Impacts of chytrid fungus and contaminants on amphibians of the Missouri river

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B.S., Benedictine College, 2007

A Thesis Submitted in Partial Fulfillment of
The Requirements for the Degree of
Masters of Science
Division of Biology

In the Graduate School
The University of South Dakota

August 2011

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ABSTRACT

Nearly one third of amphibian species are considered endangered and/ or declining. Amphibian chytrid fungus (Bd, *Batrachochytrium dendrobatidis*) is found around the world causing the death and decline of populations in some of these regions. Amphibian populations are also being impacted by land use changes, with agricultural land severely modifying and removing native habitat. As amphibians depend on runoff water from these sites, they are susceptible to contamination. Very little research has examined the interaction of contaminants on disease susceptibility in amphibians. For this study, I performed a field study to examine the contamination and disease levels along the National Recreational Missouri River and laboratory experiments to examine the impacts of atrazine (an herbicide), triclosan (an antimicrobial), Bd, and cue from natural predators on larval anurans. I found Bd along the sampled section of the Missouri River although in low prevalence. In 15 of the 20 sampled sites I found Atrazine; and in five of those sites there was additional pesticides detected: acetochlor, metalochlor DEA, and DIA. The survival of Leopard frog larvae in the atrazine experimental study were largely determined by the type of site they were collected from (with high agricultural impact sites having decreased survival) and further impacted by exposure to low (0.5 μg/L) and medium (5μg/L) concentrations of atrazine. The Woodhouse's toad larvae tested in the triclosan experimental study had a lower LC50 then the EPA's most sensitive species. An additional finding revealed that the tadpoles exposed to both a low level of triclosan (10μg/L) and Bd had an increased survival rate when compared to those exposed to only Bd suggesting a protective effect of the contaminant. From these studies, we can see that while contaminants can have both positive and negative impacts on amphibian survival in the presence of Bd. Given the prevalence of pesticides in natural areas though, and the clear impact of these areas on the survival of wild captured tadpoles, agricultural land use is likely have a large indirect effect on amphibian populations. Further research investigating what pesticides, or other factors, are driving these impacts is of great importance given the recent increases in agricultural land use and recent declines of amphibian populations worldwide.

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CHAPTER ONE

Potential Impacts of Agricultural Inputs on Amphibian Populations Along the Upper Missouri River

Abstract

Amphibians are declining worldwide with multiple factors being involved, including the loss of habitat, anthropogenic disturbances, invasive species, and disease. While studies have been conducted on each of these factors independently, their interactive impacts are still being explored. Along the 59-mile reach of the Missouri National Recreational River, there are a variety of land uses each of which affects amphibian habitat quality within the floodplain. This study examines the influence of pesticide presence on amphibian populations and how it might increase susceptibility to the amphibian chytrid fungus. During the summer of 2010, twenty sites along the 59-mile reach were monitored for water quality, pesticide contamination, amphibian population estimates, and chytrid prevalence. Over the course of the study, seven species of amphibians were detected and sampled for chytrid and the herbicide atrazine was found in 15 of the 20 sites at concentrations as high as 0.59 ppb. Five of 15 sites with atrazine also contained other herbicides such as metolachlor and acetochlor. Species richness at these multiple pesticide sites was significantly lower indicating a potential impact due to pesticide exposure. Using the information collected during the field study; I conducted a multi-factorial laboratory study exposing Leopard frog (*Lithobates pipiens/blairi*) tadpoles to three typically encountered stressors: the herbicide atrazine, the amphibian chytrid

fungus, and a predatory dragonfly cue. Tadpoles collected from sites with multiple pesticides exhibited significantly lower survival, and reduced measures of length and mass. With site origin of tadpoles incorporated as a covariate, atrazine at the low (0.5 μg/L) and medium (5 μg/L) exposure concentrations still exhibited significant lethal and sub-lethal impacts on the tadpoles. This study suggests that amphibian populations along the Missouri River are being impacted surface runoff from nearby agricultural fields. Declines in species richness might be entirely due to impacts of, or related to, higher herbicide presence at these sites.

Introduction

The leading cause of amphibian declines is habitat modification and loss (Stuart et al. 2004). The alteration of land use from grassland to agricultural areas can result in the doubly problematic stress of loss of ideal habitat along with potential contamination of nearby intact habitats. While several studies have examined the role that pesticides have on amphibians (e.g. (Fraker and Smith 2004, Relyea 2005a), very few have been able to measure these impacts in the field (Hayes et al. 2002a).

Land use along the upper Missouri River experienced a major change when several dams were built during the 1950s. Deep reservoirs were formed above the dams while reduced flooding downstream of dams resulted in decreased backwater habitats and off channel shoots, or wetlands (Yeagar 2010). Along with the reduction of the threat of flooding, agricultural usage of land near the river has increased dramatically since that time. Therefore, in addition to the reduction in

wetlands, there has been an increase in the amount of land being plowed up and planted with row crops (corn and soy beans). Unfortunately, much of the suitable amphibian wetland habitat that remains intact is adjacent to this farmland and likely to be highly influenced by agrochemicals.

Atrazine is used around the world but is the most commonly used herbicide along the corn belt of the United States. It is used as broadleaf/ grass herbicide used when growing sugar gum, corn, and soybeans. It is also the most detected contaminant in freshwater systems in the United States (Kolpin et al. 2002). It can be found in some of the most 'pristine' areas, carried there by wind, rain and water drainage (citation). Atrazine can be found in low amounts (0.05 - 1.83 μg/L) in rain water in the United States, especially along the corn belt; it can also be found across the Atlantic ocean in several European countries (Buser 1990, Trautner et al. 1992, Blum et al. 1993, Steinheimer and Scoggin 2000).

Atrazine at levels as low as 1 ppb increase the number of feminized males in both African Clawed (*Xenopus laevis*) and Northern Leopard Frogs (*Rana pippins*) (Hayes et al. 2002a, Hayes et al. 2002b, Hayes et al. 2006b, Oka et al. 2008). Atrazine also slows the metamorphic rate of African Clawed Frog tadpoles (Sullivan and Spence 2003). Several agrochemicals also have synergistic effects when paired with disease and parasites (Davidson et al. 2007, Rohr et al. 2008). For example, atrazine in particular, causes Tiger Salamanders (*Ambystoma tigrinum*) to become more susceptible to ranavirus (Kerby and Storfer 2009, Kerby et al. 2011). Wood frogs (*Lithobates sylvaticus)* exposed to atrazine also experience an increased rate of trematode infection (Kiesecker 2002). Despite these data, there is little known

regarding the impacts of atrazine on amphibian populations along the upper Missouri river.

The 59-mile reach of the National Recreational Missouri River is a stretch of river where wetland habitats are protected and created through the management of the National Park Service. It begins at the base of Gavins Point Dam, just outside of Yankton, SD, and freely flows until Ponca State Park in Nebraska. At this point, the river becomes channelized till it reaches the Mississippi River. The banks along this stretch of the river are comprised of both private and public land with land use varying from game production to farmland. This reach of the Missouri River has higher levels of agrochemicals then the Upper Mississippi River, the only other large equivalent river stretch in the United States (Lerch and Blanchard 2003). Pesticide levels found in nearby wetland habitat has been attributed to the large amount of surrounding cropland area (Frankforter 1995). Despite this heavy use of pesticides, little research has focused on amphibian populations in the region.

Only a few studies have been conducted on amphibian populations along the Missouri River (Fishbeck and Underhill 1960, Cunningham 2005). Those that do typically focus on a specific species (i.e., the Blanchard's Cricket Frog (*Acris crepitans*), Northern Leopard Frog; (Burdick and Swanson , Henrich 1968, Burdick and Swanson 2010). The 59-mile reach of the National Recreational Missouri River encompasses habitat for seven known species of amphibian some of which are experiencing documented declines in the region. The Plains Leopard Frog (*Lithobates blairi*), also natively found along the Missouri River, is listed as scarce or very rare in the State of South Dakota (http://www.sdgfp.info). In particular the

Blanchard's Cricket Frog (*Acris blanchardi*) experienced a drastic decline in the 1970's which was attributed mainly to the use of agrochemicals and habitat loss (Hammerson and Livo 1999). It is now endangered in the state of Minnesota (Berendzen 2003) and "critically imperiled" in the State of South Dakota (http://www.sdgfp.info).

Northern Leopard Frog (*Lithobates pipiens)* populations are also being monitored as their populations fall closer to the criteria for being listed as a federally threatened species. The 59-mile reach of the Missouri River is a unique area within the Northern Leopard Frog's range, within this section it has been hybridizing with the Plains Leopard Frog (*Lithobates blairi;* (Lynch 1978). The Northern Leopard Frog (*Lithobates pipiens)* is also know to hybridize with the Southern Leopard Frog (*Lithobates sphenocephalus)* in Texas, while studies have been conducted on these hybrid species (Platz 1972, Sage and Selander 1979, Parris 2001) little is known regarding the Northern and Plains Leopard Frog hybrid (Littlejohn and Oldham 1968, Dunlap and Kruse 1976, Di Candia and Routman 2007).

The Northern Leopard frog is known to carry the deadly fungal pathogen *Batrachochytrium dendrobatidis* (or Bd) known to cause chytridiomycosis in amphibians. This disease is known to decimate amphibian populations worldwide, but virtually nothing is known regarding its presence in South Dakota. Nor is there much known regarding how pesticide contamination might alter the susceptibility of amphibians to this disease.

In this study, I investigated the potential effects of pesticide contamination on the overall amphibian diversity and richness of species at each site along the 59-mile reach of the Missouri River. In a follow up laboratory experiment, I looked at the direct interactions of atrazine, Bd, and predator cue on the survival and growth of Northern Leopard Frog tadpoles.

Methods

Field Study

Map creation

The 59-mile reach of the National Recreation Missouri River stretches from the base of Gavins Point Dam in Yankton, South Dakota, to the start of channelization at Ponca State Park, Nebraska. All sites for this study were selected from within this reach of the river. Using the cropland, slope, aspect, aerial photos, and topography map layers in ArcGIS 9.3, sites were given a high or low runoff impact designation. High sites included those that had a slope and aspect facing the site, near to a farm (using a buffer of 25 meters around each identified site where those that would potentially have a high level of impact from run-off or aerial drift of pesticides applied to surrounding row crops), visible water in the aerial photo, and a disconnect from the main channel of the river. Twenty sites were selected based on potential run-off impacts from surrounding agricultural areas with half qualifying as high impact sites, and half as low (Figure 1.1, Table 1.1).

Data collection

During each site visit, I measured the standing water dimensions, and several water quality parameters: the temperature (°C), conductivity (mS/cm), pH, chlorine (mg/L), ammonium (mg/L-N), ammonia (mg/L-N), and chlorophyll (µg/L). Water quality measurements were taken using a YSI 650 MDS meter using a 6820 probe. Weather data were recorded for each visit including: air temperature (°C), cloud cover, wind, and precipitation.

Visual encounter surveys were conducted to generate an estimate of amphibian abundance and diversity at each site. Two researchers walked the edge of the water body on either side of the site counting the numbers of individuals for each species encountered. Calling individuals were also noted. Captured individuals were swabbed for Bd, measured (snout vent length, and mass), and sexed. A photo was taken of each individual for mark and recapture study. Species such as the Northern Leopard frog and Woodhouse's toad have unique patterns on their ventral side that can be identified for recapture identification (D.S. Pilliod In prep).

Tadpole sampling

During the second set of surveys in June, tadpoles began hatching from egg masses at the sites. Approximately 50 tadpoles from each site were collected for Bd analysis and diversity calculations. No specific species was targeted; the first 50 captured tadpoles were taken regardless of species.

Mid-season estimate of surface area

An approximation of surface area of water was calculated in June. At each site, an electronic range finder or standard measuring tape was used to measure the sites longest width and length. Each site was also sketched to assist in the calculation. The diameter of each survey site was traced using the measuring tool in ArcGIS 9.3, which then calculated the diameter and surface area. The aerial photos from the 2010 sampling season and reference sketches made in the field were used to obtain the actual area surveyed.

Pathogen analysis

Swabs taken in the field were analyzed for the presence of Bd. DNA extraction was done using the DNeasy kit from Qiagen. Each sample was run using the Applied Biosystems StepOne Plus real time PCR machine (Applied Biosystems, Inc., CA, USA) according to standard protocol as described by Boyle et al. (2004). Each sample was run in triplicate and was considered positive if two of the three replicates were positive.

Pesticide sampling

During the first sampling trip, pesticide grab samples were collected in 1 liter amber glass jars and put on ice while out in the field and then immediately placed in a 4°C refrigerator upon returning to the lab. The samples were taken to the University of Nebraska Lincoln's Water Quality Lab in Omaha, NE. Each sample was tested for 24 common pesticides including: acetochlor, alachlor, atrazine, butylate, chlorpyriphos, chlorthalonil, cyanazine, deethylatrazine (DEA), and deisopropylatrazine (DIA), dimethenamid, EPTC, metolachlor, metribuzin, norflorazon, pendamethalin, permethrin, prometon, propachlor, propazine,

simazine, tebupyrimfos, telfluthrin, terbufos, and trifluralin. Due to flooding and the timing of pesticide application, no additional samples were taken later in the season.

