control may be the result of the only feeding the *D. magna* the green algae: *Pseudokirschneriella subcapitata*. Recent research suggests that a mixture of the algae and a suspended yeast may be required to achieve the high reproduction in the negative control that is necessary to meet the standard method's objectives (Buratini and Aragao, 2010).

Inhibition of reproduction was observed in the atrazine treatments (up to a concentration of 2.0mg/L). This was the upper limit of concentrations tested because a previous study in this laboratory found 100% mortality in *D. magna* at an atrazine concentration of 2.5mg/L. In an acute study conducted by Hartman and Martin (1985), the LC_{50} was found to be 36 mg/L, a value much higher than determined in our previous experiment.

D. magna exposed to the atrazine and Roundup® produced more offspring than did *D. magna* in the negative control. One possible explanation for this enhanced reproduction of *D. magna* exposed to the mixture treatments is that the two herbicides are interacting and reducing toxicity. Studies of atrazine and Roundup® mixtures in soil have found increased atrazine degradation in the presence of Roundup®, a potential explanation for the results obtained here (Krutz *et al.* 2003).

Conclusions

While atrazine alone at the concentrations tested did not produce a significant effect (except for an increase in reproduction at the first concentration tested), in a mixture with Roundup®, there was an observed increase in reproduction potentially because atrazine is being degraded in the presence of

Roundup®, causing a decrease in toxicity. Further analysis to examine the life span of *D. magna* exposed to a mixture of atrazine and Roundup® could help explain this increase in reproduction.

Acknowledgements

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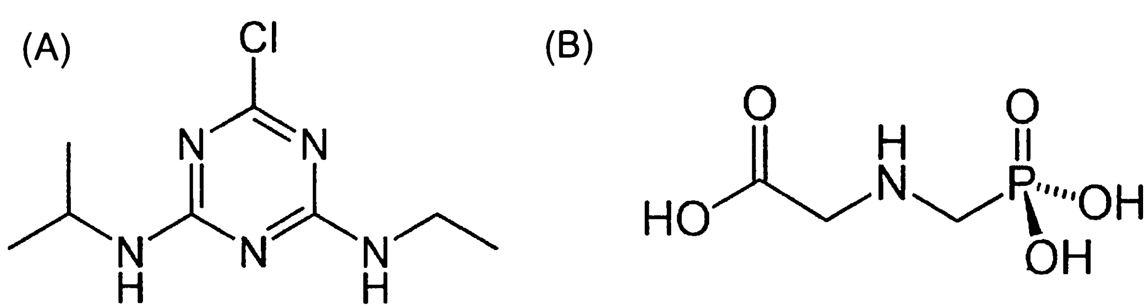

Figure 1. (A) Structure of atrazine. (B) Structure of glyphosate, the active ingredient in Roundup®.

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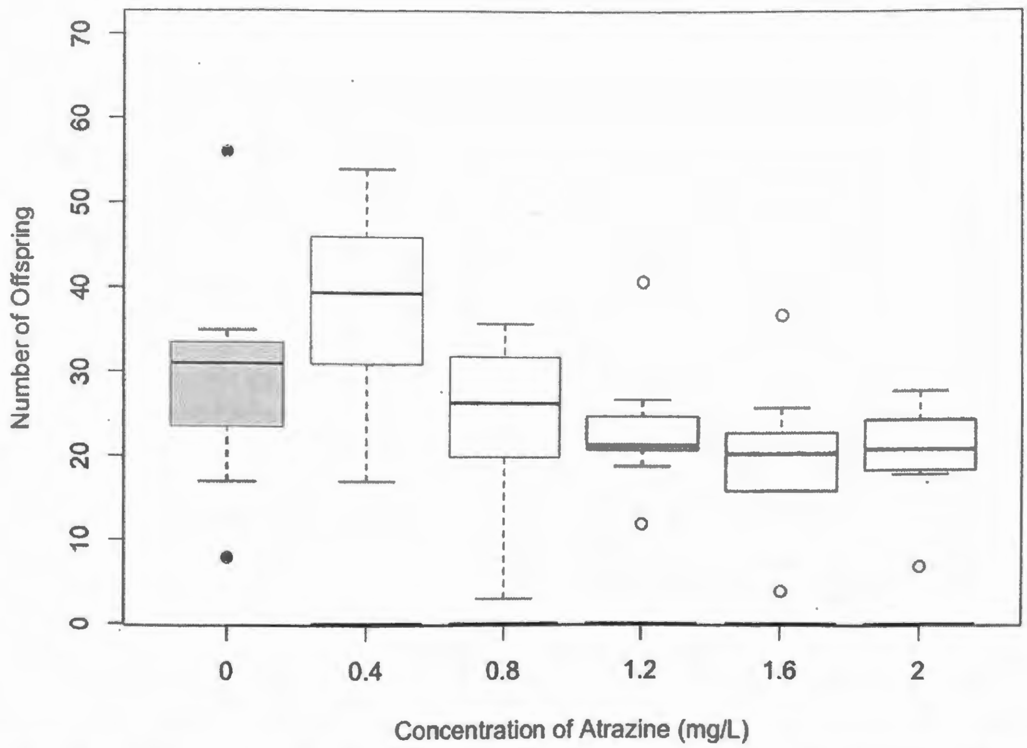

