Exploring the Sublethal Effects of Pesticide Pollution in Crayfish

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Abstract

Freshwater systems connected to agricultural practices can be prone to having pollutants (e.g. pesticides) introduced from runoff. These pollutants can have damaging effects on the biotic community assemblages throughout these systems. Low doses of pesticides can negatively affect non-target organisms, such as macroinvertebrates, by compromising their metabolic function and overall health. This project expands on how pesticide pollution impacts freshwater ecosystems by exposing crayfish to environmentally relevant combined concentrations of three common herbicides (Atrazine, Glyphosate, and 2,4-D) and documenting sublethal effects through hepatopancreas cell counts. Results reveal negative sublethal impacts of common herbicides on crayfish, and by extension indicate the possible impact the herbicides can have on other non-target organisms after exposure to herbicides.

Exploring the Sublethal Effects of Pesticide Pollution in Crayfish

Introduction

Although much research has been done on the topic of pesticides and ecological health, the full extent of the chemical effects on humans and the environment is unknown (Nicolopoulou-Stamati et al., 2016). This project is a baseline study which investigates pesticide impacts on non-target organisms, providing insight on how chemical combinations of atrazine, glyphosate, and 2,4-D are affecting a common macroinvertebrate in today's freshwater environment: the crayfish (de Abreu, 2020).

This study answers the question, "What are the sublethal effects of common herbicides on crayfish?" The pesticide effects were determined through cell count analysis of the crayfish hepatopancreas, a site of detoxification in the crayfish (Banaee et al., 2019). Before the importance of investigating sublethal effects on the crayfish can be understood, pesticide usage, its subsequent introduction to the environment, and role of crayfish in the ecosystem will be reviewed.

Background

Pesticide Usage

Various types of pesticides are used in agricultural industries worldwide. Pesticides are known to improve crop production, lowering food prices and increasing food supply worldwide; approximately $1 spent on chemicals brings a $4 crop increase (Pimental et al., 1978). However, the increase of pesticide usage has sparked concern for environmental and human health due to its potential negative effects on non-target organisms (Nicolopoulou-Stamati et al., 2016).

"Pesticide" is an umbrella term for chemical and biological compounds used to control plants (herbicides), fungi (fungicides), animals (rodenticides), and pathogens, such as bacteria and viruses (Hayes & Hansen, 2017). The largest of these pesticide categories are herbicides, which compose 50% of pesticides worldwide; approximately 3 billion pounds of herbicides are used globally every year, with 678 million pounds being used in the U.S. in 2012 alone (Atwood & Paisley-Jones, 2017). According to a 2012 EPA report, three of the five most common herbicides in the United States were atrazine, glyphosate, and 2,4-D (Atwood & Paisley-Jones, 2017).

Chemical Composition and Environmental Impacts of Atrazine, Glyphosate, and 2,4-D

Atrazine

Atrazine, or 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine, degrades into various secondary metabolites after an environmentally dependent half-life ranging from 60 to 660 days (Ribaudo & Bouzaher, 1994). In 2001, the EPA estimated that 64-75 million pounds of atrazine were applied in pre-emergent or post emergent applications (Brent et al., 2010).

Various studies have detected atrazine at high levels throughout both the terrestrial and freshwater environments (Martins et al., 2018; Ribaudo & Bouzaher 1994). Wu et al. (2010) detected a concentration of 59.57 ppb in finished drinking water, which is well above the EPA maximum contaminant level (MCL) of 3 ppb (ATSDR, 2003). Spikes exceeding 220 ppb in the freshwater ecosystem were also observed, including a sample containing 227 ppb of atrazine in a stream in Ohio (Wu et al., 2010). The presence of atrazine in the freshwater system raises interest for connecting ecosystems; recently, 30 ppb of atrazine has been recorded in a tributary

to the Chesapeake Bay, which causes concern for the intersection between contaminated freshwater ecosystems and marine environments (Britt et. al, 2020).

Exposure to Atrazine is correlated with significant health impacts in both people and the environment (ATSDR, 2003). Atrazine is listed as a possible human carcinogen under EPA standards (Ribaudo & Bouzaher, 1994). The EPA recognizes concentrations above 65 ppb as harmful to sensitive aquatic species, mentioning that this concentration was rarely detected (Ribaudo & Bouzaher, 1994).

