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Herbicides and Amphibian Populations

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Herbicides and Amphibian Populations

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An Undergraduate Thesis

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Introduction

Amphibians are a unique class of species that are found worldwide except Antarctica and Greenland. They range in size from a few millimeters to over six feet long. Amphibian's habitats are as various as their size, but one thing in common is that amphibians require an aquatic location to breed. Amphibians live in deserts, mountain prairies, rain forest, wetlands, and almost everywhere in between. Amphibians are an integrated part of most natural ecosystems across the world. Amphibians are a critical part of many food chains; they provide the important link between secondary and tertiary consumers. Amphibians are indicator species in their ecosystems, meaning their health can determine if the ecosystem is healthy or unhealthy. In there ecosystems without amphibians these food chains would be deteriorated and many species would be negatively affected.

Unfortunately various amphibian populations are starting to decline. Amphibians have already become extinct or have been placed on the endangered and threatened species list. Since 1980, declines in certain amphibian populations have occurred worldwide (Crump et al., 1992). In 2004, amphibian biologists at an international conference announced that 32 percent of amphibian species are currently, threatened, 44 percent of species are in a population decline (Stuart et al., 2004), and 120 amphibian species have likely become extinct in the last 25 years (Blaustein and Wade, 1995). Due to habitat loss and deterioration, global warming, ultraviolet light, acid rain, commercial collection, invasive species, and pesticide use have all been investigated and implicated as causes for these declines in amphibian populations.

Many amphibians inhabit both aquatic and terrestrial ecosystems, allowing them to be exposed to both terrestrial and aquatic environmental changes. Amphibians have highly permeable skin, which makes them more susceptible to toxins in the environment compared to reptiles, mammals, and fish. Amphibian exposed embryonic development, and permeable skin of amphibians heighten their susceptibility to the ramifications of pesticides.

Review/Analysis of Literature

The increasing use of herbicide in the United States, especially in the Midwest along with published research regarding herbicides and their affects on amphibian population were reviewed to determine if herbicides are adversely affecting amphibian populations.

Herbicide Use

Pesticide use has been one cause that has been under intense debate of whether it is causing decline in certain amphibian populations or if it has no adverse affect on amphibian populations. Pesticides are widely used in agriculture and in landscape management. Herbicides are a group of pesticides used to kill unwanted plants. In the U.S., herbicides account for 70 percent of all agricultural pesticide use (Kellogg et al., 2000). Herbicides were first widely used in the late 1940's. Since then herbicide use has increased over the 20th century with the introduction of Atrazine® and herbicide resistance crops in the 1990's (Gianessi, 2006). Herbicide use has started to decline in the 21st century due to more environmentally friendly farming practices; however certain herbicides have continued to increase in use. Glyphosate, the major active chemical in

Roundup® has increased in usage from 35 million pounds in 1997 to 102 million pounds used in 2002 in the United States (Gianessi, 2006). In Nebraska, 23 million pounds of herbicide active ingredients were used on crops in 2002, and 280 pounds of herbicide active ingredients in the top five used herbicides in the United States were applied to crops in 2002 (Gianessi, 2006). Herbicides were applied to 97 percent of the corn acreage in the United States in 2005, with a total of 76,470,000 acres of corn planted received herbicide application (Harris, 2006). Atrazine® was applied to 66 percent of corn planted acreages totaling 83,964,364 pounds of Atrazine® in 2002 in the United States. From these statistics, it can be concluded that the concern for amphibian populations may be due to the large amounts of herbicide use in the United States.

The primary point of exposure for amphibians associated with herbicides is related to the runoff from applications. Herbicides can accumulate in waterways across the United States. Therefore the more herbicide that is used in an area results in increased herbicide residues in waterways and can lead to wide spread accumulated contamination. The Midwest regions of the United States has the highest occurrence of herbicides in their waterways, where herbicide levels can go over EPA set containment levels in water and can be health threatening levels to humans. The allowable containment level of Atrazine® in drinking water is only 3 ppb, and short term exposure of 200 ppb is not considered health threatening to humans (Hayes, 1993). Atrazine® is found at various levels in drinking water. The average for Atrazine® is 21 ppb in ground water to 102 ppb in river basins in agricultural basins and is well above the allowable containment level for humans (Koplin et al., 1997). In midwestern states streams can be as high as 224 ppb, and tailwater pits can be as high as 2,400 ppb (Koplin et al., 1997). If

three ppb exposure of Atrazine® is harmful to humans this amount of exposure could be completely devastating to amphibians.

Published Research

With the large usage of herbicides, studies have been done to determine their affects on Amphibians. Two leading scientist have provided data indicating that herbicides are likely to cause adverse affects of amphibian populations and I concentrated most of my review on their research. Research conducted by Rick Relyea from the University of Pittsburgh, will be summarized in the study entitled, “Pesticides and Amphibians: The importance of Community Context,” Relyea tested the effects of Roundup® on three different tadpoles species: American toad (*Bufo americanus*), Leopard frog (*Rana pipiens*), and Gray Tree frog (*Hyla versicolor*). Roundup® is a commercial formulation of glyphosate (the active ingredient) that is combined with surfactant, (POEA, polyethoxylated tallowamine).

Relyea’s research is based on more natural amphibian environments. Natural environments are more complex with a variety of species and conditions. Relyea tested what affects Roundup® was having on amphibian environments as a whole and not just on the amphibians themselves. The negative affects of Roundup® related to amphibians could have an affect on the survival rates when placed with predators.

The experiment was set up with forty-five, 1200-liter cattle watering tanks containing 1000 liters of well water, to simulate compounds of natural ponds and wetlands. Over the next week dry leaves, rabbit chow, and water from nearby ponds were added to each tank. Pond water was added to serve as sources of algae and

zooplankton. A shade cloth lid was placed over the tanks to prevent insects, amphibians, and other species from entering the tank.

The algal and zooplankton communities were allowed three week to establish and then the larval amphibians were added. The American toad, leopard frog, and gray tree frog species were collected as newly deposited eggs from nearby ponds and after the eggs hatched in a separate wading pool; twenty individuals of each species were placed in each tank.

The experiment was completely randomized, with three predator treatments (no predators, adult newts, and larval beetles) and three pesticides treatments (no pesticide, insecticide Malathion, and the herbicide Roundup®). In the study, the insecticide treatment did not meet the criteria set by researcher and were not looked at. Each treatment combination was replicated five times. The tanks that had predator treatments were collected from nearby ponds and were placed in assigned tanks and added two days after the tadpoles. The predators were caged for six hours to allow the tadpoles to acclimate to the predators. It was noted that the predator numbers were selected to represent the same densities that would be found in natural wetlands. After the predators were released, herbicide was applied to selected tanks. The application of Roundup® was reduced to an application rate one-third the recommended application rate (6.4 mL AI/m²). The Roundup® was 3 percent active ingredient and with 10mL added to each tank, a concentration of 1.3 mg AI/L was added.