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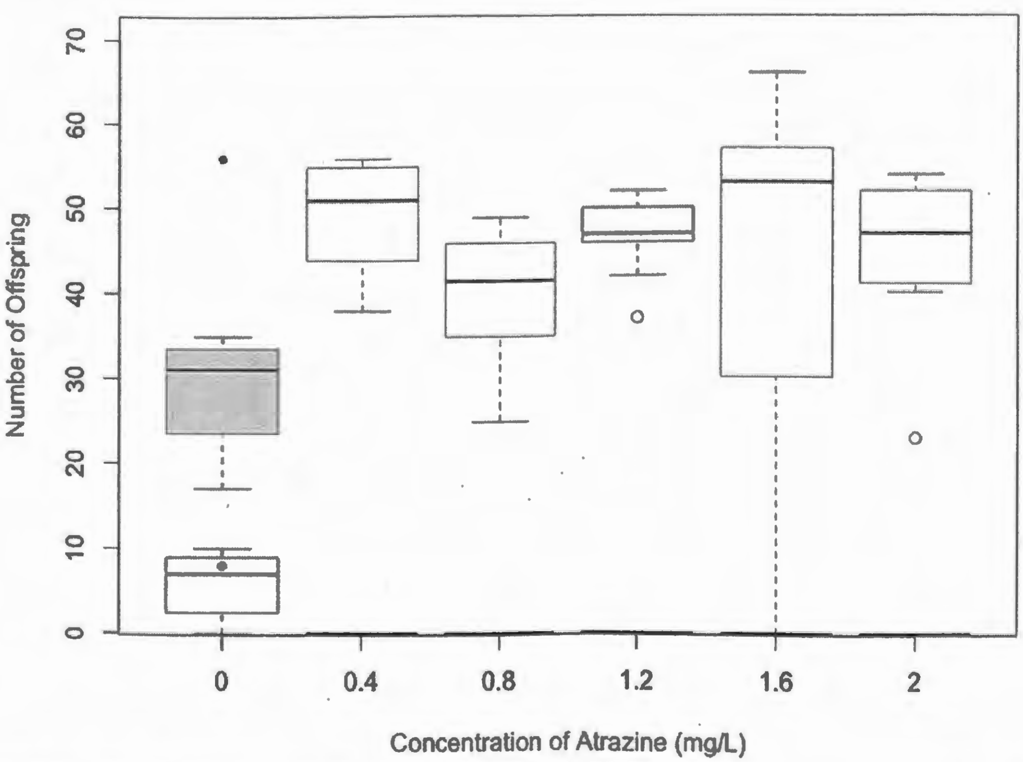