Reproductive effects have been noted in many organisms after low dose exposure to Atrazine. Concentrations at and above 0.1 ppb have shown to impact the reproductive organs of clawed frogs (Hayes et al., 2010), minnows (Tillitt et al., 2010), and even humans (Cragin et al., 2011). Atrazine has also caused endocrine disruption in crayfish (MacLoughlin et al., 2016), and enzyme misfunction in worms (Contardo-Jara et al., 2008).

Glyphosate

Glyphosate, the active ingredient in many common herbicides, is composed of the chemical N-(phosphonomethyl) glycine. The EPA estimates that 270-290 million pounds of glyphosate are used in the U.S. annually (Atwood & Paisley-Jones, 2017). The EPA limit of Glyphosate in soil samples is 700 ppb (parts per billion), but the MCL for drinking water is 70 ppb (U.S. Environmental Protection Agency, 2018). Glyphosate half-life in water ranges from 3 days to 19 weeks, and aquatic applications are permitted to control emergent aquatic weeds through inhibiting enzymatic synthesis of aromatic amino acids (Gravena et al., 2012; Wisconsin Department of Natural Resources, 2012). Surfactants used with aquatic applications of glyphosate herbicides raise concern for aquatic environments (Solomon & Thompson, 2003).

Glyphosate salts are highly soluble in the environment, and can readily permeate soil (Tóth et al., 2020). Some studies have detected glyphosate at high concentrations in areas such as wetlands or lakes, drainage ditches, and soil samples, with values reaching 450 ppb (Battaglin et al., 2014). In a study conducted by Peruzzo et al. (2008), glyphosate was found at concentrations between 100 and 700 ppb in streams near agricultural waters, with an increase in herbicide presence after rainfall.

Glyphosate has been shown to cause metabolic disruption to various organisms, including crayfish (Banaee et al., 2019). Crayfish had signs of hormone disturbance after exposure to glyphosate and showed damage to their exoskeleton (Banaee et al., 2019). Protein reduction was seen in crayfish hemolymph, the fluid in the crayfish open circulatory system, after exposure to glyphosate (Banaee et al., 2019).

2,4-D

2,4-D is another common herbicide used throughout the United States. About 30-40 million pounds of 2,4-D are used annually, and about 1500 pesticides utilize 2,4-D as an active ingredient (Atwood & Paisley-Jones, 2017; Islam et al., 2018). This herbicide is used in a variety of applications, including aquatic application, where it has been used to kill the invasive Eurasian watermilfoil (Nault et al., 2018).

2,4-D degrades into various products after a half-life of about 6 days in soil, 15 days in aquatic aerobic environments, and 41-333 days in anaerobic conditions (U.S. Environmental Protection Agency, 2005). The herbicide causes plants to produce and maintain auxin at high levels, which leads to death (Wijnja et al., 2014). 2,4-D can evaporate readily, and has been detected in soil, air, and freshwater systems (Islam et al., 2018). The chemical can penetrate soil

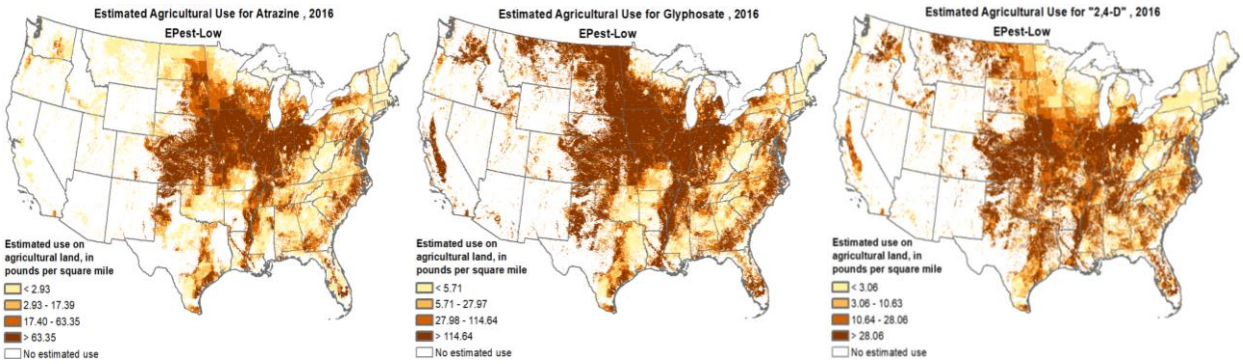
layers in a process called leaching or enter the atmosphere through volatilization (Islam et al., 2018). The chemical is prevalent in the environment; a study done in Massachusetts found 2,4-D to be the most common pesticide in environmental samples (Wijnja et al., 2014). The MCL for 2,4-D in drinking water is 70 ppb (U.S. Environmental Protection Agency, 2018).