After 23 days the tests were terminated because the number of live tadpoles was not efficient. Pesticides and predators had no effect on periphyton or phytoplankton. However, pesticides and predators have interactive effects on the total number of

tadpoles surviving in the communities and the total biomass of the tadpoles in the community (Fig 1). When no predators were present, total tadpole survival and biomass were high, but a reduction 39-41 percent was noticed in biomass and survival was reduced from 30-80 percent when exposed to Roundup® (Fig 2) (Relyea et al., 2005). When newts were added, survival and biomass were much lower than no-predator/ no-pesticide control treatments. When Roundup® was added with newts, there was significant reduction in total survival and marginal reductions in total biomass compared to tanks containing newts with no pesticides (21 percent addition reduction in leopard frogs) (Fig 1) (Relyea et al., 2005). Beetles also reduced survival and biomass compared to control treatments, however when beetles and Roundup® were applied in the tank there was no significant difference in survival and biomass of tadpoles compared to tanks with just beetles.

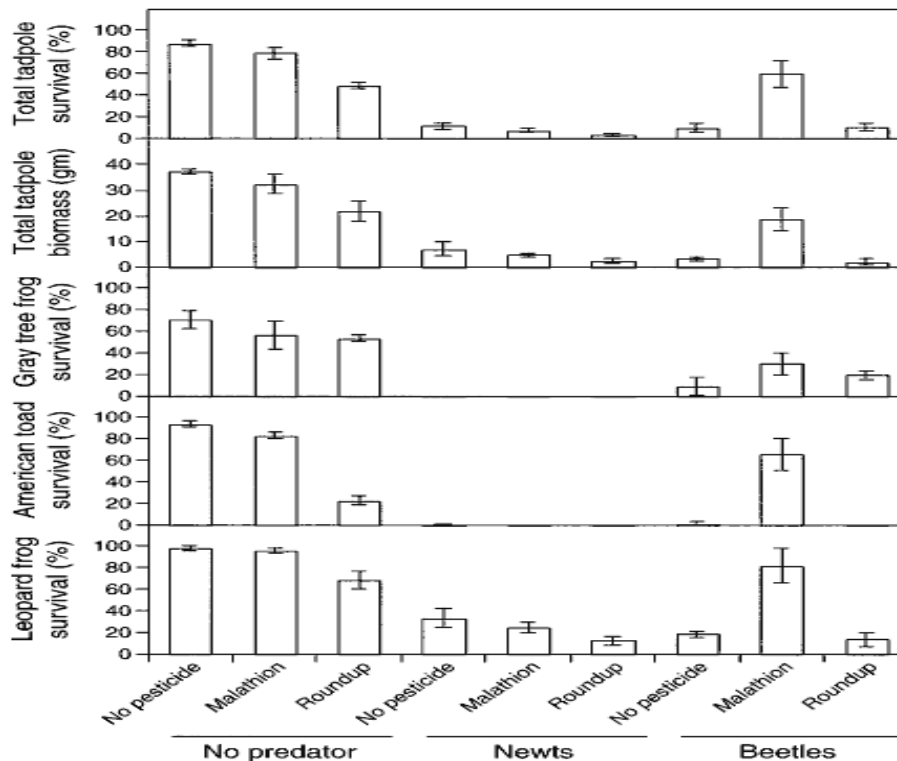


Figure 1: Relyea Roundup® Study 1- Relyea et al., 2005

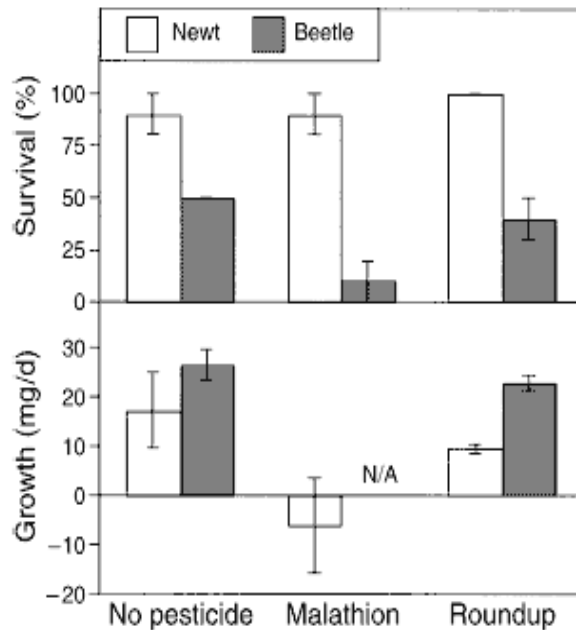


Figure 2: Relyea Roundup® Study 2 - Relyea et al., 2005

This study demonstrated that herbicides could have a diverse direct and indirect effect on aquatic communities, because Roundup® caused a 40 percent reduction in total tadpole survival and biomass (Fig 2) (Relyea et al., 2005). In previous experiments higher concentrations of Roundup® (3.8 mg AI/L) were used and caused a 70 percent reduction in amphibian diversity (Relyea et al., 2005). Tadpole survival rates were reduced ten-fold, on average from 97 percent to two percent. These results show that amphibians are affected by the toxicity of the herbicides. When both herbicides and predators were added, a reduction in survival and biomass were compared to the results when just predators were present, a reduction in survival and biomass comparable to when just predators were present shows that there is a combined effect on mortality. Amphibians affected by herbicides had reduced their ability to evade predators. The data indicates that herbicides are having a negative effect on amphibian population levels and

when combined with other negative affects it causes even a greater reduction in survival rates.

It is interesting to note from the experiments that indicated newts were not adversely affected by the herbicide. One would assume that if frogs were affected by herbicides then newts would be included in that assumption. Furthermore, the herbicides had different affects on survival rates and biomass on each of the frog species.

Relyea's work brings up interesting ideas for further research to concentrate on and a variety of theories to test. Conducting research in natural conditions versus in the laboratory will allow scientists to analyze how herbicides can adversely and directly affect the foci species and the bio-accumulated impact that may have on the surrounding ecosystems. Relyea's methodology using a variety of predators and varying the concentrations of herbicides gives a better idea of impact in specific regions where predators and herbicide concentrations are similar to those found in an area. Reylea is successful in mimicking natural conditions in order to achieve the most accurate results.