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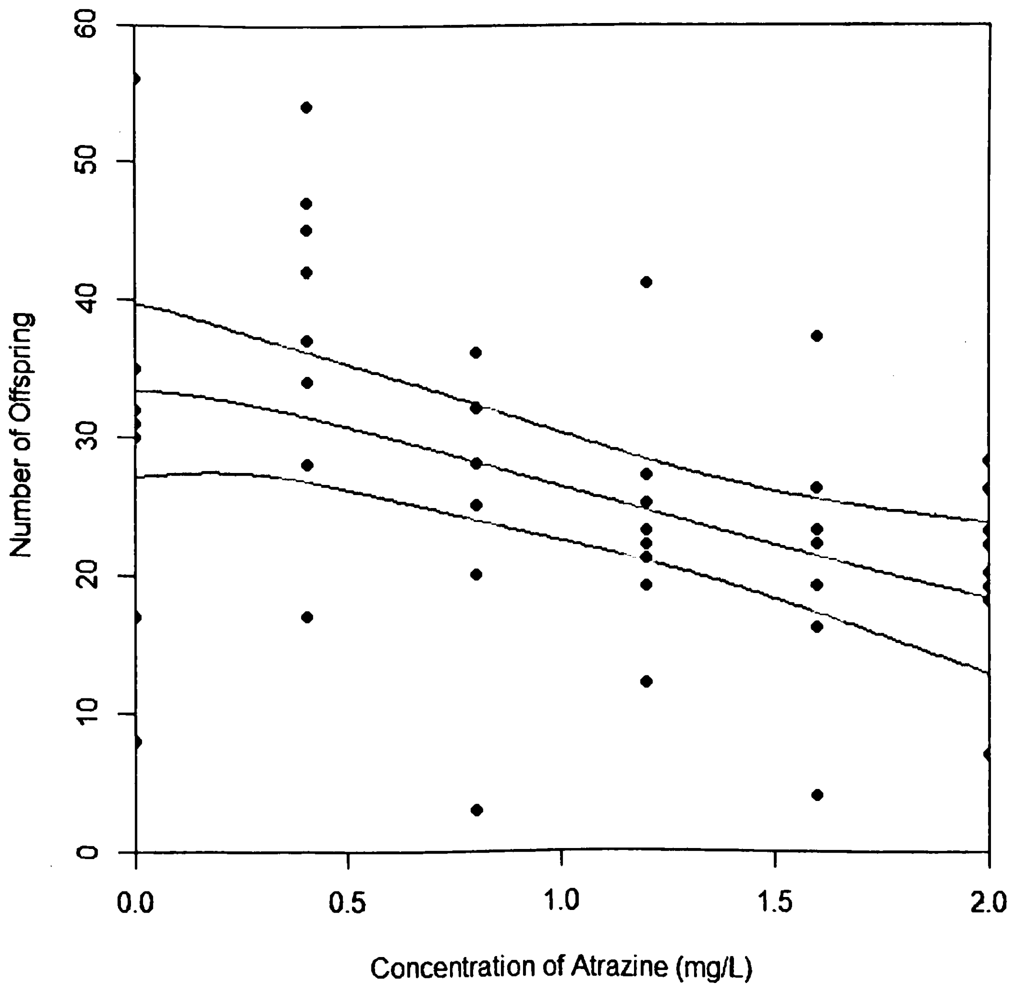

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Discussion

The relatively low average reproduction observed in the negative control may be the result of the only feeding the *D. magna* the green algae: *Pseudokirschneriella subcapitata*. Recent research suggests that a mixture of the algae and a suspended yeast may be required to achieve the high reproduction in the negative control that is necessary to meet the standard method's objectives (Buratini and Aragao, 2010).

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Reproductive Impacts of Atrazine and Roundup® on *Daphnia magna*

Jen Stiles

WWU Institute of Toxicology

WESTERN WASHINGTON UNIVERSITY

HUXLEY COLLEGE OF THE ENVIRONMENT

Overview of Toxicology Research

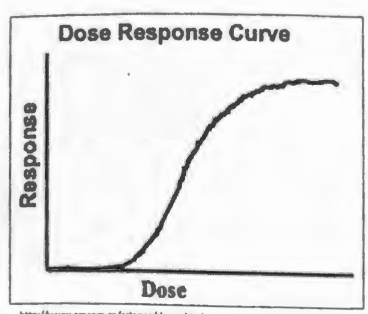
Chemical characterization from the environment