2,4-D has been shown to cause various sublethal effects in organisms. In one study, crayfish exposed to 2,4-D had difficulty finding food, potentially due to damaged chemoreceptors (Browne & Moore, 2014). Earthworms exposed to 2,4-D in soil experienced weight loss and other sublethal effects, with increased weight loss corresponding to increased 2,4-D concentration and exposure time (Correia & Moreira, 2010).

The chemicals described above are used concurrently with each other (Figure 1). Due to multiple chemicals being used in the same proximity throughout the United States, it is likely that chemicals from surrounding areas mix during runoff events (Carpenter et al., 2008).

Figure 1

U.S. Usage Maps for Atrazine, Glyphosate, and 2,4-D



Note. The usage maps show the proximity atrazine, glyphosate, and 2,4-D usage throughout the U.S. Adapted from "Pesticide National Synthesis Project" by USGS, 2016 (https://water.usgs.gov/nawqa/pnsp/usage/maps/compound_listing.php?year=2016&hilo=L). In the public domain.

Chemical Introduction into Freshwater Ecosystems

Because different types of pesticides are widely used throughout the United States (Figure 1), it has been proposed that combinations of these chemicals can enter freshwater ecosystems during stormwater runoff events, where rainwater washes chemicals from agricultural fields into freshwater ecosystems (Solomon et al., 1996). Combinations of chemicals have been found in freshwater ecosystems, with one study finding atrazine and glyphosate to be the most frequently detected pesticides in the St. Lawrence River and its tributaries; atrazine and glyphosate were detected in 82% and 84% of samples, respectively (Montiel-León et al., 2019).

Biodiversity and the Role of Crayfish in Stream Ecosystems

Biodiversity is crucial for healthy ecosystems and is important for environmental stability and efficiency (Cardinale et al., 2012). Freshwater diversity is said to be declining faster than

marine biodiversity (Richman et al., 2015), which is concerning for freshwater ecosystem health. Some crayfish populations are declining globally, causing additional concern due to the large part they play in stream ecosystems (Richman et al., 2015); crayfish are involved in multiple trophic levels, and declines in crayfish populations could cause disproportional impacts on the freshwater ecosystem (Browne & Moore, 2014; Reynolds et al., 2013; Weinländer & Füreder, 2016).

Crayfish are described as good ecological health indicators due to their importance in stream ecosystems and sensitivity to chemical pollution (Creed & Reed, 2004; Kholodkevich et al., 2008). Crayfish can play a part in engineering stream ecosystems for other organisms by breaking down leaves and other organic matter for other organisms to process (Helms & Creed, 2005; Creed & Reed, 2004). Energy enters wooded, headwater streams through organic matter, namely leaf litter (Figure 2), and crayfish break down leaf matter into smaller particles useful to other organisms, recycling matter for use later downstream (Huryn & Wallace, 1987).

Crayfish are benthic organisms and interact with the sediment deposited in water systems. Since herbicides can settle into sediment (Islam et al., 2018), crayfish may be exposed to these sediment-bound chemicals and negatively impacted as they ingest the chemicals. Therefore, this study will investigate how combined treatments of atrazine, glyphosate, and 2,4-D are affecting crayfish through analyzing the hepatopancreas.