It is also an imperative factor to do determine herbicide impacts on amphibian predators. To understand how herbicides are affecting the whole ecosystem will allow scientists to determine if there are levels of herbicide contamination that will affect the health of amphibian predators. This could provide insight into what is happening to amphibian populations due to the populations of their predators. The experiments could also provide data to why newts were not affected by the herbicides and if there is something in the internal chemistry that makes them more resistance to herbicides.

In continuing with the newt resistance to the herbicide, experiments need to be conducted to see why herbicides affected amphibian species at different levels.

Experiments need to be done to figure out why the Leopard frog had higher survival rates than the American toad when exposed to herbicides. Studies done on tadpole size and how that relates to survival and biomass when tadpoles are exposed to herbicides could provide data that helps indicate which amphibian species may be more susceptible to herbicides. If these experiments are done, research and protection could be addressed to those species that need the most help. Overall, Relyea's data demonstrates that herbicides and their adverse affects on amphibian populations is a very complex issue and is in need of further research.

Another leading scientist in this study area is Tyrone Hayes. Hayes has conducted studies on the effects of Atrazine® on amphibians and in particular if Atrazine® is causing endocrine disruption on amphibians. One major study conducted and published by Hayes entitled, "Atrazine-induced Hermaphroditism at .1 ppb in American Leopard Frogs (*Rana pipens*): Laboratory and Field Evidence," tested the effects of Atrazine® on Leopard frogs and produced some alarming results.

Leopard frogs were first examined in laboratory conditions. Eggs were allowed to hatch and then placed in rearing tanks. Thirty larvae were placed in each tank and fed rabbit chow. The tanks were treated with Atrazine® at levels of .1, 1, and 25 ppb. The treatments lasted for three days and were repeated three times. To do field studies, eight field sites were selected across the United States based on Atrazine® sales in that area. Four sites (counties in Utah; Wisconsin; Nebraska, and Iowa) had Atrazine® sales lower than .4 kg/m² and four sites (Cache County, Utah; York County, Nebraska; and two sites in Iowa) had Atrazine® sales higher than .4 kg/m² (Fig 3). From each site 100 animals

were collected and sent to the lab for testing. Chemical analysis of the water was collected at these sites to determine herbicide levels.

The results from the lab studies indicated that 12 percent of the males treated with .1 and 25 ppb Atrazine® suffered from gonadal dygenesis, underdeveloped testes with poorly structure or closed lobules, and low to absent germ cells (Fig 4) (Hayes et al., 2003). 29 percent of the animals exposed to .1 ppb Atrazine and 8 percent of the animals exposed to 25 ppb Atrazine® showed signs of sexual reversal (Fig 4) (Hayes et al., 2002). The control males showed only two cases of sex reversal and one case of gonadal dygenesis. The results show a strong correlation of herbicides, in particular Atrazine®, showing signs of making male Leopard frogs hermaphroditic, which means having reproductive organs of both male and female. This procedure was done to only males and for accurate understanding of the effects Atrazine® on sex organs and systems, I believe that further research of the effects on females must be conducted.

The field data results were similar to the lab results except in the field there was no way to make a control experiment for their was no site sampled that had no trace of Atrazine® however, results seemed to correlate that the increased concentration of Atrazine® use in an area directly correlates with more cases of gonadal dygenesis and sex reversal. Sex reversal was found at seven of the eight sites. All the sites with Atrazine® sales greater than .4 kg/m² and contamination levels exceeding .2 ppb showed signs of sex reversal. In York County, Nebraska, which has high Atrazine® use, had 28 percent of the males examined had gonadal dygenesis (Fig 4) (Hayes et al., 2003)