↓

Identify the toxicity pathways of interest and do targeted testing

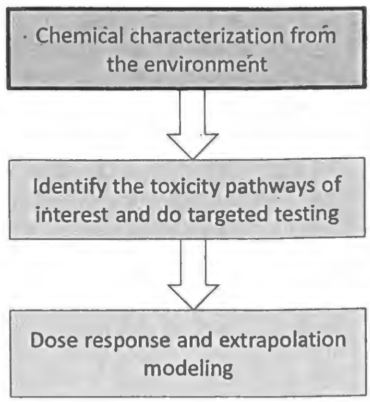
↓

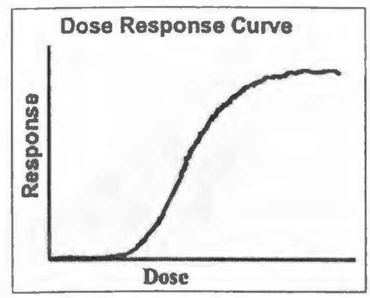
Dose response and extrapolation modeling



http://www.emcom.ca/science/dose.shtml

Overview of Toxicology Research

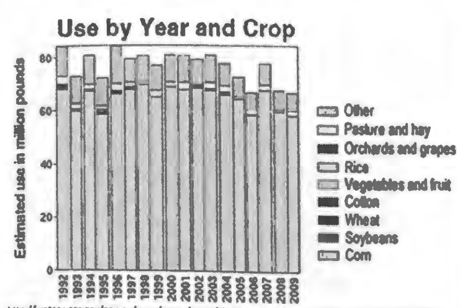




http://www.emcom.ca/science/dose.shtml

Atrazine

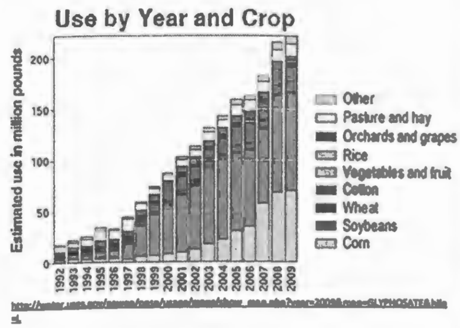
- Targets broadleaf weeds and grasses
- Commercial use for corn production
  - U.S. is the number one producer of corn (EPA 2013)
- Controversial



http://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2009&map=ATRAZINE&hilo=L

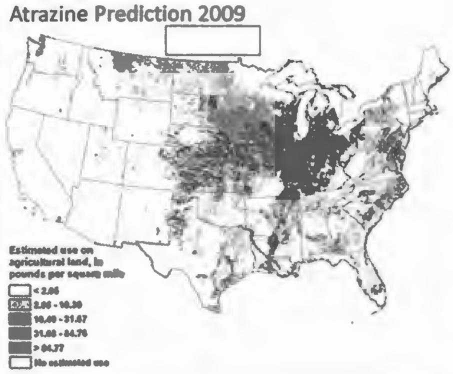
Roundup®

- Active ingredient: glyphosate
- Targets broadleaf weeds and grasses
- GMO Controversy

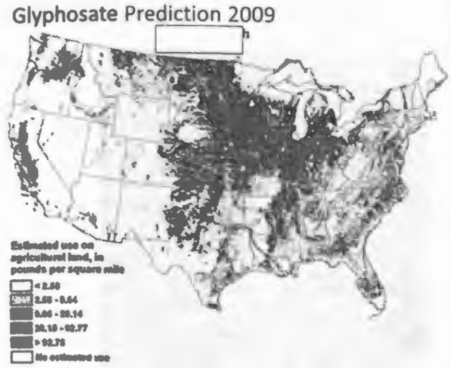


Distribution and Potential for Mixing

Atrazine Prediction 2009



Glyphosate Prediction 2009



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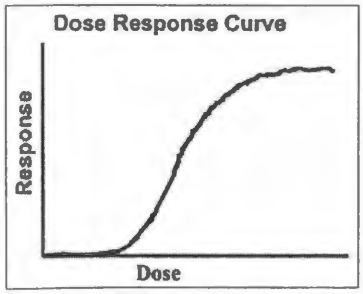

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What are *Daphnia magna*?