Data Analysis

Statistical Analysis

The water quality parameters were examined by month by site designation and compared for seasonal and impact level differences. Water quality was then compared to the amphibian diversity and richness to determine any impacts. For the site type analyses, each site was averaged across the entire field season (May, June, July), and overall averages were used in a generalized linear model. To look at effect by month, I used a linear model with each water quality parameter as a response variable.

Amphibian Species Richness

A species count was calculated for the total number of species per site over all three visits. Using a generalized linear value model, this value was then run as the response variable with the impact of run-off type as the independant variable. This test was run in order to determine if the pesticides presence was associated with the number of species at a site.

The species diversity for each site was determined using a Shannon Weiner index. Each species value was standardized by dividing the frequency of individuals at each site for each species by the distance walked for each survey. This value was then used to determine the diversity for each site. The diversity value was then run as a response value in a generalized linear model using pesticide presence or

absence as the independent factor to determine if pesticide presence was associated with amphibian diversity at the sites.

The parameters of each site testing positive for Bd were considered with a focus on the water and air temperature, month of capture, and pesticide impact level for each sample.

To look at the difference between the sites modeled runoff designation to the pesticide detection I grouped all sites with positive detections for pesticide as high impacted sites and those without any detections as low. A chi-square test was used to compare the run-off expected contamination with the sites that had pesticides in them.

All statistical analyses were performed on the software package R version 2.11.1 with the R commander interface.

Lab Study

Understanding the interactions of different stressors the tadpoles encounter at the field sites is important to understanding the dynamics they are up against. This study looks at a few key parameters in the lab based on field observations. I conducted a multiple-stressor experiment looking at the impacts of atrazine, Bd, and predator cue on Leopard frog (*Lithobates pippins/ blairi*) tadpoles.

Leopard frog (*Lithobates pippins/ blairi*) tadpoles were collected from the aforementioned field study sites, noting from where each was collected. All tadpoles were kept in the laboratory for a minimum of 7 days prior to experimentation for acclimation. Since both species breed in the same sites and readily hybridize at

these sites, I am combining the species designation for the tadpoles since hybrids cannot be reliably distinguished at this developmental stage.

Tadpoles were fed ground up fish flakes (TetraMin ProCare Fish Food Flakes) on a weekly interval (24 hours prior to water changes). Each tadpole was individually housed in a circular prewashed plastic container in-200 mL of treated water (see below). The tadpoles were randomly assigned to positions on shelving units and split between the high and low run-off designation from the field study. The tadpoles were raised and the experiment was conducted at 22 ± 1° C with a 12: 12 hour light: dark photoperiod.

A full factorial 2 (Bd/ no Bd) x 5 (0 μg/L, 0.5 μg/L, 5 μg/L, 50 μg/L atrazine and a solvent control) x 2 (predator cue/ no predator cue) design was implemented for 20 treatments that were replicated 11-12x with a total of 228 animals. All water used in the experiment was reverse osmosis water treated with R/O Rite (Kent Marine R/O Rite, Walnut Creek, CA) to create a consistent pH and mineral content and avoid inadvertent pesticide contamination. The atrazine and predator cue treatments were applied on day 1 and reapplied after a water change on day 8. The experiment was run for a total of 15 days. On the initial day and at subsequent weekly water changes photos of the tadpole and scale were taken for measurements including: mass (g), snout-vent length (SVL, mm), and total body length (mm). Daily, deceased tadpoles were noted, removed, and stored in ethanol for future Bd testing.

Bactrachochytrium dendrobatidis

Bactrachochytrium dendrobatidis (JEL 215 isolate) was grown on 2 % tryptone-agar according to standard procedure (Longcore et al. 1999). After a week of growth, each plate was flooded with reverse osmosis water, to suspend the Bd zoospores. The spores were counted using a phase contrast microscope and a hemocytometer. To apply the Bd treatment, 1 mL of zoospore-laden water was added to each container resulting in 70,000 Bd zoospores per 200 ml of water. The tadpoles were exposed to the Bd only on the initial day of the experiment.

Atrazine

The second stressor the tadpoles were exposed to was atrazine. It was dosed as a water control, solvent control (Dimethyl sulfoxide, DMSO), low (0.5 μg/L), medium (5 μg/L), and high (50 μg/L). Stock solutions were created for each desired concentration, for application, 1 ml of each stock solution was added resulting in the desired final concentrations.

Predator cue

The final and natural stressor used in this study, predator cue was created using dragonfly larvae (*Anax sp*.) taken from the same location as the tadpoles. Dragonflies were fed tadpoles 36 hours prior to being placed in cue water. The dragonfly larvae were left in the water for an additional 36 hours prior to use with the tadpole to create enough cue signal strength. Eleven dragonfly larvae were used to create the cue in 94.6 L of treated water.

qPCR analysis

At the end of the experiment, the mouthparts of the tadpoles were dissected off the tadpoles and used for Bd analysis. The DNA extraction and qPCR was conducted using the same methodology stated above for the Bd field analysis.

Statistical Analysis

I ran the statistics using a generalized linear model with binomial distribution and logit link function, with tadpole survival as the response variable and each treatment (atrazine, Bd, and predator cue) as a factor. For this model, the site the tadpoles were collected from was run as a covariate, each site was categorized as high (more than one pesticide detected) or low (one or none pesticides detected) prior to running the model.

As a measure of sub-lethal impacts, SVL and mass were both analyzed using a linear model with the site type collected from as a covariate. SVL and mass values were normalized by taking the difference between the initial and final measurements and then dividing by the initial measurement ((final- initial)/initial). This value is is referred to as the 'adjusted mass/ SVL' for the remainder of the manuscript.

All statistics were run using R version 2.11.1 with the R commander interface.

Results

Field Study

Water Quality

Average conductivity increased significantly during June and remained high through July ($F_{2, 22}$=5.18, P=0.0143; Figure 1.2). NH_3, NH_4, chlorine, chlorophyll, and pH did not have any seasonal trends (Table 1.2). There was no significant difference between sites when looking at conductivity, NH_3, NH_4, chlorine, chlorophyll, nor pH (Table 1.3).

Amphibian Richness

Field sites that were modeled to be highly impacted by runoff from agriculture were found to have a lower level of amphibian richness then those that were modeled to have a low level of runoff ($F_{1, 18}$=5.3455, P = 0.03282; Figure 1.3). While I was able to detect this difference in richness, I was unable to detect a difference in amphibian diversity between sites ($F_{1, 18}$= 2.1363, P= 0.1611).

Pesticides

During the field study, each of the sites was sampled for 24 commonly found pesticides (listed in methods; Table 1.4). Fifteen of the twenty sites were contaminated with atrazine. Out of those, five samples also contained varying combinations and concentrations of Acetochlor, DEA, DIA, and Metolachlor (Table 1.4). There was a significant difference in where I expected to find agrochemical contamination (high run-off sites) to where the pesticides were detected. Atrazine was detected in 3/4 of the sites that was significantly higher that the 1/2 expected rate of site contamination estimated via runoff (Figures 1.1 and 1.4, Table 1.4).

Bd Prevalence

Only 4/128 swabs tested positive for Bd. Two of these four detections occurred on Northern Cricket Frogs (*Acris crepitans*) during May sampling at separate sites. At each of the sites, the swabbed individual was the only amphibian captured and swabbed during the visit. The third and fourth positive samples were Woodhouse's Toads (*Anaxyrus woodhousii*) both from the site 25, which has a high level of impact from agrochemical run-off. An additional two Bd free Woodhouse's Toads were caught at this site during the same sampling period (Table 1.6).

During the June sampling 607 tadpoles were caught and tested for Bd, only one Boreal Chorus Frog (*Pseudacris maculata*) tadpole was positive. The tadpole was collected from a highly impacted runoff site and had multiple pesticides. This site also has the highest detection frequency of Boreal Chorus frogs (Table 1.6).

<u>Lab Study</u>

Collection sites

Tadpoles collected from different sites exhibited significant differences in mass, SVL, and overall survival. Initial measurements showed that tadpoles collected from high impact pesticide sites (sites that contain two or more pesticides) had reduced SVL (F_1= 4.9886, P=0.0266, Figure 1.5) but there was no difference in initial mass (F_1= 2.9906, P=0.0853). Tadpoles collected from sties with low pesticide detection had a higher survival rate, a greater SVL increase and a lower mass loss (Figures 1.7, 1.8, and 1.9; Table 1.7, 1.8, and 1.9) over the course of the lab experiment.

Treatment

By running the collection site as a covariate, I was able to detect the impacts of the applied treatments of the study. Here I see that the low (0.5 μg/L) and medium (5 μg/L) concentrations of atrazine had a significant impact on the survivorship, SVL changes, and differences in tadpole mass; when compared to the control treatment, there was an increased death rate, lower SVL growth, and a decrease in mass (Figures 1.10, 1.11, and 1.12; Table 1.7, 1.8, and 1.9).

The difference seen in the SVL and mass measurements could be indicative of a more rapid development of those tadpoles collected at the high sites or exposed to atrazine. So, we also looked at the difference in developmental stages where we did not find any significant differences in tadpole development over the course of the study ($F_{15, 212}$= 1.118 P=0.3414). Tadpoles exposed to Bd and random selections of non-exposed tadpoles were tested for the establishment of Bd on the tadpoles. There was no Bd was detected on the tadpoles, even in the Bd treatments, nor did the tadpoles exposed to Bd exhibit any lethal nor sub-lethal effects when compared to the controls.

Discussion

Several studies suggest that pesticides can have significant impacts on amphibian populations (Hayes et al. 2002a, Relyea et al. 2005, Forson and Storfer 2006, Hayes et al. 2006a, Oka et al. 2008). Almost none of these studies are field-based surveys and rely entirely on mesocosm and laboratory based approaches. This study provides some evidence that the presence of pesticides in amphibian

habitat not only reduces survival rates of tadpoles at those sites, but also might ultimately be leading to reduced species richness at highly impacted sites. This potential for exposure of tadpoles to several pesticides early in development also seems to increase their subsequent susceptibility to environmentally relevant concentrations of atrazine (0.5 μg/L and 5 μg/L) (Figures 1.10, 1.11, and 1.12; Table 1.7, 1.8, and 1.9; (Graziano et al. 2006). These findings are of great importance and stress the additional need for field survey work in amphibian habitats on or adjacent to agricultural areas.

Field Study

Water Quality

While each water quality parameters were modeled against season and site type, the only significant water quality variable was the variation in conductivity across the season (Figure 1.3, Tables 1.2 and 1.3). This is likely due to high rainfall events between the May and June sampling periods that caused flooding across the region. These flood conditions were sustained through the July sampling period as well. Thus, the conductivity was lowest during the May sampling and statistically equal during the June and July sampling periods.

Species Richness

There are seven known species inhabiting the area I surveyed and I detected all seven. Some caution was taken in site selection to attempt to avoid too many confounding factors. Since species diversity (the number and frequency of species at each site) is likely to be affected by a whole host of variables, I did not expect to be able to detect differences in diversity between high and low impact sites. This

variable has been examined in other studies with no significant differences apparent (Pope et al. 2000, Sparling et al. 2000). Therefore, it is quite interesting that I found a small yet significant difference in species richness (the number of each species at each site) between low and high impact sites. Upon closer examination of species identities I found no distinct patterns regarding more or less sensitive species that disappeared at high impact sites. Again, since species present at each of the sites were variable according to site, I expect that several other biotic and abiotic factors are influencing species presence. Nonetheless, I found that sites with a low level of run-off impacts had greater species richness signifying that there was not a particular sensitive species to the high impact sites, but rather some overall decrease in habitat quality.

Amphibian- Bd

Bd was found in three of the seven detected species but only on very small numbers of individuals (Table 1.3). The low prevalence of Bd in this area is likely due to the predominately warm temperatures that occur during the summers. My sampling exhibits this temperature linked idea in that the amphibians that tested positive for Bd were collected during the May and early June sampling. The water temperatures during this sampling period were at their lowest during the study, providing a more favorable environment for the growth and reproduction of Bd (Berger et al. 2004). Interestingly, all of the positive samples came from sites with a high level of agrochemical runoff with atrazine as a common contaminant in all of them. Unfortunately, as the number of infected individuals is so low, it is unlikely that Bd is having an interactive effect at these field sites during the times sampled.

Further investigation in the susceptibility of the metamorphosed amphibians to disease in the presence of agrochemical contamination would be intriguing.