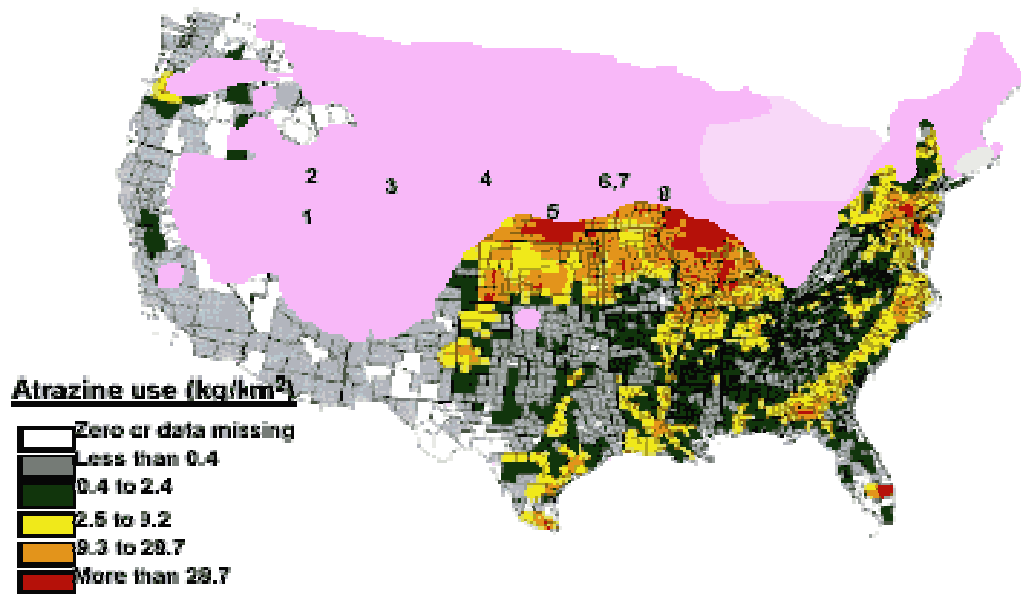


Figure 3: Hayes's Field Sites - Hayes et al., 2003

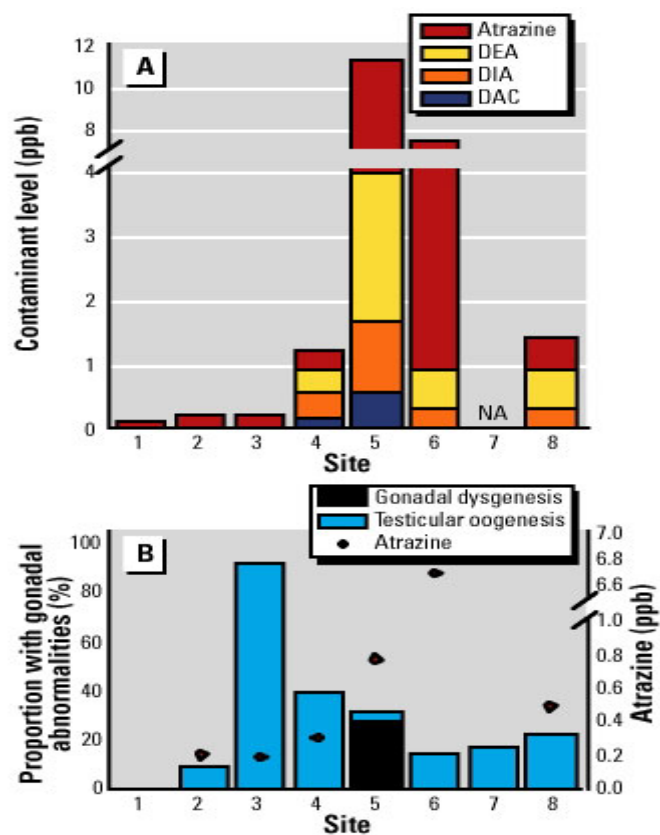


Figure 4: Hayes's Study - Hayes et al., 2003

Overall the data indicates that amphibian exposed to Atrazine® disrupted gonadal development in larvae. Gonadal dygenesis and hermaphroditism were noticed in amphibians that were exposed to Atrazine®. The data concluded that amphibians in the wild when exposed to Atrazine® experience the same effects as in a lab setting. There was also a low-dose effect. Amphibians exposed to .1 ppb Atrazine® showed higher percentages of individuals becoming hermaphroditic and gonadal dygenesis than animals exposed to 25 ppb Atrazine®. The data also seemed to indicate a chemical analysis correlation between usage and residue levels. At the site in York County, Nebraska, Atrazine® levels were found over .2ppb in irrigation ditches when Atrazine® had not been applied for over a year (Hayes et al., 2003). Also at this location rainwater and tap water had contamination levels of .4 ppb and .3 ppb, which would both cause hermaphroditism in Leopard frogs (Hayes et al., 2003). Hayes does go on to comment that the timing of Atrazine® applications could play a huge factor in affecting amphibian populations during vital development periods. These studies do not indicate that amphibian populations will be effected by Atrazine®; however one would deductively conclude becoming hermaphroditic would directly impact mating behavior potentially causing less mating to occur causing populations to decline. Due to long-term exposure, populations could become resistant to Atrazine® but modulated affects will need further research.

Hayes's research could be improved by broadening the parameters of the experiments allowing Atrazine® to be added at different levels of concentration to different developmental stages from egg to adult. These experiments could show at what levels of amphibian development are at the greatest risk from herbicides and at what

levels of concentrations. Then herbicide applications could have more restrictions on when they can be applied based of amphibian life cycles.

Hayes's experiment also brings up concerns if the EPA should readdress how herbicides are applied. Atrazine® was present at locations where previous research concluded that no Atrazine® levels should have been present. Atrazine® and other herbicides need to be retested on how they interact in the environment and it's rate of decomposition.

Additional chemical analysis needs to be conducted in order to understand all the different complexities about amphibians and their interactions with herbicides because there are still critical intricate components yet to be answered. Many inquiries arose during the research process including; Why was a lower concentration of Atrazine® causing higher levels of hermaphroditism than higher concentrations? How levels of Atrazine® effect the endocrine and sexual reproduction of amphibians? Can amphibians become resistant to herbicides at very high levels but can not at lower levels of concentration? Hayes's experiment indicated that herbicides were negatively affecting the reproductive systems of amphibians; however, the results did not demonstrate that herbicides were directly adversely affecting amphibian populations.

Hayes has also published another experiment entitled, "Hermaphroditic, demasculinized frogs after exposure to the herbicides Atrazine at low ecologically relevant doses." The experiment tested the affects of Atrazine® on the metamorphosis and sex differentiation at the ecologically relevant low doses via endocrine-disrupting mechanisms. The results indicated that Atrazine® had no effect on mortality, time to metamorphosis, length, or weight at metamorphosis. However there were affects on

sexual differentiation, larynges size, and testosterone levels. Up to 20 percent of the animals had multiple gonads (up to six in a single animal) or were hermaphrodites (with multiple testes and ovaries) (Hayes et al., 2002). Control males had larger larynges than females at metamorphosis, but males exposed to Atrazine® (>1 ppb) had reduced larynges (Hayes et al., 2002).

In another experiment, Hayes looked at plasma testosterone levels in sexually mature African Clawed frogs (*Xenopus laevis*). The effects after 46 days indicated that experimental animals suffered 10-fold decrease in plasma testosterone (Hayes et al., 2002). The loss of masculine features, such as decreased laryngeal sizes, may be a result of decreased androgens, whereas the induction of ovaries may be a result of increased estrogen synthesis and secretion. The possible common mechanism underlying the abnormal sexual development in Hayes's studies and reproductive abnormalities will have significant implications for the ecosystem and its counterparts. The data indicated that the effective doses of Atrazine® (.1-3 ppb), which can have very high occurrence in nature, are having very harmful affects on amphibians and likely reducing amphibian populations.

Hayes also conducted a study on an herbicide called Bicep 11 Magnum®, which is a combination of Atrazine® and S-metolachlor. The combination of two herbicides induced damage to the thymus of African clawed frog. The damage to the thymus caused immunosuppression and contraction of flavobacterial meningitis, which reduces the ability of the immune system to function and allow infections from bacteria. This bacterium affects the brain and spinal cord. The membranes affected are called meninges and when they become inflamed the brain cannot function properly. The herbicides also

retarded larval growth and development due to longer metamorphosis. The longer it takes to metamorphosis, the smaller the frog will be and the more susceptible it will be to predation. This study also indicates that combinations of herbicides will have negative affects on amphibians and demonstrates a more realistic concentration of herbicides in nature, where more than one herbicide is usually found in waterways and can have a conglomerated affect. This experiment also indicates that there may be other indirect negative affects due to herbicides. If herbicides cause amphibians to be more susceptible to diseases, amphibian populations could be greatly reduced. All of Hayes's experiments demonstrated that herbicides are adversely affecting the health of amphibians ranging from: hermaphordism, gonadal dygenesis, sexual differentiation, laryngeal size, testosterone levels, and immunosuppression. However Hayes' does not directly say results indicated that herbicides were adversely affecting amphibian populations but the statistics can support this statement. Studies need to be conducted to be able to connect these health issues with a decline in amphibian populations to prove that herbicide are adversely affecting amphibian populations.

There have been many studies conducted to see the affects on amphibians due to herbicides. These studies are broad and numerous but I have reviewed a few more that show a variety of the effects of herbicides on amphibians.