Figure 2

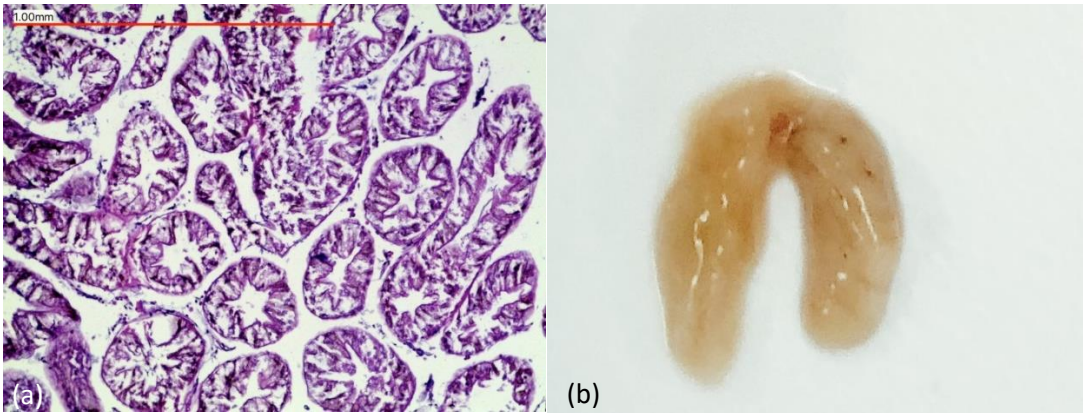
Leaf Litter in a Wooded Headwater Stream

Hepatopancreas as a Site of Detoxification

In this study, the crayfish hepatopancreas was used to test for sublethal effects in the form of cell degradation (Figure 3), as one purpose of the organ is processing toxins (Banaee et al., 2019). The hepatopancreas is composed of a tubular system containing different cell layers (Vogt, 2019). The cells of the layers serve different functions, including nutrient absorption, respiration, fat storage, and digestive enzyme production (Vogt, 2019). The hepatopancreas also produces compounds functioning in crayfish immunity, and chemical pollutants impacting this organ can negatively impact crayfish health (Wei and Yang, 2015).

Figure 3

Hematoxylin and Eosin (H&E) Stain of Crayfish Hepatopancreas Cross Section (a) and Dissected Hepatopancreas Organ (b)



Note: The hepatopancreas cross section (a) shows the tubular nature of the organ, and (b) is a tissue sample used for analysis. Used with permission from Dr. Kyle Harris.

Physiological changes in the hepatopancreas have been observed in correlation with environmental pollution; toxins such as heavy metals can be seen absorbing into hepatopancreas cells (Vogt, 2019), oxidation of proteins in hepatopancreas cells has been observed in crayfish exposed to pollution (Wei & Yang, 2015), enzymatic changes have been discovered in the hepatopancreas after exposure to pesticide pollution, and significant changes in macromolecule concentrations have been observed in the crayfish hepatopancreas after exposure to glyphosate (Banaee et al., 2019).

Although research has been done on the chemical changes in the hepatopancreas after exposure to environmental pollutants, the full effect of herbicides on the hepatopancreas and subsequent crayfish health is unknown (Banaee et al., 2020; Wei and Yang, 2015). Because the hepatopancreas serves many important functions to crayfish health, the effects of herbicide pollution on this organ should be investigated in attempt to understand their effects on the

surrounding ecosystem. This study investigates the effects of the atrazine, glyphosate, and 2,4-D herbicide combination on the cell counts of hepatopancreas cross sections.

Previous Studies on Herbicides and Freshwater Invertebrates

This research project was preceded by two preliminary experiments. The first experiment tested the effects of atrazine on juvenile crayfish growth. Young crayfish from a rural stream were chronically exposed to low doses of atrazine at 0.05 and 0.5 ppb for five months (Chandler et al., 2017). Results indicated that the crayfish were negatively impacted; growth was stunted, and hepatopancreas cell counts were significantly lower than the control groups (Figure 4).

Another pesticide experiment done by Youngbar et. al (2020) tested the effects of herbicide concentrations on crayfish ectosymbionts called branchiobdellidan worms, which benefit crayfish by consuming bacteria cleaned from gill filaments (Brown et. al., 2002). Due to the mutualistic relationship between the annelid and the crayfish (Creed et al., 2015), the worm was tested to investigate pesticide influence on another biological factor impacting crayfish health (Youngbar et al., 2020).