Pesticides

The half-life of all the detected pesticides during this study are less than a year so they reflect the level of applications for that growing season. The overall trend of lower levels of metolachlor and acetochlor in rivers along the corn belt of the United States has been attributed to largely to the lower levels of application (Vecchia et al. 2009). Three of the twenty sites I looked at had both metolachlor and acetochlor, and one additional site for each pesticide also tested positive for a total of four sites testing positive for each pesticide (Table 1.4). I found metolachlor in 15 % of all the sites tested, while in 1994-95 it was only detected in 3% of the streams sampled in northern Missouri despite all sites being sampled adjacent to row crops (Blanchard and Lerch 2000). This difference might be due to regional or temporal patterns of metolachlor usage. Use of herbicides can vary by area and depending on sampling time, higher prevalence of pesticides can be detected at different times of year.

Atrazine is the most commonly found pesticide in rivers along the corn belt of the United States (Frankforter 1995, Blanchard and Lerch 2000, Lerch and Blanchard 2003). When atrazine and its metabolites (DIA and DEA) were found at a site, the atrazine was always found at a higher concentration, other studies within the region report similar findings (Kolpin et al. 2000). The concentrations I found in our study also mirror concentrations found in a study conducted in southern Missouri and northern Iowa streams, but the frequency of detection in my study was

lower. Atrazine was found 15 of 20 sites I tested (Table 1.4), on the other hand during Blanchard and Lerch's pre-emergent period (March-April), they found Atrazine in 90% of their sites in 1994 and 99% of their sites in 1995. This difference in detection rates may be due mainly to our focus on finding sites with a low level of run-off contamination, while they had a more generalist approach, taking into consideration only the location of pesticide dealerships which could act as point source polluters (Blanchard and Lerch 2000). This difference in approach demonstrates the intensity at which atrazine is contaminating the water of the Midwest.

Pesticide Sampling /Agro Chemical Runoff Designation Differences

The pesticide grab samples were taken only during the month of May at each of the study sites. This provides a snap shot of the sites at that point in time but is insufficient to determine the true level of pesticide contamination at the sites across the whole season. The detections I found were comparable to concentrations and frequencies detected in similar studies in the region (Frankforter 1995, Blanchard and Lerch 2000). The contaminants detected during this study are typically used as pre-emergent pesticides and is reflective of the early sampling time within the growing season. Further sampling, including some taken later the season, would provide information on post-emergent herbicides and insecticides being used on the surrounding cropland and eventually contaminating the study sites. This would aid in understand of the 'true' contamination level of each site. Further pesticide sampling did not occurring during this study due to flood stage conditions during the June and July sampling periods. Sampling later in the season would increase the

likelihood of detected both post-emergent herbicides and insecticides. This would help in understanding the potential impacts of interacting pesticides on amphibian populations.

Lab Study

Pre-exposure

Quite unexpectedly, the largest significant driving force in tadpole survival had almost nothing to do with treatment, but rather was mostly dependant on the amount of pesticides detected at the sites from which the tadpoles were collected. Van Bursirk (2000) emphasized the importance of using tadpoles raised from eggs to avoid any influence of environmental factors on the experimental laboratory study. In this study, I was interested in whether these environmental factors played a role on how individuals might respond to differing and combined stressors. I found that tadpoles from sites with multiple pesticides had significantly lower survival rates and were shorter.

The designation of few and multiple pesticide sites should be clarified. Since my sampling only took place at a single point in time, I cannot be certain that sites that did not exhibit any pesticide presence were in fact pesticide free the entire season. Also, due to the overwhelming contamination of atrazine at most of the sites, I actually cannot be certain that atrazine was also absent from sites that it was not detected from. For this reason, I grouped the collection sites into two broad categories of few and multiple pesticides. Few pesticide sites had no detectable pesticides or only low (<0.08 ng/L) levels of atrazine. Interestingly, sites with multiple pesticide detections always had atrazine present (along with its degradates

DIA and DEA), and typically in a higher concentration (mean= 0.34 ng/L+0.13). The multiple pesticide sites had comparatively lower concentrations of acetochlor, metolachlor, and atrazine (Frankforter 1995, Blanchard and Lerch 2000, Lerch and Blanchard 2003, Vecchia et al. 2009). The tadpoles collected from these sites seem to have been negatively impacted by the increased levels of pesticides or some other strongly associated factor.

Treatments

After controlling for the covariate of origin site for tadpoles, I found that atrazine exposure in my experiment had significant effects. Atrazine at the low (0.5 μg/L) and medium (5 μg/L) concentrations had significant lethal and sub-lethal impacts on the tadpoles. While I did see changes in SVL and mass, there was no significant difference in the change of developmental stage, regardless of treatment. Atrazine has been found to change the feeding behavior of tadpoles (Allran and Karasov 2001), this could lead to the changes I saw in survivorship, SVL, and mass. It is interesting to note that we also had an atrazine treatment level at 50 μg/L that did not significantly impact the tadpoles. This hormetic effect is oddly common in atrazine studies for amphibians, where the low concentrations of atrazine had the greater impacts (Larson et al. 1998, Hayes et al. 2002a, Hayes et al. 2002b, Storrs and Kiesecker 2004, Hayes et al. 2006b).

Exposure to multiple pesticides is impacting the Leopard frog's ability to survive and grow and increasing its susceptibility to atrazine. In another study, Northern Leopard frog tadpoles had a low survival rate (1%) when exposed to the mixture of low concentrations of commonly used pesticides (Relyea 2009). It has

also been found that the increasing concentrations of a mixture of multi-pesticides correlates with an increase in the amount of lung worm found in Leopard frogs (done in a controlled laboratory setting; (Gendron et al. 2003). More studies combining relevant concentrations of various contaminates are important to understanding their impacts when they interact with each other and any synergistic type effects that may occur.

By looking at both the field parameters and laboratory-based parameters we were able to determine that being raised in an environment with multiple pesticides present had significant 'carry over effects' in the laboratory study. This is not a new concept, salamanders exposed to low levels of atrazine have decreased survivorship even when long removed from the contaminant exposure when compared to unexposed individuals (Rohr et al. 2006). This can also be broadened out to the impacts exposure to other chemicals making them more susceptible to atrazine (included in the control). Further looking into whether it is the exposure to agrochemicals that makes them more susceptible, or if it is just a stressor and if the tadpoles are more susceptible to just atrazine or agrochemicals or to stressors in general, would be interesting venues to explore.

Amphibian ecotoxicology studies often relate their test concentrations to a general location tested in a different study, average across a large area and habitat types; or LC50 values established for other organisms (Relyea 2005b, Rohr et al. 2006, Relyea 2009, Relyea and Jones 2009). It is important to be able to relate the findings in the laboratory to the field. Laboratory studies tend to simplify a complex habitat and all its interactions into just a few variables. While this is valuable, it is

important not to lose the link of the laboratory study to what is happening out in the 'real world.' Taking the variables from what we see happening in the field to the lab, to make sure what we are perceive to be happening in the field is the truly happening. This study is unique in that I conducted the study in the field and within the same season brought the variables into the lab to look at the interactions in a controlled lab setting. The complexity of the whole habitat can be difficult to replicate, but it is this multi-factorial dynamic and its interactions that make up the habitat that the amphibians are striving to survive in. The more we understand each part of the habitat, the better we can understand the whole, but it is important not to lose the larger picture. Future lab based studies should strive to balance their research with field based data. Field based studies and conclusions drawn from field observations should be backed up and solidified with the help of more controlled lab based studies. With a balance of both field and lab studies a better understanding can be achieved.

CHAPTER TWO

Interactive effects of chytrid, triclosan, and predatory stressors on Woodhouse's toad (*Anaxyrus woodhousii)* tadpoles

Abstract

Population declines of amphibians are of great concern worldwide. Two significant factors impacting these declines are emerging infectious disease and anthropogenic contaminants. Chytrid (*Bactrachochytrium dendrobatidis (Bd)*) is an emerging fungal pathogen known to cause rapid declines worldwide. The antimicrobial triclosan is commonly used in household soaps and can ultimately contaminate freshwater systems. The interactions of novel stressors with natural stressors, such as predation have not been well researched. We conducted a 2 x 4 x 2 factorial design to examine the potential interactive impacts of Bd, triclosan (0, 10, 100, 1000 μg/L) and dragonfly predator cue on Woodhouse's Toad (*Anaxyrus woodhousii*) tadpoles. We measured the impacts of these stressors on survivorship, growth, and development of the tadpoles over a four-week time span. There was a significant toxic effect of triclosan exposure on the tadpoles. All tadpoles in the 100 and 1000 μg/L concentrations died within 24 hours of exposure. Interestingly, there was a non-monotonic survival response at the lowest concentration (10 μg/L) where tadpoles survived better at low concentrations as compared to controls. This pattern was driven by a significant interaction between the factors Bd and triclosan. Tadpoles in non-Bd treatments exhibited only a marginal decrease in survival between control (84.1%) and low triclosan (10 μg/L; 77.3%) treatments. Tadpoles

exposed to only Bd (no triclosan) had a markedly low survival rate (67.5%). Although, those exposed to both a low dosage of triclosan (10μg/L) and Bd had a survival rate of 91.1%. A proposed mechanism for this increased survival is the potential toxicity of triclosan to Bd itself. No significant effects of predation were detected, as were no significant impacts to the sub-lethal measures of growth and development.

Introduction

Worldwide amphibian declines have been described as a mark of the 6th great extinction (Wake and Vredenburg 2008). These declines are proposed to be from several different yet potentially interactive anthropogenic stressors (Stuart 2004). While habitat destruction is the leading cause of amphibian decline, contaminants and the spread of disease are also large contributions (Hayes et al. 2010)). Any given population of amphibians is going to be subjected to a varying combination of the above-mentioned factors and each species will have differing abilities to cope with them. It is key to amphibian conservation to understand not only the effects of these factors, but also any synergistic or antagonistic effects that interacting factors might display. In this study, we look at the interactive effects of three stressors larval amphibians are commonly exposed to: disease, contaminants, and predation.

A significant disease contributing to amphibian declines is chytridiomycosis caused by the parasitic chytrid fungus: *Bactrachochytrium dendrobatidis* (Bd; Rachowicz et al. 2006). While Bd is known for wiping out whole populations and

even entire species of amphibians; this epizootic dynamic is not being seen in all populations infected with Bd (Green and Dodd 2007). Several amphibian populations maintain a low prevalence of Bd suggesting more of an endemic dynamic (Retallick et al. 2004). Bd is able to infect anuran species at both the adult and larval stage, utilizing the keratin in the mouthparts of the tadpoles (Marantelli et al. 2004). An infection in a tadpole will alter their feeding and forging behaviors which can lead to negative impacts on their ability to obtain sufficient energy for growth and metamorphose (Venesky et al. 2010a, Venesky et al. 2010b).

Another significant factor in the amphibian declines stems from contaminant exposure. Contaminants varying in types and concentrations can be found in some of the most "pristine" areas of the globe (McConnell et al. 1998). Amphibian species have varying levels of susceptibility to contaminants, not only between species but also between populations within the same species (Bridges and Semlitsch 2000). For example, a study in California was able to show that the contamination due to mere pesticide drift is linked to population declines across several anuran species (Davidson 2004). Exposure to these anthropogenic contaminant not only impacts the survival of the tadpoles, it can also cause immune depression, making them more susceptible to diseases (e.g. (Davidson et al. 2007, Rohr et al. 2008, Kerby and Storfer 2009).

In a review of toxicity tests on aquatic organisms, amphibians were revealed to exhibit particular sensitivity to phenolic compounds (Kerby et al. 2010). Triclosan is one such compound that has been found effective against gram positive and gram-negative bacteria as well as some viruses and fungi (McMurry et al. 1998,

Levy et al. 1999). Due to the potency of this compound, it has gained popularity in common household items ranging from hand soaps and toothpaste to cutting boards and clothing. As humans use these products, the triclosan is absorbed through the skin and what does not break down in our bodies is excreted. In the United States, the amount of triclosan found in urine is on the rise. It was found in 74.6% of tested samples at concentrations ranging from 2.4 to 3,790 μg/L in 2004 (Calafat et al. 2007). Triclosan was also in three out of five samples collected from Swedish mothers' milk (Adolfsson-Erici et al. 2002).

With triclosan's abundant use, it lends itself to a high level of contamination in the environment. In 1999-2000, triclosan was found in 56.7% streams sampled within the United States at a median concentration of 0.14 μg/L (Kolpin et al. 2002). Research on the impacts of triclosan on freshwater organisms has begun but much work is still needed. Concentrations as low as 0.12 μg/L triclosan decrease algal diversity in river systems, eliminating some of the rarer species first (Wilson et al. 2003) . While the triclosan is decreasing the food base the tadpoles depend on it is also affecting them directly. Levels as low as 0.15 μg/L of triclosan quicken the development of the hind limbs and decrease the total body weight of Northern Leopard frog (*Lithobates pipiens*) tadpoles (Veldhoen et al. 2006).