- Freshwater cladoceran
- Indicator Species (commonly used in toxicity tests)
- Asexual reproduction

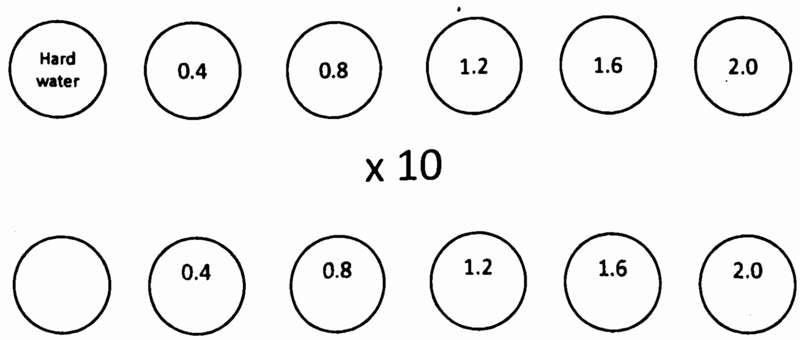
http://commons.wikimedia.org/wiki/File:Daphnia_magna-female_adult.jpg

Goal of this project

- Observe the reproduction of *D. magna* exposed to atrazine and Roundup®, alone and in a mixture

Atrazine (mg/L)
Roundup (mg/L)

Methods



x 10

Methods

- 21 day chronic test
- Counted offspring
- Statistical Analysis – R (agricolae and drc)

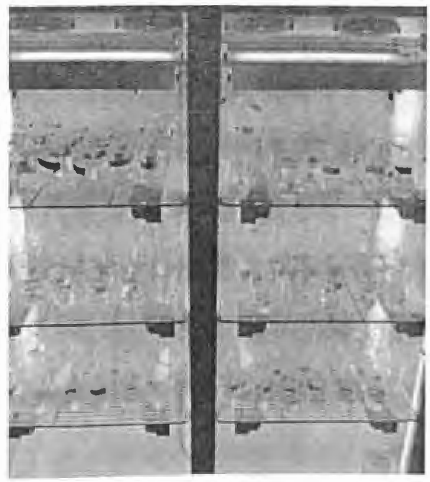
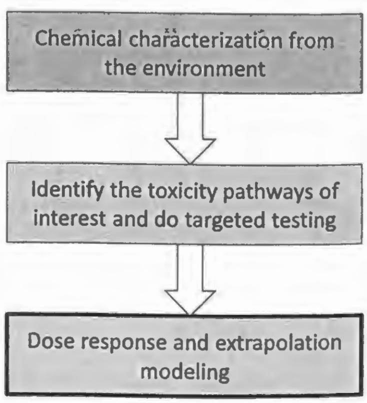
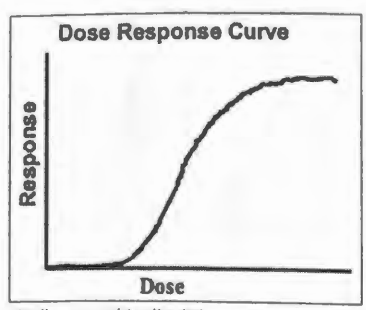


Image taken by Geoff Lowery (April 2012)

Overview of Toxicology Research





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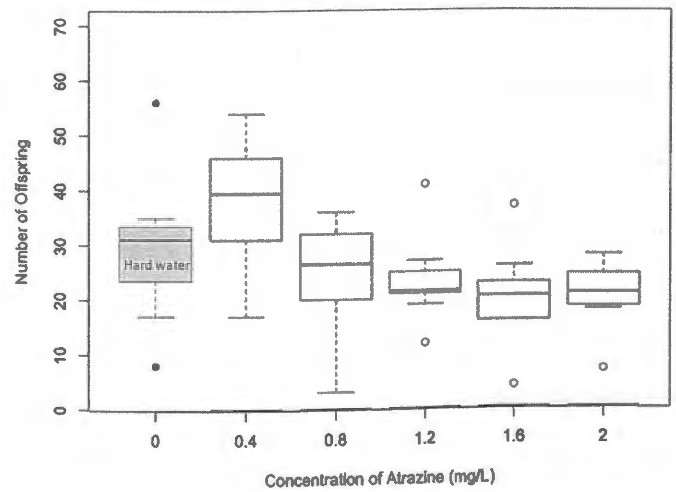
Results – Atrazine only