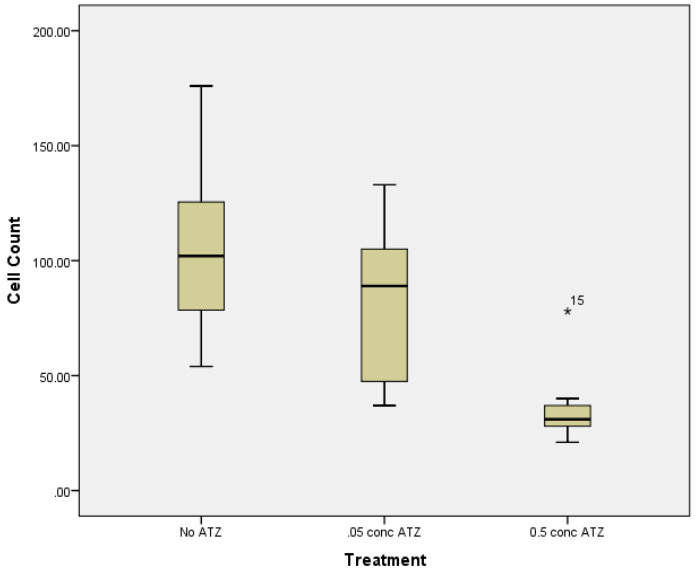
In branchiobdellidan worms exposed to individual treatments of atrazine and glyphosate, only sublethal affects were observed (Figure 5); no branchiobdellidan worms died in individual 100 ppb and 1000 ppb treatments of atrazine and glyphosate, although cell degradation was observed (Youngbar et al., 2020).

However, in combined 100 ppb and 1000 ppb treatments composed of 50 ppb and 500ppb of atrazine and glyphosate (Table 1), 100% branchiobdellidan mortality was observed (Youngbar et al., 2020). This indicates that combinations of chemicals could cause greater harm than individual herbicide treatments, even if their combined concentration was lower than

individual treatments. The present study continues to explore the combined sublethal effects of the same three herbicides on crayfish.

Figure 4

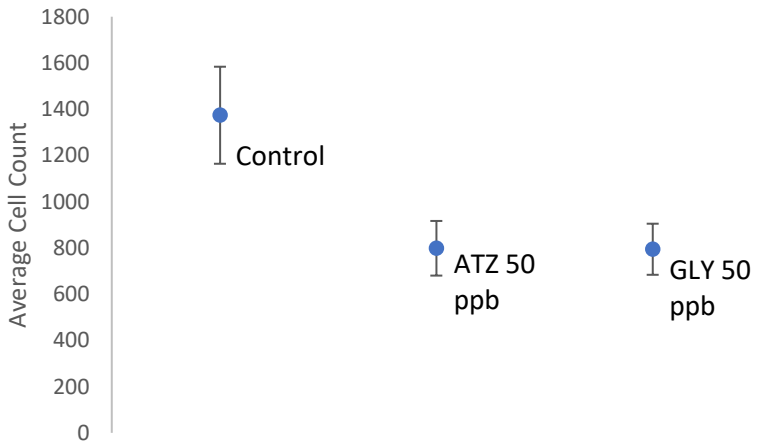
Box Plots for Hepatopancreas Cell Counts from Juvenile Crayfish Exposed to Two Concentrations of Atrazine



Note. ANOVA analysis showed a significant decline in cell counts between the control (No ATZ) and 0.5 ppb concentration of atrazine (0.5 conc ATZ). Reprinted from "The Effects of a Common Herbicide (Atrazine) on Juvenile Crayfish Growth and Development" by Chandler, N. T., Minuto, A. M., Froese, S., Suttle, A. H., Owens, S., Grant, J., Sooklal, S., Allen, T., Blais, M., & Harris, K. J., 2017. *Research Gate.* (https://doi.org/10.13140/RG.2.2.23683.20002). Reprinted with permission.

Figure 5

Mean Cell Counts of Branchiobdellidan Worms Indicate Significance Decline Between Treatments and Control



Note. Mean cell counts of branchiobdellidan worms after exposure to atrazine (ATZ) and glyphosate (GLY) indicate significant decline in degradation between each 50 ppb treatment and the control. Error bars are 2× standard error. Reprinted from "Exploring the Lethal and Sub-Lethal Effects of Pesticide Pollution in Crayfish and their Ectosymbionts" by Youngbar, Z., St. Claire, K., Pizzo, M., Carnathan, B., and Harris, K. *Liberty University.* (https://digitalcommons.liberty.edu/research_symp/2020/posters/70/). Reprinted with permission.