In addition to anthropogenic stressors and invasive disease, there are natural stressors that have negative impacts on the survival of tadpoles, namely predators (Kats and Dill 1998). Tadpoles primarily use chemical cues to detect the presence of a predator (Kiesecker et al. 1996). Under the stress of a predator cue the tadpoles

display a decrease in activity levels and earlier metamorphosis at a smaller size (Skelly and Werner 1990, Relyea 2001).

Recently, researchers have begun looking at the impacts of a combination of multiple stressors on tadpoles. The combination of predator cue and the pesticide carbaryl reduces the level of survival in Gray Treefrogs (*Hyla veriscolor*; (Relyea and Mills 2001). Atrazine increases the rate of infection of trematodes in Northern Leopard frogs (*Lithobates pippins*; (Rohr et al. 2008). Similar to the exposure to single stressors, species have varying responses to multiple stressors. In one study, two out of six species tested by exposure to carbaryl and predator cue had a synergistic effect on survival, the remaining four species had a lower tolerance to carbaryl making the interactive effects harder to detect (Relyea 2003).

In this study I am testing the main and interactive effects of triclosan, Bd, and predator cue on the on Woodhouse's toad tadpoles. I expect there to be a decrease in tadpole survival for each treatment. With increasing numbers of stressors, I expect to see an increase in developmental rate and decrease in the survival, body length, and mass of the tadpoles.

Methods

Experiment design

All Woodhouse's toad (*Bufo woodhousii*) tadpoles used in this study were collected from backwaters along the Missouri River near Vermillion, South Dakota. A subset of tadpoles was kept through metamorphosis for species verification. Tadpoles were fed ground up fish flakes (TetraMin ProCare Fish Food Flakes) on a weekly interval (24 hours prior to water changes). Each tadpole was individually

housed in 9.5 cm x 12 cm x 5 cm cups with 200 mL of treated water (see below) and randomly assigned to positions on shelving units. The tadpoles were raised and the experiment was conducted at 22 ± 1° C with a 12: 12 hour light: dark photoperiod.

A full factorial 2 (Bd/ no Bd) x 4 (0 μg/L, 10 μg/L, 100 μg/L, 1000 μg/L triclosan) x 2 (predator cue/ no predator cue) design was implemented for 16 treatments that were repeated 11 times for a total of 176 animals. All water used in the experiment was reverse osmosis water treated with R/O Rite (Kent Marine R/O Rite, Walnut Creek, CA) to create a consistent pH and mineral content and avoid inadvertent contamination. The triclosan and predator cue treatments were applied on day 1 and with subsequent water changes (days 8 and 15). The experiment was run for a total of 21 days. On the initial day and at subsequent weekly water changes measurements were taken including: mass (g), snout-vent length (SVL, mm), total body length (mm), and developmental stage (Gosner 1960). Dead tadpoles were removed daily and stored in ethanol for future Bd testing. At the end of the experiment, surviving tadpoles were killed and stored in 95% ethanol until they could be examined for Bd infection.

Bactrachochytrium dendrobatidis

Bactrachochytrium dendrobatidis (JEL 215 isolate) was grown on a 2 % tryptone-agar according to standard procedure (Longcore et al. 1999). After a week of growth, each plate was flooded with reverse osmosis water, to suspend the Bd zoospores. The spores were counted using a phase contrast microscope and a hemocytometer. To apply the Bd treatment, 1 mL of zoospore-laden water was

added to each container resulting in 70,000 Bd zoospores per 200 ml of water. The tadpoles were exposed to the Bd only on the initial day of the experiment.

Triclosan

The second stressor the tadpoles were exposed to was the antimicrobial-triclosan. It was dosed with water control, the solvent control (Dimethyl sulfoxide, **{**DMSO}), low (10 μg/L), medium (100 μg/L), and high (1000 μg/L). Stock solutions were created for each desired concentration, for application, 1 ml of each stock solution was added resulting in the desired final concentrations. The Water Science Laboratory at the University of Nebraska, Lincoln, verified the stock solution concentrations as being the same as nominal concentrations.

Predator cue

The natural stressor, predator cue, was created using dragonfly larvae (*Anxa sp*.) taken from the same location as the tadpoles. Dragonflies were feed Woodhouse's toad tadpoles 36 hours prior to being placed in water for cue creation. The larvae were left in the water for an additional 36 hours prior to use for with the tadpole to create a potent cue. A total of 11 dragonfly larvae were used to create the cue in 94.6 L of treated water. Predator treatments were applied with water changes starting on day one (days 1, 8 and 15).

qPCR analysis

Mouthparts of the tadpoles were dissected off the tadpoles and used for Bd analysis. The DNA extraction was done using the DNeasy kit from Qiagen. Each sample was run using an Applied Biosystems StepOne Plus real time PCR machine (Applied Biosystems, Inc., CA, USA) according to standard protocol as described by

Boyle et al. (Boyle et al. 2004). Each sample was run in triplicate and was considered positive if two of the three replicates were positive.

Statistical Analysis

We ran the statistics using a generalized linear model with a binomial distribution. Total body length, SVL, mass and devilment stage were all ran as response variables with Bd, the four levels of triclosan, and predator cue the treatments. The model was run using R version 2.11.1 with the R commander interface. An estimated LC50 was calculated based on the survival rate of the tadpoles when exposed to triclosan.

Results

There was not any significant difference in the mass ($F_{7,96}$ =0.6349), SVL ($F_{7,96}$ = 0.5095), and total body length ($F_{7,96}$ =1.528) measurements taken for the study. There was also no detectable interactive nor main effect on survival caused by predator cue (Table 2.1). As a main effect, Bd had no effect on the survival of the tadpoles. All tadpoles exposed to Bd were tested for infection using qPCR techniques as described above and did not display positive results of infection. A subset of the control tadpoles was also run to confirm no previous infection of Bd.

Triclosan was lethal to the Woodhouse's Toad tadpoles within 24 hours of exposure at 1000 μg/L and 100 μg/L (Figure 2.1). The LC50 was calculated to be at 23.921 μg/L (SE=9.360) over a 21-day exposure.

Interestingly, there was a non-monotonic survival response at the lowest concentration (10 μg/L) where tadpoles survived better at low concentrations as

compared to controls. This pattern was driven by a significant interaction between the factors Bd and triclosan. Tadpoles in non-Bd treatments exhibited only a marginal decrease in survival between control (84.1%) and low triclosan (10 μg/L; 77.3%) treatments (Table 2.1). Tadpoles exposed to only Bd (no triclosan) had a markedly low survival rate (67.5%). Although, those exposed to both a low dosage of triclosan (10μg/L) and Bd had a survival rate of 91.1%.

Bd exposed to triclosan at all concentrations utilized in this experiment significantly impacts fungal survival. Similar to the tadpole's reaction, triclosan causes complete inhibition at the 100 and 1000 μg/L and a significant decrease in growth at 10μg/L (Miiller et al. 2011).

Discussion

In this study, we looked at the main and interactive effects of triclosan, predator cue, and Bd on both the sub-lethal and lethal impacts on Woodhouse's toad tadpoles. Overall, we found triclosan to be deadly to the tadpoles at high concentrations, but actually protective to the tadpoles at lower concentrations due to its inhibitory effect on Bd growth.

The most interesting interactive effect we found was when the tadpoles were exposed to both low levels of triclosan (10 μg/L) and Bd. Compared to Bd only exposed treatments, there was increased survival in tadpoles exposed to both Bd and triclosan. This is most likely due to triclosan acting as an antimicrobial on Bd. In a follow up experiment, we were able to determine that triclosan was completely lethal to Bd itself at 100 μg/L and significantly inhibitory at 10 μg/L (Miiller 2011).

Thus, the Bd has a higher susceptibility to triclosan than the Woodhouse's toad tadpoles used in this study creating protective effect for the tadpoles.

These low concentrations of triclosan are likely not all beneficial for tadpoles. Green algae species overall have some of the highest levels of sensitivity to triclosan with effective concentrations (EC50s) of triclosan on algal densities ranging from 1.4 to 66 µg/L (Orvos et al. 2002, Capdevielle et al. 2008). At even lower concentrations, triclosan has been shown to lower the diversity of algae within a river system (Wilson et al. 2003). The low levels of triclosan, found in our freshwater rivers are likely negatively affecting the diversity and density of the algae on which the tadpoles rely on for food. Although in the end, it might be better to have a lower variety of food in exchange for the elimination of a deadly pathogen.

Despite being less sensitive than Bd or algae, Woodhouse's toads were surprisingly more sensitive than several of the model species used for regulatory decisions. For instance, Fathead minnows (*Pimephales promelas*) have a relatively high tolerance (1000 ug/L and 3000ug/L) to triclosan exposure, and yet have been deemed the most sensitive species. In a separate study, Woodhouse's toad had the lowest LC50 out of several tested species at 152 ug/L over 96 h period (4 days) (Palenske et al. 2010). We calculated the 21 day LC50 for Woodhouse's Toad tadpoles to be 23.92 µg/L. While there might be regional variation in susceptibility, likely the prolonged exposure accounts for the greater sensitivity. Given that triclosan is known to bioaccumulate for short periods of time in Zebrafish (*Danio rerio*), data on these long term exposures are critical for our understanding of impacts on amphibians and other wild species (Orvos et al. 2002) .

Further investigation of the impacts of triclosan and other contaminants on both hosts and pathogens should provide a better understanding of the survival of amphibian species living in a multiple stressor environment. Other studies have shown the importance of these negative additive effects (Kerby and Storfer 2009). Studies that examine these community interactions between the algal food source, tadpole herbivores, and potential predators are critical to understanding how these emerging contaminants can alter and affect freshwater ecosystems.

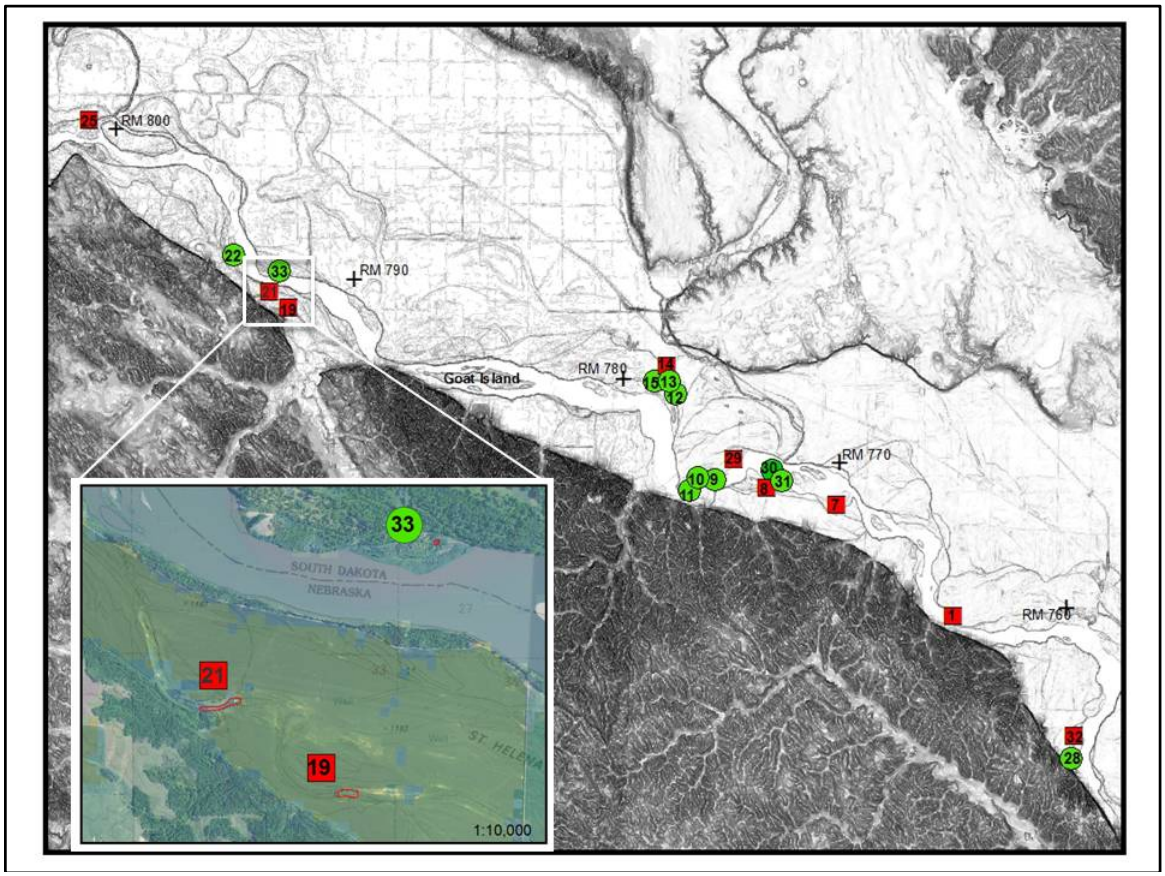

Figure 1.1. **Agrochemical impact designation of wetland sites**. Using ArcGIS 9.3, 20 sites were selected along the 59-mile reach of the Missouri River for agrochemical impact levels and amphibian habitat. Sites estimated to have a high level of contamination are indicated by a red square. Sites modeled to have a low agrochemical contamination are green circles. The pop-out box shows a subset of sites with of the *land use* layer overlaying the *2009 areal photo* with the wetland site outlined in red.