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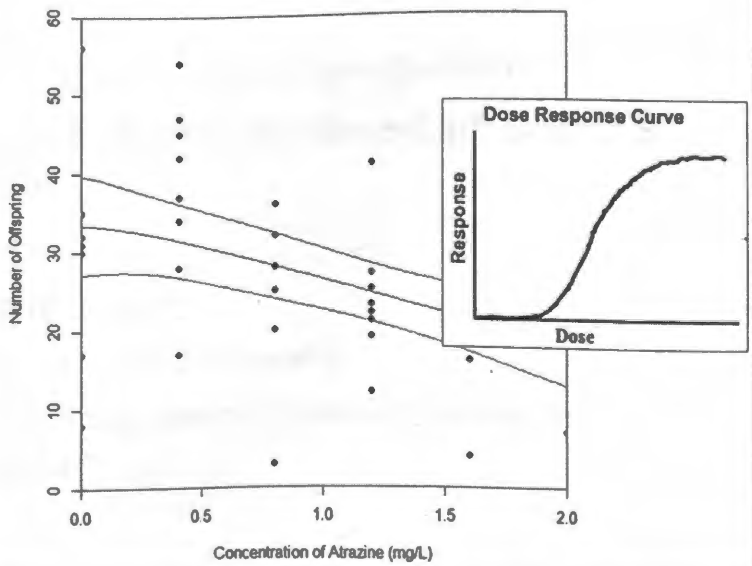
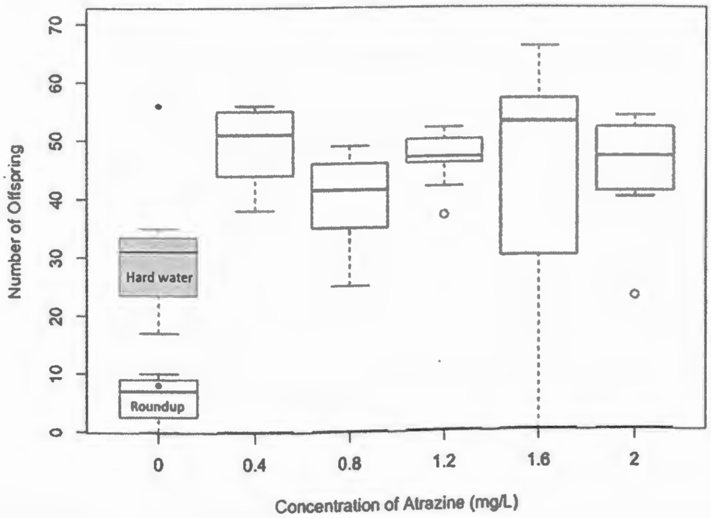


Results – Mixture



Conclusions

- Atrazine alone inhibits reproduction
- Mixture of Atrazine and Roundup® promotes reproduction

- Future research:
  - 2nd generation survivorship
  - Atrazine degradation in water in presence of Roundup®

Acknowledgements

- Geoff Lowery & Charles Hixon
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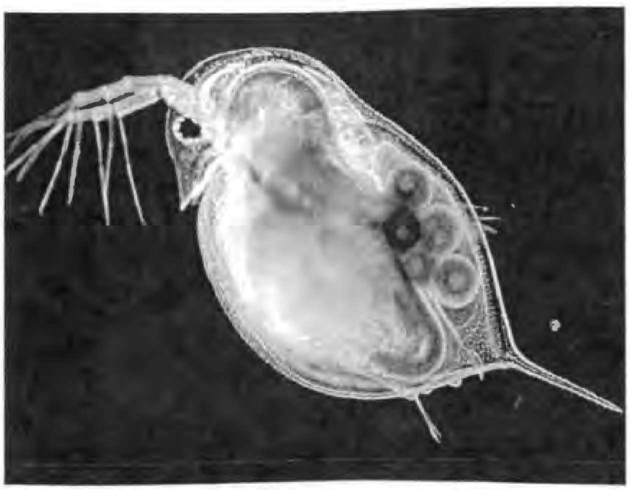
- April Markiewicz & Dr. Wayne Landis

- Institute of Toxicology & Huxley College of the Environment

Questions?



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