Table 1

Mortality of Branchiobdellidan Worms

Treatment Group	Time (hours) Percent Dead Over Time					Total number out of 10 dead after 48 hours
	0	8	24	32	48	
Control	0	0	0	0	0	0
ATZ 50 µg/L	0	0	0	0	0	0
ATZ 500 µg/L	0	0	0	0	0	0
GLY 50 µg/L	0	0	0	0	0	0
GLY 500 µg/L	0	0	0	0	0	0
ATZ 50 & GLY 50 µg/L	0	10	10	10	100	10
ATZ 500 & GLY 500 µg/L	0	10	90	100	100	10

Note. No mortality was observed in any individual herbicide concentration, but 100% mortality was observed after 48 hours in combination treatments. Reprinted from "Exploring the Lethal and Sub-Lethal Effects of Pesticide Pollution in Crayfish and their Ectosymbionts" by Youngbar, Z., St. Claire, K., Pizzo, M., Carnathan, B., and Harris, K. *Liberty University.* (https://digitalcommons.liberty.edu/research_symp/2020/posters/70/). Reprinted with permission.

Current Study

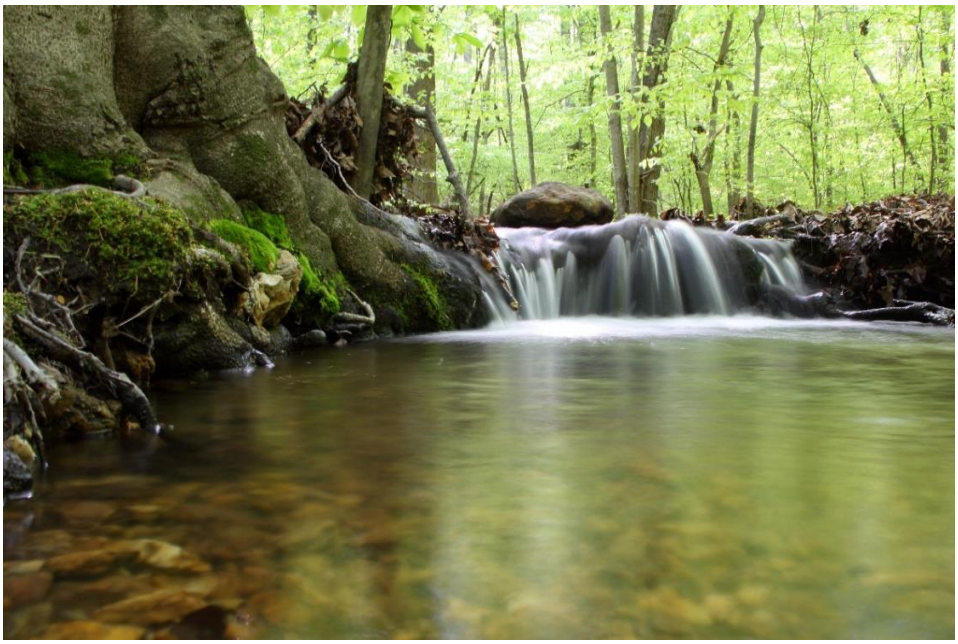
Methods

Field Collection

Crayfish were collected from Opossum Creek, a rural second order creek in Central Virginia (Figure 6). The stream was chosen due to its distance from agricultural areas; therefore, it should have minimal exposure to pesticides. A kick-seine technique was used to collect the crayfish *Cambarus sp*. by kicking rocks upstream from the seine. The crayfish carried into the seine were placed in whirl packs and transported to the lab. In the laboratory, crayfish were placed in tanks at room temperature to acclimate to captivity for a minimum of two weeks before being separated into treatment groups.

Figure 6

Opossum Creek Collection Site



Note. Crayfish used in this study were collected from Opossum Creek, a rural headwater stream in Central Virginia.