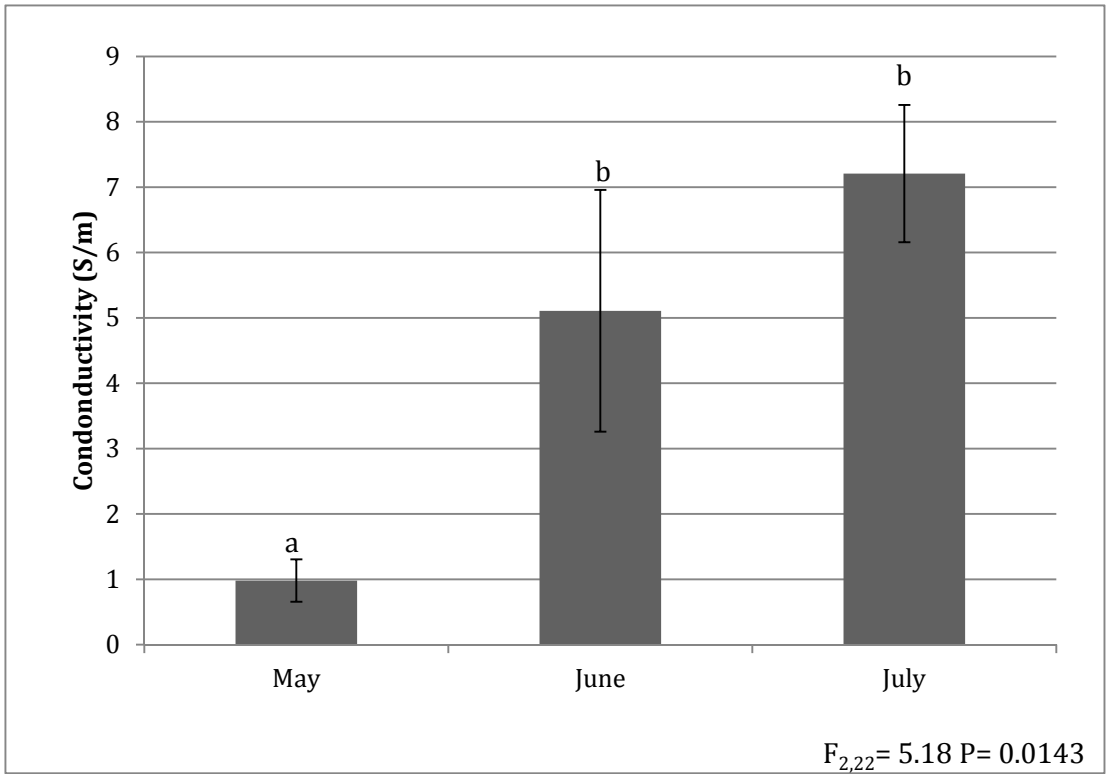

Figure 1.2. Seasonal trends of conductivity in wetland sites along the 59-mile reach of the National Recreational Missouri River. There was an increase from May to June yet remained statistically insignificant through July. This change is most likely due the flood that occurred during the summer.

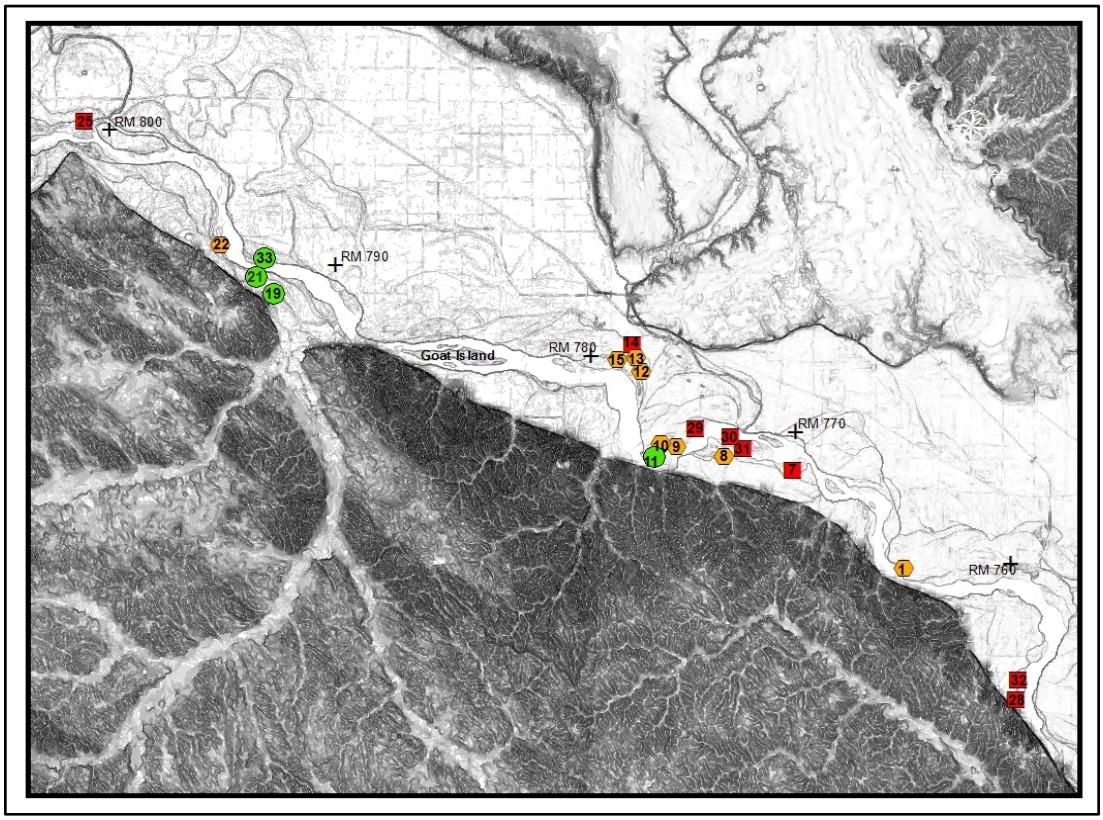

Figure1.3. Pesticide contamination levels. The designation of pesticide impact based on water grab samples taken during the month of May. Red squares are sites where two or more pesticides were detected, orange hexagons are sites that only Atrazine was detected, and green circles had no detectable pesticides.

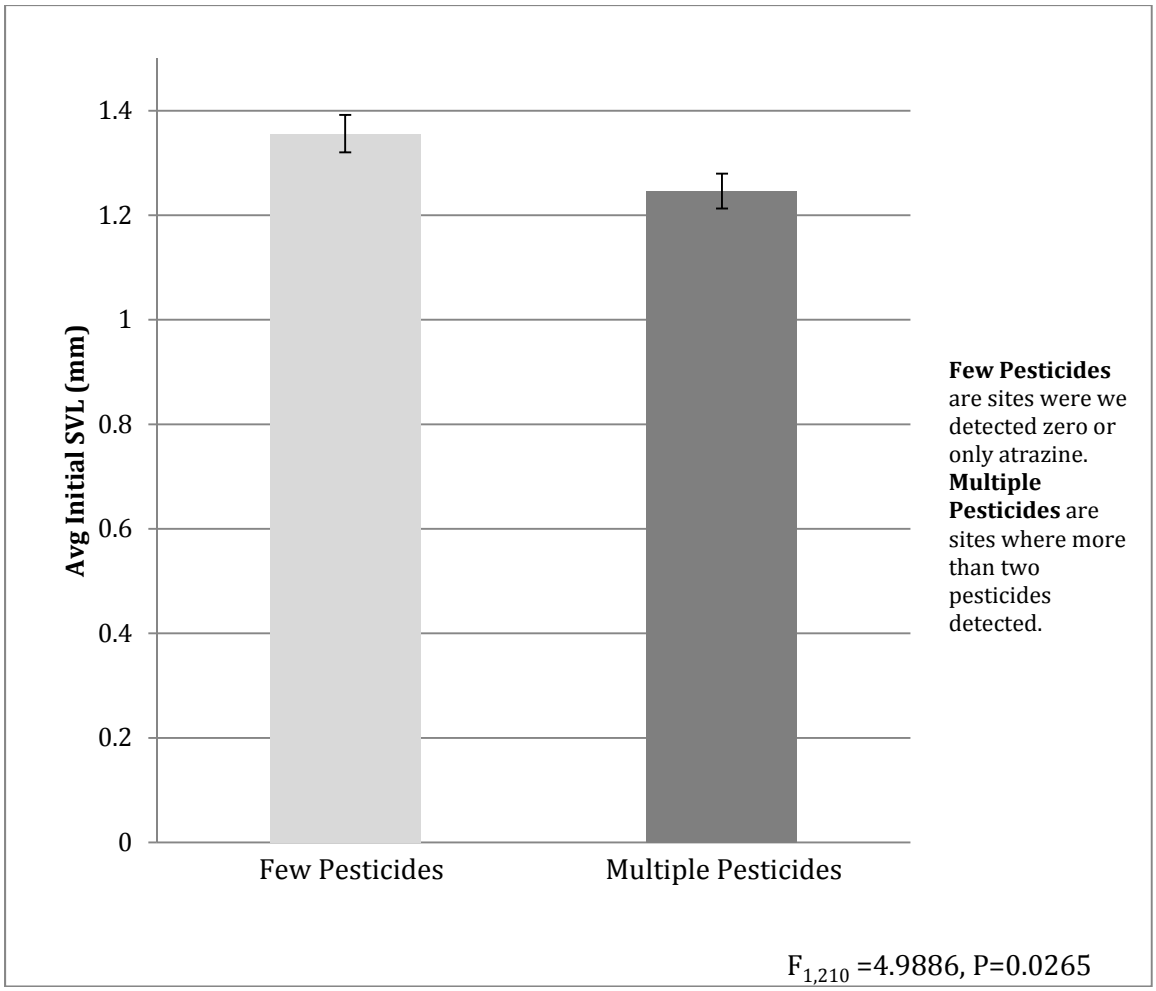

Figure 1.4. Initial average snout vent length (mm) of tadpoles by pesticide contamination. Tadpoles collected from sites with a low agrochemical frequency (<2) were initially significantly longer then those from sites with greater then two agrochemical contaminates.

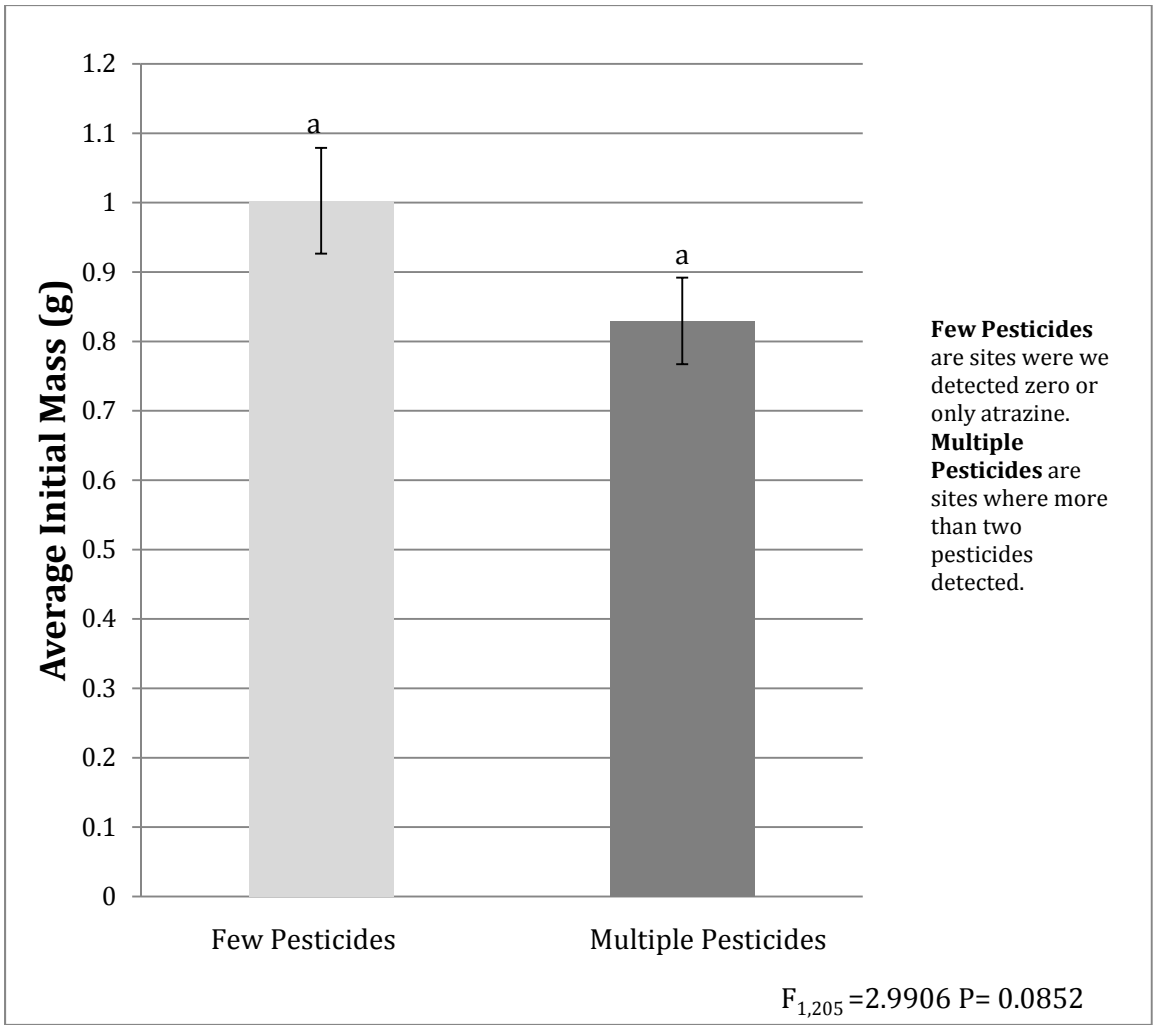

Figure 1.5. Initial average tadpole mass (g) by pesticide contamination. Tadpoles collected from sites with varying levels of contamination did not have significantly different masses.