Studies have also been conducted on Streamside salamanders (*Ambystoma barbouri*) and the affects of Atrazine® on them. The results indicated that these salamanders exposed to Atrazine® exhibited greater activity, fewer water-conserving behaviors, and accelerated water loss four to eight months after exposure to Atrazine® compared to controls (Rohr et al., 2005). This experiment also indicated negative affects

after long periods of time after exposure. After 14 months post exposure to Atrazine®, these salamanders had significantly lower survival rates than did control animals (Rohr et al., 2006). This data indicated that not just anuran species of amphibians are being affected by exposure to herbicides but this exposure to herbicides can continue to affect an animal months after exposure. Longer time parameters of the monitoring period of the long-lasting effects are needed. Also research needs to be conducted to see the affects of herbicides on amphibians that have been exposed for months to determine long-term affects.

Herbicides have also been shown to cause deformation in amphibians. Malformations seem to be the result of environmental factors affecting frog development during early tadpole stages. From the studies reviewed prior it has been indicated that herbicides are affecting the development of amphibian larvae and are causing significant developmental problems. The studies have also indicated that herbicides are making amphibian species more susceptible to infection and parasites, which can increase their probability of becoming deformed. These deformations cause decreased fitness levels, more susceptible to predators, and less likely to mate in amphibian species. All of these problems due to malformations would likely cause lower amphibian populations.

Forty-four states have known reported frog malformations and in some areas 60 percent of certain amphibian populations showed some form of malformation (USGS website, 2001). A study conducted by Deborah Cowman tested the affects of agricultural pesticides on tadpoles in California. Tadpoles were collected along transects at three determined sites. The tadpoles were placed in field-tested design cages in ponds in three

parks (Lassen, Yosemite, and Sequoia National Park). All three parks showed signs of malformations due to herbicide runoff in the area (Cowman et al., 2002).



Figure 5: Deformed Frogs- www.science.psu.edu/alert/Kiesecker7-2002.htm

Studies have also been looked at to see the affects that herbicides combined with other stressors linked to amphibian decline are having on amphibians. The overall belief is that these combined effects could be having a greater affect on amphibian decline. Also these joint effects represent a more likely affect on amphibian populations in nature.

One study conducted by Joseph M. Kiesecker entitled, “Synergism between trematode infection and pesticide exposure: A link to amphibian limb deformities in nature?”, examined to see if there was a link between chemical contaminant exposure and trematode infection causing amphibians limb deformities. To conduct the research, Kiesecker used field experiments and laboratory experiments to test four hypotheses on wood frogs, *Rana sylvatica*. The hypotheses were: 1. Limb deformities are associated with trematode infections at natural breeding sites, 2. Trematode-mediated limb

deformities at natural breeding sites are a function of proximity to agricultural runoff, 3. Exposure to chemical contaminants will increase rates of trematode infection, 4. Contaminant-mediated increases in trematode infection are consistent with a decrease in the ability of developing amphibians to elicit an immune response that would prevent infection.

To test these hypotheses field experiments were conducted first. The field experiments had six ponds that varied in exposure to agricultural runoff. Pond water samples were tested for presence of chemical contaminants using EPA standards. The test revealed that three of the ponds had no detectable chemical contaminants, while the other three ponds had detectable levels of Atrazine and Malathion. All six ponds had measured and recorded populations of the snail, *Planorbella camponalata*, which were infected with a trematode *Riberorra sp.* During the spring of 2000, six field enclosures were placed at each of the six ponds. Three enclosures at each site were designed to allow the trematode cercariae to enter from the pond, while the other three allowed no entry.

Wood frog embryos were collected from three ponds in Centre County, PA, and allowed to hatch in 120-liter outdoor pools. After the embryos hatched, larvae were mixed together from different ponds and randomly assigned to the field enclosures. All animals in the experiment were Gosner stage 25 (Gosner, 1960) and size matched.

After 29 days the first metamorphic frog was observed. Enclosures were monitored daily and individuals were removed as they metamorphosed. Metamorphs were removed to the lab. At the lab they were weighed and preserved for later determination of limb deformities and metacercarial cysts.

To further evaluate potential links between contaminant exposure, immunocompetency, and trematode infection Kiesecker conducted a series of laboratory experiments. The experiments examined the impacts of low concentrations of three commonly used pesticides on the susceptibility to infection and a measure of immune response of larval wood frogs.

Tadpoles used in the experiment were from the same clutches used in the field experiment. Individual tadpoles were raised in polyethylene tubes containing 3.5 liters of dechlorinated tap water under a 16:8 light/dark cycle. Tubs were randomly assigned one of the four treatments and replicated 12 times. The treatments were negative control (water), a solvent control, and two levels of contaminant addition for each contaminant tested. Exposure levels were chosen to mimic conditions that may be found in nature. Wood frogs were exposed to Atrazine at 3 and 30 I ¼ g/liter, to Malathion at 200 and 2000 I ¼ g/liter, and Esfenvalerola at 180 and 1800 I ¼ g/liter. During the experiment the water was changed every other day and chemical treatments were reapplied after water changes.

After four weeks, all tadpoles were moved into 75-ml containers and exposed to either 50 *Ribeiroroe sp.* cercariae or 50 *Telorchis sp.* cercariae. After exposure tadpoles were moved into a small volume of water to eliminate the influence of tadpole behavior on exposure rates. Tadpoles were exposed to cercariae for four hours and then returned to their original containers. No cercariae were found in the tadpole containers indicating that they had all penetrated the tadpoles. After one week after exposure the tadpoles were killed and the animals preserved for later examination. Immediately before tadpoles were

killed blood samples were taken and leukocytes (white blood cells) were counted and identified.