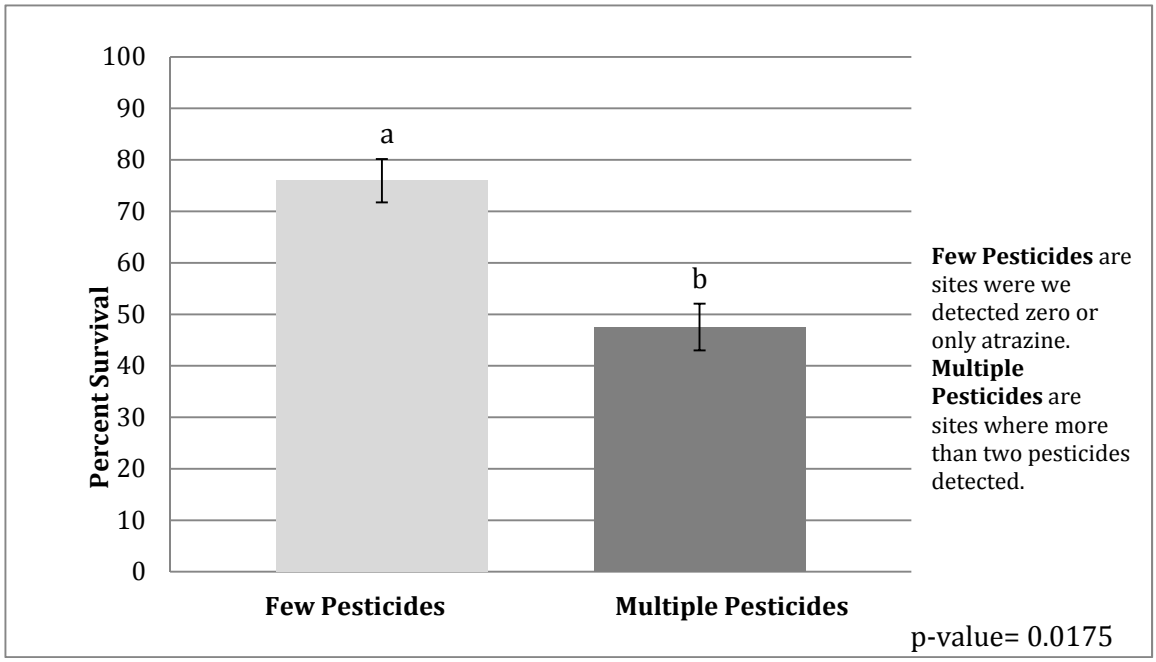

Figure 1.5. Percent survival of tadpoles by the pesticide presence at the site collected from. Tadpoles collected from sites with greater then two pesticides detected at were significantly more likely to die then those collected from sites with less then two detected pesticides.

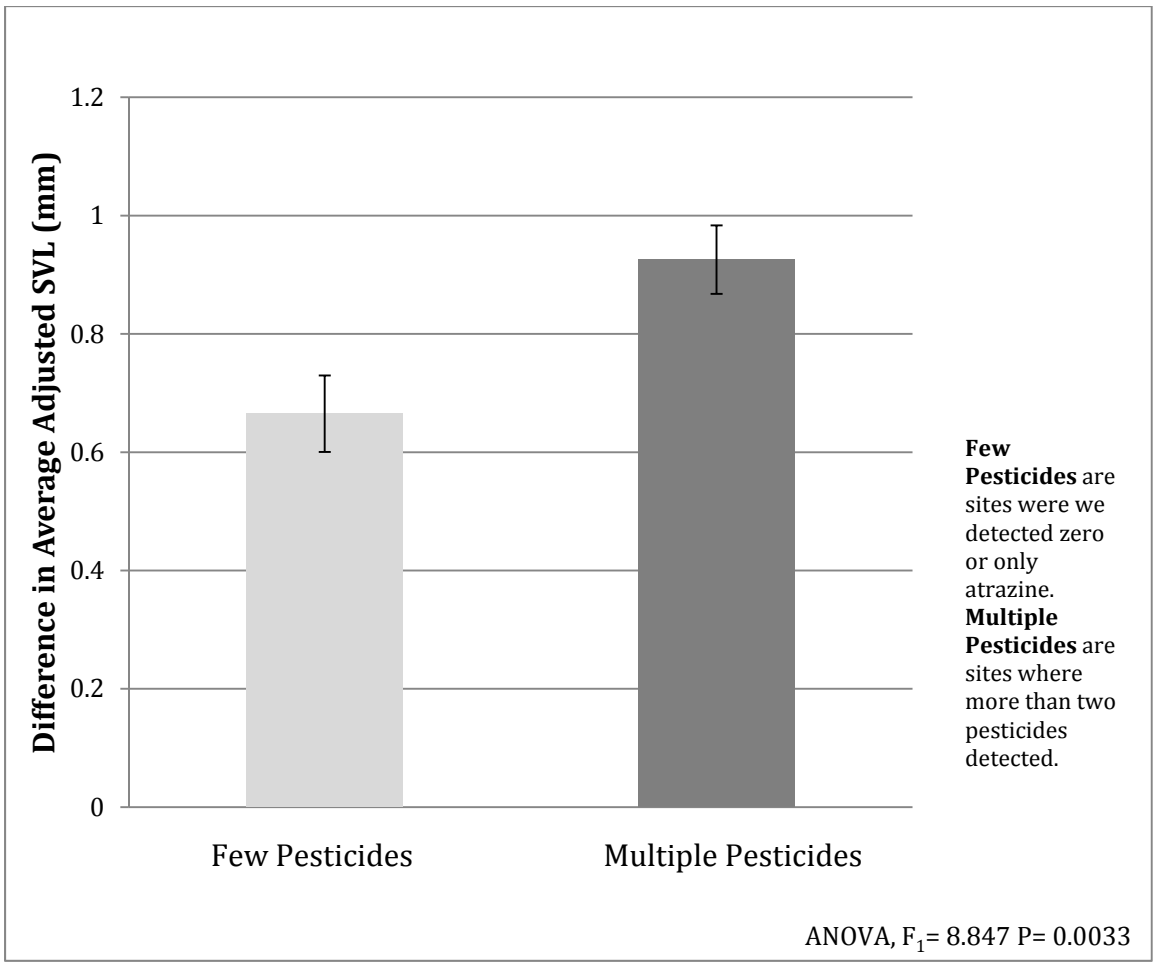

Figure 1.7. Difference of average adjusted snout vent lengths (SVL) of tadpoles collected at sites with varying levels of pesticides. Tadpoles exposed to two or more pesticides had a lower SVL growth rate then those not exposed to lower number pesticides prior to the experiment.

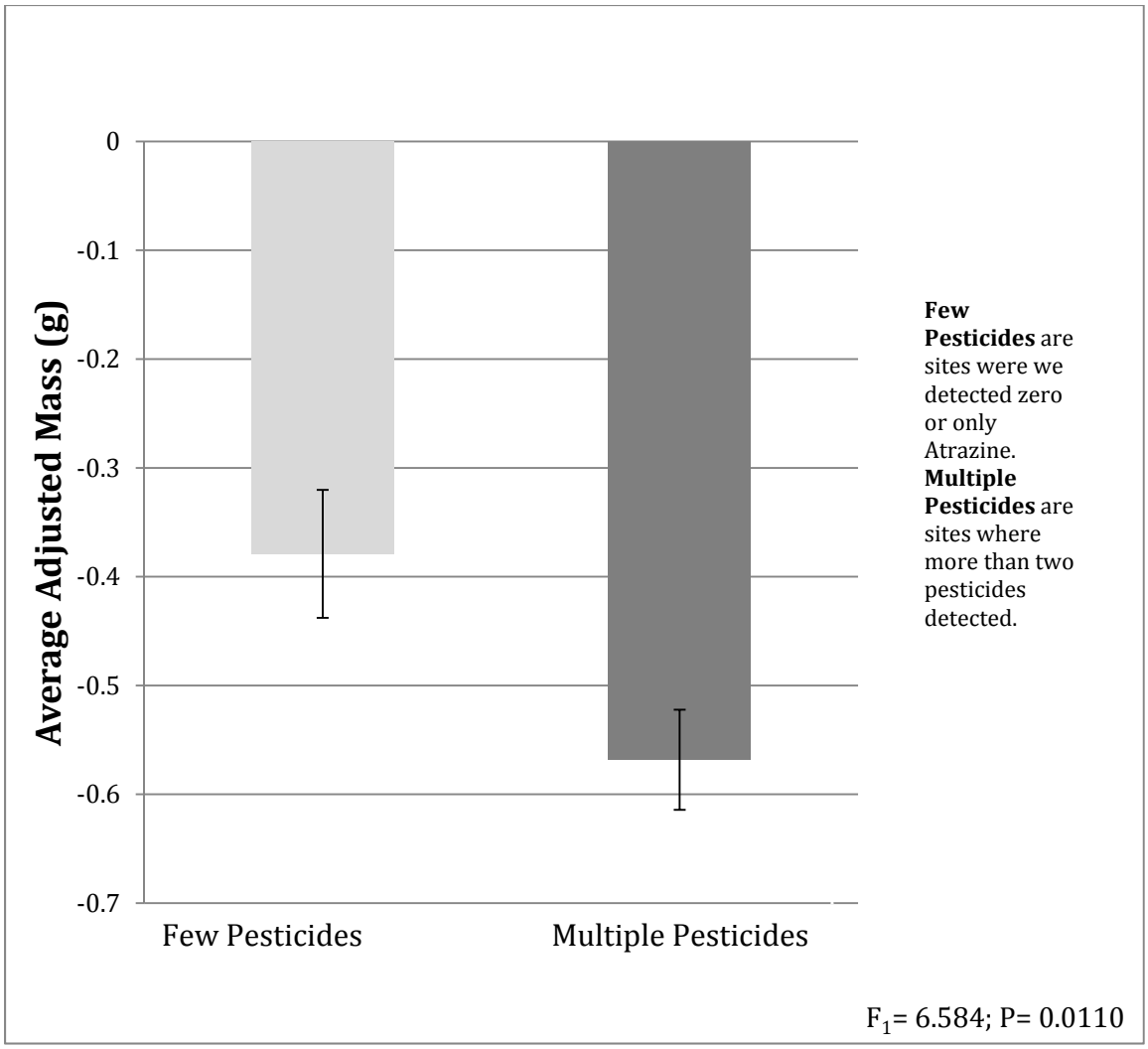

Figure 1.8. Average difference in mass (g) of tadpoles by pesticide contamination. Tadpoles collected from field sites with greater then two pesticides lost more mass then those collected from sites with less then two pesticides.