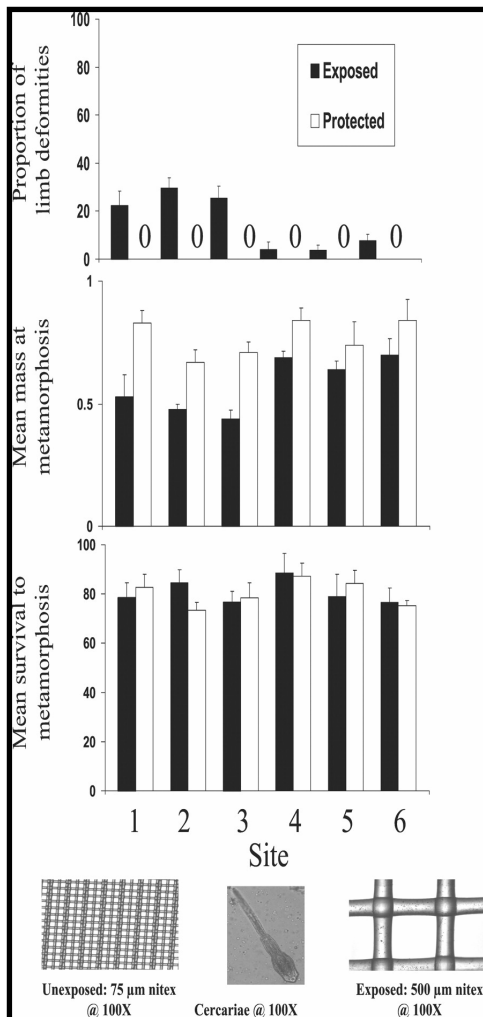


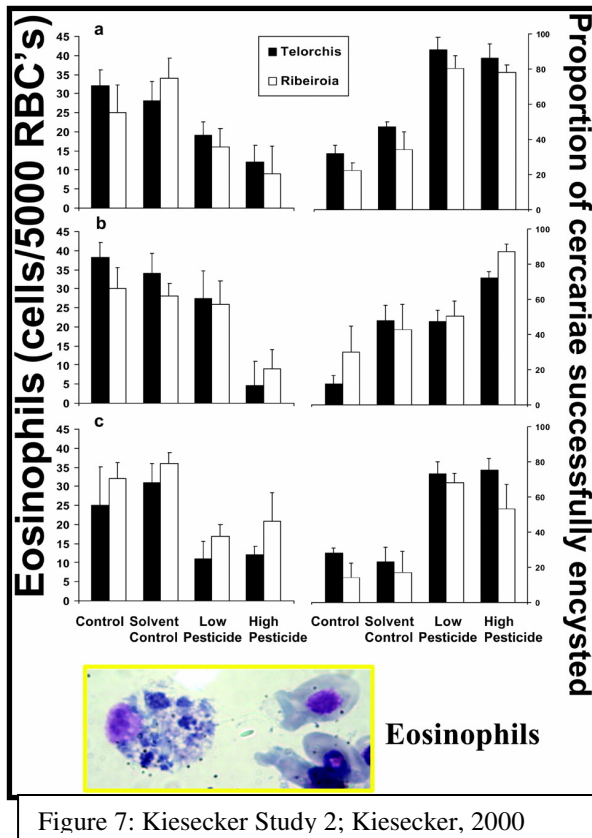
Figure 6: Kiesecker Study: Kiesecker, 2000.

The results indicate that the occurrence of limb deformities at breeding sites is directly related to trematode infection of developing amphibians (Fig 6). The analysis also revealed strong main effects of exposure to trematode infection and exposure to agricultural runoff (Kiesecker, 2002) (Fig 6).

There was also a strong interaction between the factors for limb deformities and mass. There were a significant number of limb deformities in wood frogs adjacent to agricultural fields. Wood frogs were also 37% smaller in agricultural runoff ponds exposed to cercariae than wood frogs not exposed to cercariae in agricultural runoff ponds (Fig 6) (Kiesecker, 2002).

In agricultural runoff ponds, 28.6% of the wood frogs exposed to trematode infection developed limb deformities compared to 0% among wood frogs not exposed to trematode infection (Fig 6)(Kiesecker, 2002). Wood frogs not exposed to agricultural runoff were 22% smaller in cercariae exposed specimens than wood frogs not exposed to cercariae, and only 4% developed limb deformities (Fig 6) (Kiesecker, 2002).

The laboratory experiments also revealed that cercarial encystments increased when wood frogs were exposed to chemical contaminants (Fig 7). The leukocytes were also reduced in wood frogs exposed to chemical contaminants, indicating an alteration in the immune system. The immune systems seemed to be weaker and not able to fight the infection as well when the wood frogs were exposed to chemical contaminants.



The results indicate that the combination of chemical containments and trematode infection caused a decrease in wood frog size and an increase in limb deformities. This occurrence could be because the chemical contaminants altered the immune systems and reduced the ability to fight the infection. The animals that were able to fight off the infection did so at a cost of size. Smaller frogs would be more susceptible to predation and other

factors. Frogs with weaker immune systems would be less likely to fight infections and have limb deformities. Overall, the combination of chemical contaminants and trematode infection decreases the fitness level of amphibians by decreasing size, increasing infection rates, and altering immune systems. The combination of these factors would decrease the chance of survival and reduce amphibian populations.

Multiple stressors when combined can have catastrophic affects compared to when singular stressors are tested at a time. This study shows that multiple stressors have

a greater effect on amphibians and show a more realistic view of what is affecting amphibians in nature. Continued studies where multiple stressors are needed to get a better idea of the magnitude of what herbicides and other factors are affecting amphibians and amphibian populations.

Another factor linked to decline in amphibians is the increase of UV-B radiation. The intensity of UV-B radiation reaching aquatic ecosystems is increasing due to human influences including: ozone depletion, acidification, and climate change (Herman et al., 1996). UV-B radiation has shown to be lethal to amphibian embryos and larvae (Anzalone et al., 2000). Slower growth and development in amphibians has also been caused by UV-B radiation (Belden et al., 2000). The effects of UV-B radiation on amphibians can be very harmful, but what happens when multiple stressors that presents an accumulated affect? Throughout my review I had sited the harmful affects on amphibians due to herbicides. Amphibians exposed to herbicides have suffered death, limb deformities, hermaphrodism, biomass reduction, gonadal dygenesis, sex reversal, and alteration of the immune systems to name a few. What is happening to amphibians that are exposed to herbicides and UV-B radiation, which more accurately represent what amphibians face under natural conditions? That is why a study conducted by Audrey C. Hatch and Andrew R. Balustein was done to see the effects of UV-B radiation and nitrate on amphibians. The study entitled, “Combined effects of UV-B radiation and nitrate fertilizer on larval amphibians”, was done to get a better understanding of stressors amphibians are dealing with in the wild.

The study looked at two species, Pacific treefrog (*Hyla regilla*) and long-toed salamanders (*Ambystoma macrodactylum*), and tested the affects of UV-B radiation and

nitrate on them. Two separate experiments were conducted in Willamette Valley and the Cascade Mountains. In Willamette Valley each species' larvae were exposed to variable conditions. There were two levels of UV-B radiation (with, without) and three levels of nitrate (0, 5, 20 mg/L). In the Cascade Mountains the species were kept in 4 liter plastic buckets and exposed to the same variable conditions, except only two levels of nitrate (0, 10 mg/L).

The results from the experiment implied that it wasn't one stressor alone but when stressors were combined survival rates dropped significantly. In the Willamette Valley Pacific tree frog mass was constant when UV-B radiation was not present; however, when UV-B radiation was present mass dropped at each level of nitrate concentration. At nitrate levels of 20 mg/L and UV-B radiation, the mean mass dropped by .12 grams. Similar findings were reported in the Cascade Mountain experiments on long-toed salamanders. Without UV-B radiation long-toed salamanders had an increase in mean mass when the concentration of nitrate went up, but when UV-B radiation was present mean mass dropped with the increase in nitrate concentration.

Survival rates were also affected by the combined affect of UV-B radiation and nitrate. In the Cascade Mountain experiment survival rates were right about 100% without UV-B radiation no matter what the nitrate concentration was. When UV-B radiation was present and nitrate levels were at 0 mg/L survivals rates were still about 95%, but when nitrate concentrations were raised to 10 mg/L the survival rates dropped to 20% for Pacific tree frogs.

This study indicates that the combine affects of the two stressors (UV-B radiation and nitrate) were more harmful than one stressor alone. This study represents a more

realistic view of the natural conditions for amphibians in nature facing multiple stressors. The combination of multiple stressors on amphibian reduced their mass and survival rates in this study. A reduction in mass can leave amphibians more susceptible to predation.

Studies need to be designed that test the effects of UV-B radiation, herbicide exposure, trematode infection, and predation, which would represent a more realistic view to what is happening in nature to amphibians. Amphibians are resilient creatures, but if too many stressors are affecting amphibians they will not be able to survive. It is possible that the addition of herbicides and added stressors combined with other factors is too much for successful amphibian survival. This study would seem to indicate that herbicides are “the straw that broke the camels back” or the final stressor that along with other accumulated affects are responsible for the severe decline.

There have been studies concerning my research topic that do not agree with each other. John Allran from the University of Wisconsin published a studies entitled, “Effects of Atrazine on embryos, larvae, and adults of anuran amphibians”, and “Effects of Atrazine and nitrate on Northern Leopard Frog (*Rana pipiens*) larvae exposed in the laboratory from posthatch through metamorphosis.” Allran’s data indicated at high concentrations Atrazine® there was increased buccal and thoracic ventilation, which indicated respiratory distress in adult Northern Leopard frogs and data indicated increased deformation with increased Atrazine® concentrations (Allran and Karasov, 2001). Studies are conducted by Allran using Atrazine® concentrations found in North American water, 20 ppb and 200 ppb. Results indicated that there were no affects on developmental rate, percent metamorphosis, time to metamorphosis, percent survival, or mass at metamorphosis of the larvae (Allran and Karasov, 2001). So from the data

Allran concluded that direct toxicity of Atrazine® was probably not a significant factor in recent amphibian decline because of Atrazine®. Atrazine® concentrations found to be harmful to amphibians are considerably higher than concentrations found in surface waters in North America. The study does indicate respiratory distress, which could cause negative health effects on amphibians; however, Allran does not believe that it would affect amphibian populations.

There are also studies that indicate that herbicides have no affect on amphibian populations. Gary Thompson and colleagues performed studies in Ontario, Canada in wetlands to quantify the probability and magnitude of contamination by a Glyphosate herbicide Vision®. 51 different wetlands were monitored and labeled as either: oversprayed, adjacent, or buffered depending on their location and amount of Vision® used in the area. Caging amphibian larvae in each of these wetlands was the monitoring technique performed. The results indicated there was no significant difference among mean mortality or no correlation between exposure of the Northern Leopard frogs or Green frog tadpoles to Vision® (Thompson et al., 1998). Thompson concluded that exposures typically occurring in forest wetlands are insufficient to induce significant acute mortality in native amphibian larvae. One thing to consider is In Gary Thompson's study his methodology of site sampling is occurring among different wetland ecosystems, which have higher filtration abilities of herbicide contaminants land adjacent to agriculture and run-off.

Monsanto® and other herbicide manufactures have done their own studies on the harmful affects of herbicides and the environment. All of their studies, including Thompson's study, indicated results that herbicides are not harming the environment or

amphibian populations. Monsanto® has worked very hard to tell the science community that studies done by Tyrone Hayes, Rick Relyea, and others who have done studies that indicate that herbicides are causing negative health affects to amphibians are not realistic studies.

Monsanto®'s major points to why recent studies that have indicated that herbicides are causing negative health affects on amphibians are numerous. Monsanto® states there are no Roundup® brand formulations approved in the U.S. of Canada for application over water. All Roundup® brand labels specifically prohibit application over water and any Glyphosate-containing products used for water application have been formulated to enhance their safety for aquatic organisms. Monsanto® believes that the 'over water' application method used in studies is not realistic and the results obtained in studies are not representative of results that would be obtained from the terrestrial application of Roundup® brand herbicides. Monsanto has also said that application rates in studies are unrealistically high for the typical use rates for agricultural applications. Finally, previous studies conducted with realistic application methods have shown no adverse effects on aquatic organisms.

Rick Relyea has spoken up and rebutted the comments made by Monsanto®. It is true that Roundup® is not intended for aquatic use; however, there is evidence that herbicides get in aquatic habitats. Either through runoff, inadvertent or unavoidable aerial overspray herbicides are showing up in waterways sometimes at alarming concentrations. Herbicides are no applied to large ponds or lakes, but many amphibians reproduce in small temporary wetlands, where thousands of tadpoles can be living. Relyea says, "These are the areas that are not avoided or unavoidable and where the most

harmful herbicide exposure occurs.” In response to Monsanto® that Relyea’s experiments use a to high an application rate, Relyea points out that he used application rates recommended directly from Monsanto®. It is also common knowledge that for many weeds that have become resistant to herbicides, application rates up to four times higher than recommended are used. Also studies done by Hayes have indicated concentrations well below recommended levels have caused negative health affects for amphibians. The studies that Monsanto® talks about in previous studies were conducted in cooperation with herbicide companies and done in the past when data was limited on the subject. Studies done by Monsanto® were on selected species in Australia and Africa. New data indicates that North American species are more sensitive to herbicides than the species previous studies studied. In Thompson’s study he only allowed tadpoles to be in contaminated waters for 48 hours, which is not sufficient time for the tadpoles to be contaminated. An additional thing to consider is in Gary Thompson’s study his methodology of site sampling is occurring among different wetland ecosystems, which can have higher filtration abilities of herbicide contaminants that will have different stressors than land adjacent to agriculture and run-off. Monsanto® and other chemical manufacture companies have done little to disprove that herbicides are negatively affecting amphibian’s health and possibly adversely affecting amphibian populations. Scientific evidence that shows that herbicide regulations need to be looked at to insure amphibian health and survival; however, this is a decision left up to the EPA.