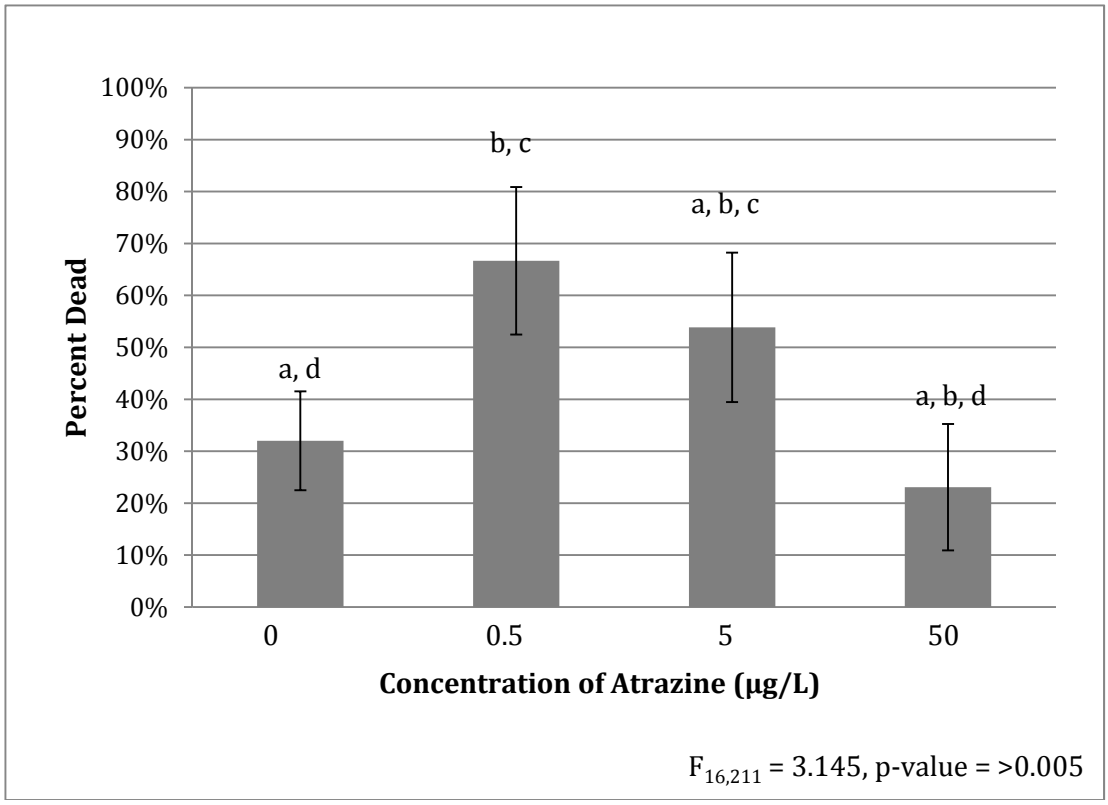

Figure 1.9. Percent survival of tadpoles exposed to varying concentrations of Atrazine. Tadpoles exposed to 0.5 and 5 µg/L of atrazine had a significantly higher death rate then the controls and those exposed to 50 µg/L of atrazine.

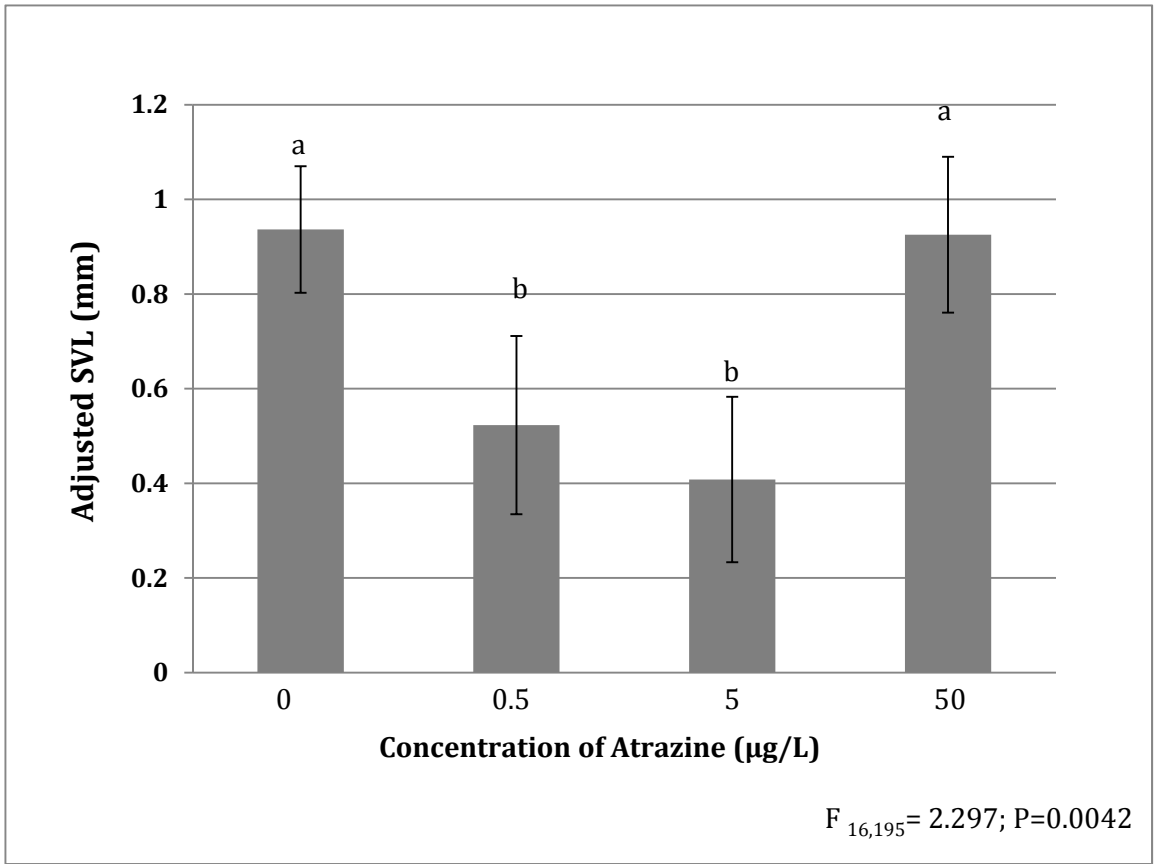

Figure 1.10. Average differences in snout vent length (SVL, mm) of tadpoles when exposed to varying levels of atrazine. Tadpoles exposed to 0.5 and 5 µg/L of atrazine had a significantly lower increase in SVL then the controls and those exposed to 50 µg/L of atrazine.

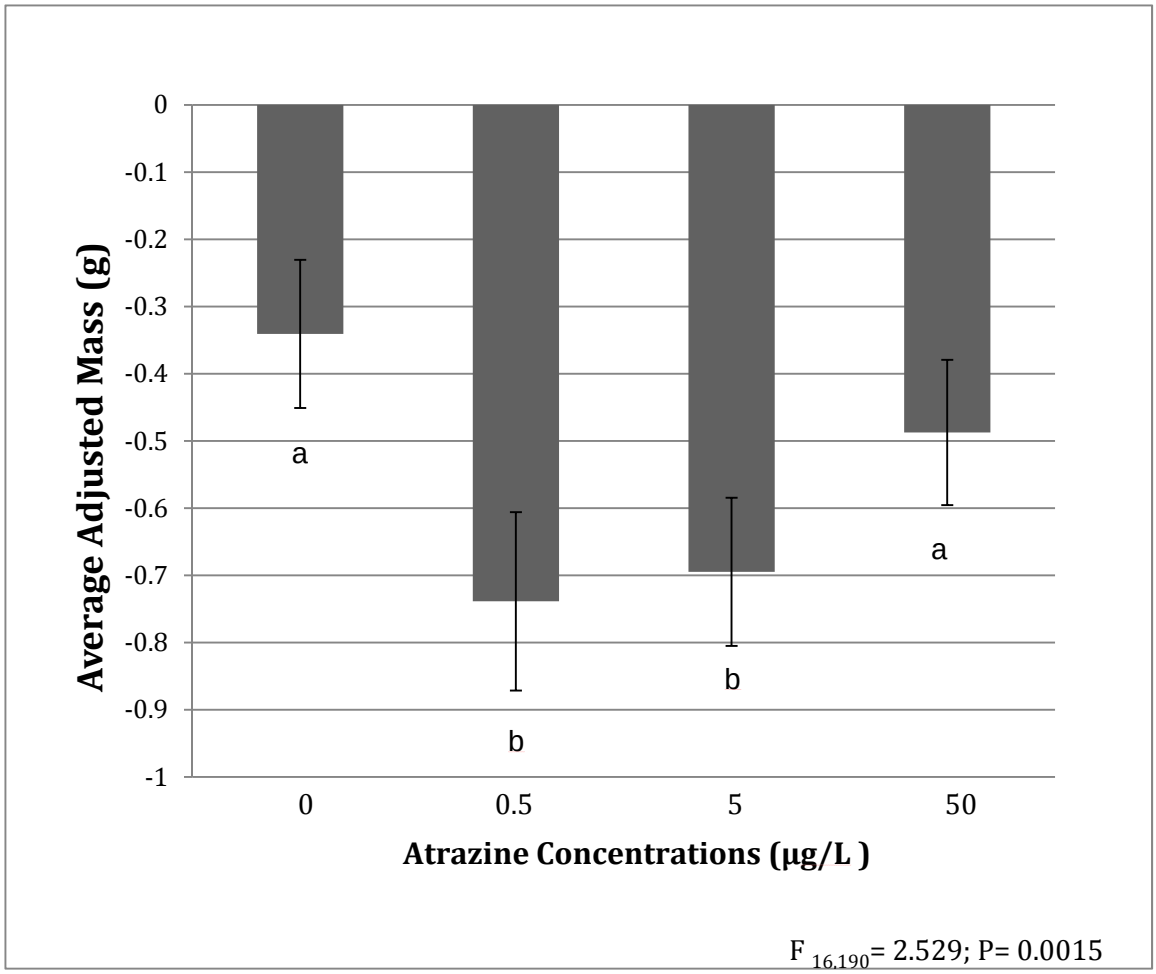

Figure 1.11. Average difference of mass (g) in tadpoles exposed to Atrazine. Tadpoles exposed to 0.5 and 5 µg/L of atrazine had significantly greater loss of mass the control tadpoles and those exposed to 50 µg/L of atrazine.

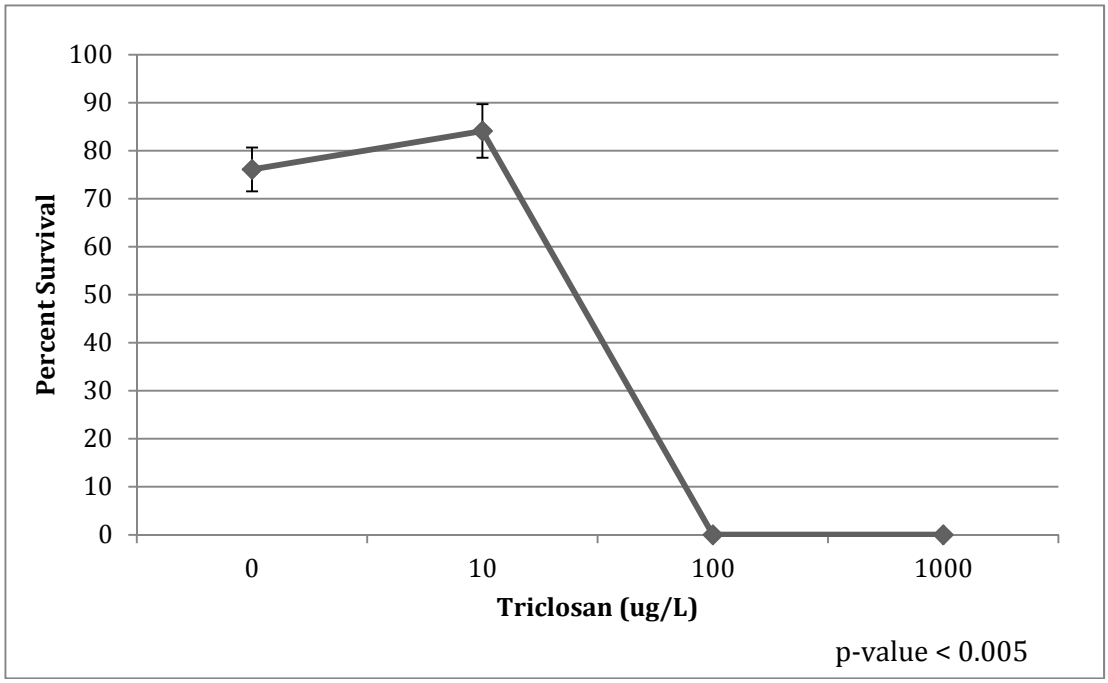

Figure 2.1. Percent survival of Woodhouse's toad tadpoles exposed to triclosan. Tadpoles exposed to 100 and 1000 µg/L died within 24 hours. Tadpoles exposed to 10µg/L did not have a significantly different survival percentage than the controls.