The EPA, which controls herbicide applications regulations and laws, has concluded in 2003 that there was sufficient information to formulate a hypothesis that Atrazine® exposure can affect amphibian gonadal development; there was not sufficient

information to refute or confirm the hypothesis. The EPA reviewed 17 laboratory and field studies and said that the available studies had deficiencies and uncertainties that limited their usefulness in interpreting potential Atrazine® effects. The EPA had identified additional information necessary to evaluate the potential casual relationship between Atrazine® exposure and gonadal development in amphibians and the nature of any associated response relationship. According to the Interim Reregistration eligibility Decision for Atrazine, the EPA believes that more studies need to be done until sufficient evidence can be proven beyond a doubt that Atrazine is negatively affecting amphibians.

Conclusion

It seems that scientists that have been able to support their findings that herbicides along with other anthropocentric causes negatively affecting amphibians have been able to disprove Monsanto® remarks that their experiments are unrealistic. In review of all the data, there are severe negative health effects caused by amphibians being contaminated by herbicides. Tyrone Hayes has indicated that herbicides are causing male frogs to become sterile and hermaphroditic. Male frogs are also losing masculine features that would help them attract mates when being exposed to herbicides. These results would seem to indicate a reduction in reproduction and therefore a reduction in populations. Combinations of herbicides have also reduced larval growth and time to metamorphosis. These smaller sizes make these amphibians more susceptible to predators, reducing their numbers and decreasing populations. Rick Relyea has indicated that herbicides are causing direct losses in survival and biomass when tadpoles are contaminated. These direct losses would lower population numbers and could create

dramatic losses in the future. Kiesecker has also indicated that herbicides can make amphibians more susceptible to infection and malformations. These susceptibilities lower chances of survival and reproduction, which in turn reduces populations.

Experiments indicating that herbicide exposure combined with other stressors, UV-B radiation and trematode infection, lead to a decrease in biomass and survival. These combined stressors have far greater effects than single stressors alone and show a more realistic representation of what is happening to amphibians in the wild. Studies have indicated that herbicides cause endocrine disruption and other internal changes in amphibians that reduced their overall fitness. A decrease in fitness would reduce an individual's chances to avoid predation, feed, reproduce, and survive. All around a decrease in fitness in large numbers overtime would reduce amphibian populations.

The current research and data indicates that herbicides are adversely affecting amphibian health and survival rates; however, no studies have shown that herbicides are actually reducing amphibian populations. So at this current time based on my data and statistics, I cannot say with confidence that herbicides are adversely affecting amphibian populations but I believe further investigation needs to be conducted on the subject. There are many ideas for continued research and ways that concerned citizens can help save amphibians from being destroyed by herbicides.

The EPA did not look at the studies based on whether Atrazine® is causing adverse affects is reducing amphibian populations; however, to be able to prove that herbicides are causing adverse affects that are reducing amphibian populations these studies must first be accepted by the EPA. Studies that can prove that herbicides are causing negative health affects on amphibians in the areas of: time to metamorphosis,

growth, gonadal abnormalities, sex ratios, laryngeal muscles area, and plasma steroid concentrations must be proven beyond a doubt. Once herbicides have been proven to negatively affect the health and fitness of amphibians, studies can then be done on how these effects are affecting amphibian populations.

So at this moment in time, more studies need to be done according to EPA standards. Continued research on the areas of: tadpole survival, growth, time to metamorphosis, gonadal abnormalities, sex ratios, laryngeal muscles area, and plasma steroid concentrations must be continued to prove the harmful affects herbicides are having on amphibians. The more data that indicates the harmful affects of herbicides to amphibians, the more likely actions will be taken by the EPA and other concerned parties.

Amphibian population surveys need to be done now, so that if the EPA accepts that herbicides are negatively affecting amphibians there can be population surveys to base future amphibian population data with. To do these surveys, experienced individuals who can identify amphibians by sight and sound are needed. Volunteers will need to go through some sort of training course to be able to correctly identify amphibian species. Courses and programs need to be started to get individuals prepared to do these surveys. Helpful CD's or computer programs might also provide easy and effective training tools.

Environmental and animal groups need to become more involved on the issue. Groups need to start public awareness on the topic and explain to the public the problems and issues that herbicides are causing on amphibians. Awareness of issues on why these issues are problems for human health and the impacts that herbicides are causing to other

species and the impacts that the reduction of amphibians will have on other species. I believe that groups like the Sierra Club, Herpetological Societies, farming groups, and even public health groups need to come together to make herbicide safer for amphibians, the environment, and everything on this planet.

If it is proven that herbicides are causing adverse affects on amphibian populations, which will lead to the negative affects herbicides are causing on other species and the environment. Studies conducted by Tyrone Hayes have shown that herbicides have not been correctly studied. That half-life times and the interaction with soil and water might not have been correctly studied. New studies need to be done on how herbicides and for that matter pesticides are interacting in natural settings with variable conditions.

To help persuade farmers to stop using herbicides or use them more efficiently, it is necessary for integrated pest management, IPM, practices nee to be instituted on every single farm in the world. IPM is an effective and environmentally sensitive approach to pest management that relies on a combination of common-sense practices. IPM programs use current and comprehensive information on the life cycles of pest and their interaction with the environment. For IPM to work, action thresholds must be set. A point at which weed populations or environmental conditions indicate that pest control action must be taken. A farmer needs to monitor and identify weeds. Education is necessary to be able to identify weeds; however, the time and money spent on education is worthwhile. Prevention and control methods need to be established to best suite the farmer and the crop needs. Farming practices including: precise application timing, cover crops, mulches (plant and plastic), cultivation, soil solarization, and grazing animals are

all ways to control weeds. These ways have been shown to reduce weeds to manageable levels where herbicides are not needed. These farming practices have shown to be profitable because reduction in time and input costs. To persuade farmers to use IPM, studies need to show that IPM is economically profitable. Also new technology can also help reduce herbicide use. A new robot device can be programmed to the crops in your fields and be able to identify which ones are weeds and crops. The robot will be able to use its arm to actually remove the weed.

In Conclusion, I believe that the reviewed research indicates that herbicides are adversely affecting amphibian populations; however it does not prove my hypothesis. There is significant data that demonstrates that herbicides are negatively affecting amphibian health ranging from areas of: time to metamorphosis, growth, gonadal abnormalities, sex ratios, laryngeal muscle area, plasma steroid concentrations, tadpole survival rates, and water retention. Continued research in these areas needs to be done so that actions are taken to reduce herbicide use and exposure to amphibians. Amphibian surveys need to be completed to be able to set up population counts. These counts can be used to compare population numbers over time and be able to correlate herbicide use to amphibian decline. Herbicide use needs to be reevaluated and better farming practices needed to be used. IPM is an example of good farming practices that reduce the amount of herbicide use on the farm. Finally, concerned citizens and organizations need to be proactive on the subject and let the general public know it is aware of what is going on. If everyone does their part, Amphibians will not be destroyed by herbicides and our environment will be a safer and greener place.

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