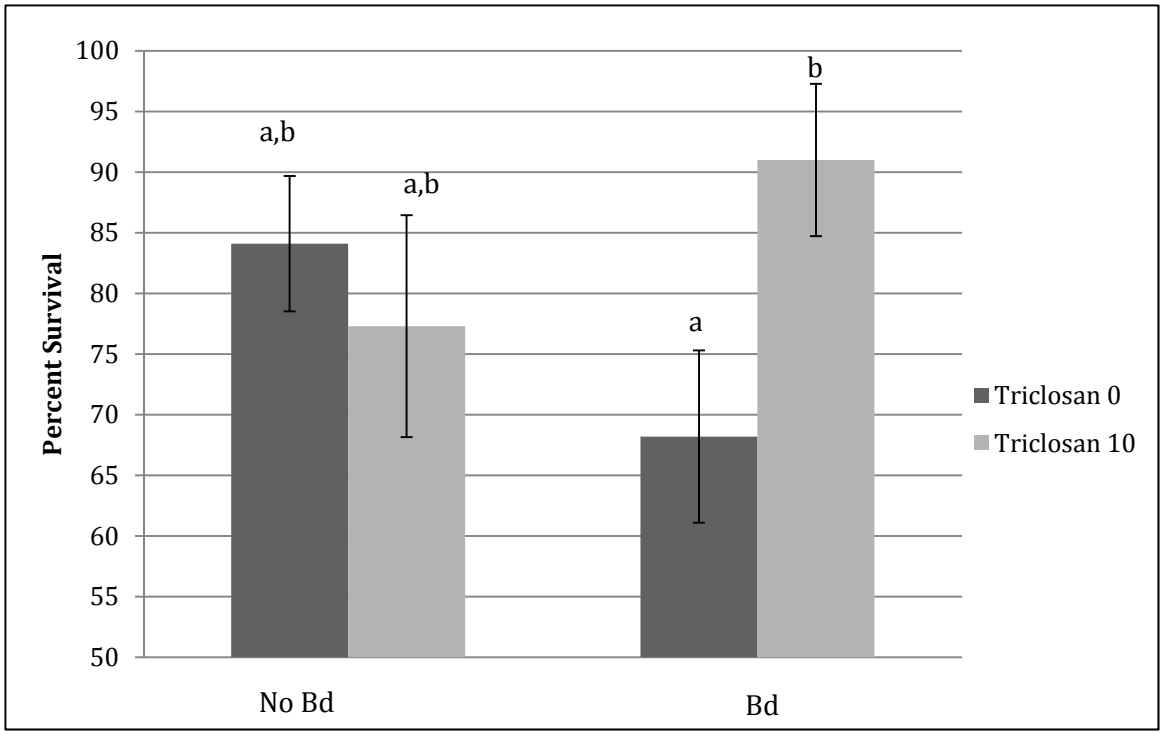

Figure 2.2. **Combined effects of Triclosan and Bd on Woodhouse Toad tadpole survival**. Control tadpoles, those exposed to only 10 µg/L and those exposed to both 10µg/L and Bd did not have a significant difference in survival. Tadpoles exposed to only Bd had a significantly lower survival rate then those exposed to 10µg/L and Bd.

Table 1.1. UTM coordinates for sample sites along the Missouri River.

Site	Zone	Northing	Easting
1	14T	4731040	189611
7	14T	4736707	183315
8	14T	4737334	180653
9	14T	4754559	101378
10	14T	4737559	177072
11	14T	478145	668145
12	14T	4741853	176112
13	14T	4742453	175793
14	14T	4742787	175712
15	14T	4736800	666112
19	14T	4739524	647722
21	14T	4747532	155773
22	14T	4748935	155077
25	14T	4744273	147506
28	14T	4719814	687313
29	14T	4738880	179246
30	14T	4732743	672267
31	14T	4737742	181113
32	14T	4720989	687112
33	14T	4747900	157588

Table 1.2. Water quality parameters by seasonal trends. The average of each water quality parameter were run in a separate model to determine any differences within the season. This was done by using a generalized linear molded with the parameter run as the response variable and to the month the measure was taken.

Parameter	df	F	P-value
Chlorophyll a	2, 18	1.237	0.3137
Ammonium	2, 19	0.0202	0.9800
Ammonia	2, 19	0.0704	0.9322
pH	2, 23	2.929	0.0735
Conductivity	**2, 22**	**5.18**	**0.0143***
Water temperature	2, 23	2.894	0.0756

Table 1.3. Water quality parameters by run-off site type. The average of each water quality parameter were run in a separate model to determine any differences between site types. This was done by using a generalized linear molded with the parameter run as the response variable to the run-off site type.

Parameter	df	F	P-value
Chlorophyll a	1, 16	1.087	0.3125
Ammonium	1, 16	2.14	0.1628
Ammonia	1, 16	0.7594	0.3964
Chlorine	1, 12	0.1179	0.7372
pH	1, 17	1.653	0.2157
Conductivity	1, 17	0.7396	0.4017
Water temperature	1, 17	0.2713	0.6092

Table 1.4. Pesticide concentrations detected from water samples taken from locations along the Missouri River. Water samples were taken during the May sampling period and tested for 24 commonly detected pesticides in Nebraska. Testing was conducted by University of Nebraska Lincoln's Water Center.
*All measurements are reported in ppb.
**Alachlor, Butylate, Chlorpyriphos, Chlorthalonil, Cyanazine, Dimethenamid, EPTC, Metribuzin, Norflorazon, Pendamethalin, Permethrin, Prometon, Propachlor, Propazine, Simazine, Tebupyrimfos, Telfluthrin, Terbufos, and Trifluralin were tested for but not detected at any of the sites.

Site	Atrazine	DEA	DIA	Acetochlor	Metolachlor	Runoff	Pesticide
1	0.06	0	0	0	0	H	M
7	0.2	0	0	0	0.07	H	H
8	0.07	0	0	0	0	H	M
9	0.07	0	0	0	0	H	M
10	0.09	0	0	0	0	L	M
11	0	0	0	0	0	L	L
12	0.07	0	0	0	0	L	M
13	0.08	0	0	0	0	L	M
14	0.13	0.07	0.13	0.07	0.11	H	H
15	0.09	0	0	0	0	L	M
19	0	0	0	0	0	H	L
21	0	0	0	0	0	H	L
22	0.08	0	0	0	0	L	M
25	0.18	0.06	0	0.08	0	H	H
28	0.3	0.11	0	0.09	0.05	L	H
29	0	0	0	0	0	H	L
30	0.1	0	0	0	0	L	M
31	0.12	0	0	0	0	L	M
32	0.59	0.35	0.26	0.09	0.11	H	H
33	0	0	0	0	0	L	L

Table 1.5. Amphibians testing positive for Bd along the Missouri River. Five amphibians tested positive for the field portion of the study. All positives were collected by June 6 and from sites with relatively lower temperature. All the sites had a positive Bd samples were high impact and had at least one atrazine contamination and three of the five had multiple contaminants.

Species	Water Temp(°C)	Date	Life Stage	Runoff	Contaminant
Northern Cricket Frog	20.35	May 21	Adult	High	Atrazine
Northern Cricket Frog	24.63	May 28	Adult	Low	Atrazine
Woodhouse Toad	21.9	June 2	Adult	High	Multiple
Woodhouse Toad	21.9	June 2	Sub-adult	High	Multiple
Boreal Chorus Frogs	26.14	June 6	Tadpole	High	Multiple

Table 1.6. Survival of Leopard frog tadpoles when exposed to atrazine, predator cue and Bd. Collection type was run as a covariate and split into two designations: multiple pesticides and few pesticides. Where multiple pesticides sties are locations where more than two pesticides detected and few pesticides are locations were we detected zero or only atrazine.

Treatment	df	P-value
Atrazine [low]	**1**	**0.0247 ***
Atrazine [medium]	**1**	**0.0429 ***
Atrazine [high]	1	0.9473
Bd	1	0.2308
Predator	1	0.7774
Collected Type	**1**	**<0.005 ***
Atrazine [high]: Bd	1	0.0520
Atrazine [low]: Bd	1	0.5601
Atrazine [medium]: Bd	1	0.0973
Atrazine [high]: Predator	**1**	**0.0180 ***
Atrazine [low]: Predator	1	0.4695
Atrazine [medium]: Predator	1	0.6479
Bd: Predator	1	0.8066
Atrazine [high]: Bd: Predator	1	0.0675
Atrazine [low]: Bd: Predator	1	0.4301
Atrazine [medium]: Bd: Predator	1	0.1086

Table 1.7. Difference in snout vent length (SVL, mm) of Leopard frog tadpoles when exposed to different levels of pesticides. Collection type was run as a covariate and split into two designations: multiple pesticides and few pesticides. Where multiple pesticides sties are locations where more than two pesticides detected and few pesticides are locations were we detected zero or only atrazine.

Treatment	df	P-value
At [low]	**1**	**0.047606***
At [medium]	1	0.594639
At [high]	1	0.144434
Bd	1	0.081155
Predator	1	0.093967
Collection Site	**1**	**0.048736***
At [low]: Bd	**1**	**0.010718***
At [medium]: Bd	1	0.277195
At [high]: Bd	1	0.546999
At [low]: Predator	**1**	**0.010078***
At [medium]: Predator	1	0.718572
At [high]: Predator	1	0.198310
Bd: Predator	**1**	**0.019426***
At [low]: Bd: Predator	**1**	**0.000329***
At [medium]: Bd: Predator	1	0.207631
At [high]: Bd: Predator	1	0.449451

Table 1.8. Difference in mass (g) of Leopard frog tadpoles exposed to different levels of pesticides. Collection type was run as a covariate and split into two designations: multiple pesticides and few pesticides. Where multiple pesticides sties are locations where more than two pesticides detected and few pesticides are locations were we detected zero or only atrazine.

Treatment	df	P-value
At [low]	1	0.3530
At [medium]	1	0.2992
At [high]	1	0.2847
Bd	1	0.2847
Predator	1	0.1086
Collection Site	1	0.1949
At [low]: Bd	1	0.1275
At [medium]: Bd	1	0.4117
At [high]: Bd	1	0.8670
At [low]: Predator	1	0.1234
At [medium]: Predator	1	0.3611
At [high]: Predator	1	0.4801
Bd: Predator	1	0.1678
At [low]: Bd: Predator	**1**	**0.0192***
At [medium]: Bd: Predator	1	0.3059
At [high]: Bd: Predator	1	0.9443

Table 1.9. Difference in snout vent length (SVL, mm) of Leopard frog tadpoles when exposed to atrazine, predator cue and Bd. Collection type was run as a covariate and split into two designations: multiple pesticides and few pesticides. Where multiple pesticides sties are locations where more than two pesticides detected and few pesticides are locations were we detected zero or only atrazine.

Treatment	df	P-value
At [low]	**1**	**0.04446 ***
At [medium]	**1**	**0.00658 ***
At [high]	1	0.76081
Bd	1	0.29834
Predator	1	0.82079
Collection Site	**1**	**0.00114 ***
At [low]: Bd	1	0.06960
At [medium]: Bd	1	0.16721
At [high]: Bd	1	0.11549
At [low]: Predator	1	0.19952
At [medium]: Predator	1	0.56575
At [high]: Predator	1	0.59652
Bd: Predator	1	0.68783
At [low]: Bd: Predator	1	0.52984
At [medium]: Bd: Predator	1	0.64439
At [high]: Bd: Predator	1	0.06074

Table 1.10. Difference in adjusted mass (g) of Leopard Frog tadpoles when exposed to atrazine, predator cue and Bd. Collection type was run as a covariate and split into two designations: multiple pesticides and few pesticides. Where multiple pesticides sties are locations where more than two pesticides detected and few pesticides are locations were we detected zero or only atrazine.

Treatment	df	P value
At [low]	**1**	**0.03191 ***
At [medium]	**1**	**0.02299 ***
At [high]	1	0.24762
Bd	1	0.13013
Predator	1	0.82422
Collection Site	**1**	**0.00254 ***
At [low]: Bd	1	0.07321
At [medium]: Bd	1	0.3824
At [high]: Bd	1	0.41925
At [low]: Predator	**1**	**0.02804 ***
At [medium]: Predator	1	0.96274
At [high]: Predator	1	0.969
Bd: Predator	1	0.89752
At [low]: Bd: Predator	1	0.12396
At [medium]: Bd: Predator	1	0.4027
At [high]: Bd: Predator	1	0.07542

Table 2.1. Percent survival of Woodhouse toad tadpoles when exposed to four concentrations of triclosan, Bd and a predator cue.

Treatment	Df	P-value
Bd	1	0.0591
Predator	1	0.1561
Triclosan [low]	1	0.4393
Triclosan [medium]	**1**	**>0.001***
Triclosan [high]	**1**	**>0.001***
Bd: Predator	1	0.7376
Bd: Triclosan [low]	**1**	**0.0295***
Bd: Triclosan [medium]	1	0.2744
Bd: Triclosan [high]	1	0.2744
Predator: Triclosan [low]	1	0.7844
Predator: Triclosan [medium]	1	0.4121
Predator: Triclosan [high]	1	0.4121
Bd: Predator: Triclosan [low]	1	0.5618
Bd: Predator: Triclosan [medium]	1	0.8466
Bd: Predator: Triclosan [high]	1	0.8466

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