Tank Study

After acclimation, crayfish were exposed to one of three different treatments. In the first treatment, crayfish were placed in individual tanks containing an herbicide concentration of 150 ppb. This concentration was composed of 50 ppb of atrazine, 50 ppb of glyphosate, and 50 ppb of 2,4-D. Crayfish were left in the solution for 48 hours. The second treatment was executed in the same manner, except the herbicide concentration totaled 1500 ppb, composed of equal parts atrazine, glyphosate, and 2,4-D. A chronic exposure ranging from 32-49 days was created with an herbicide concentration of 2000 ppb. This solution was composed of 1000 ppb of glyphosate, 500 ppb of atrazine, and 500 ppb of 2,4-D. After exposure, each crayfish was sacrificed and the hepatopancreas was immediately removed through dissection. The apical end of each side of the hepatopancreas was removed. Slides were then prepared from the tissue.

Slide Preparation

In order to standardize the same hepatopancreas region, the apical end of each hepatopancreas was removed and inserted into Cryo-Embedding Compound gel (PELCO) and frozen at -80 °C. After the gel froze, the block containing the tissue was sectioned into 7 µm slices using a cryostat. Multiple sections from each crayfish sample were melted onto a charged slide in preparation for staining to ensure that a sufficient number of replicates from each hepatopancreas was obtained.

After mounting the frozen tissue slices onto slides, the slides were stained using DAPI (4′,6-diamidino-2-phenylindole). The DAPI solution was created using a 1:100 ratio of DAPI to phosphate buffer saline (PBS). After covering the slides with DAPI during a wash, the slides were wrapped with aluminum foil and left in a dark drawer for 10 minutes. Next, the slides were

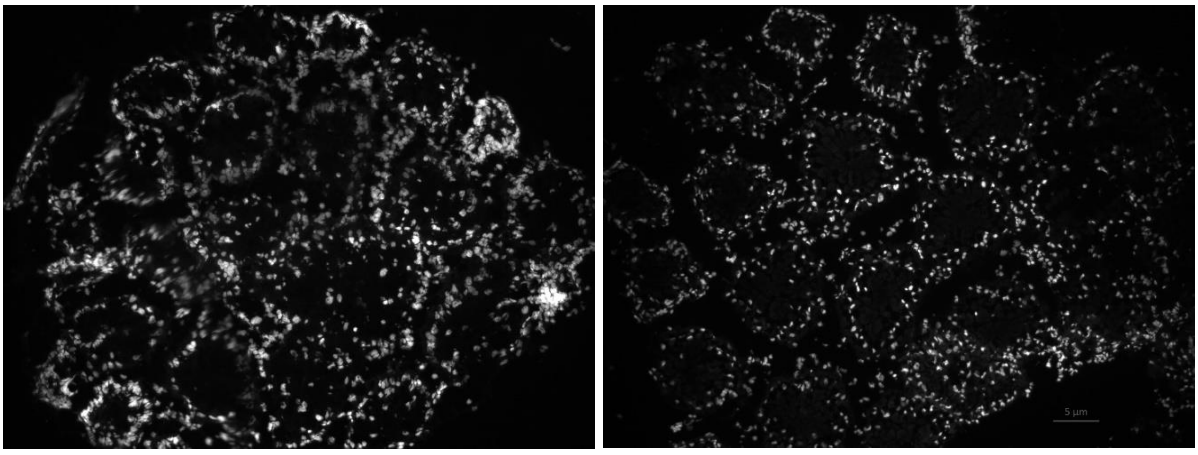
rinsed using PBS. The wash was repeated two more times, for a total of three washes. The slides were dried and stored in a dark container prior to being visualized with microscopy.

Data Collection

After the histological process described above, the slides were viewed under an EVOS M5000 microscope (Figure 7). The microscope counted cells in a fixed, predetermined area of tissue (Figure 8). Cell counts from apical slices of the same hepatopancreas were obtained from multiple cross sections at 10× magnification, and these counts were averaged for each crayfish. This averaged number from each crayfish was compared across pesticide treatments using a one-way ANOVA (IBM SPSS). After ensuring data normality using a Shapiro-Wilk test and homogeneity of variances using Levene's test, a Bonferroni multiple comparison test was used to compare pesticide treatments in a pairwise fashion using an experiment wise error rate of 0.05.

Figure 7

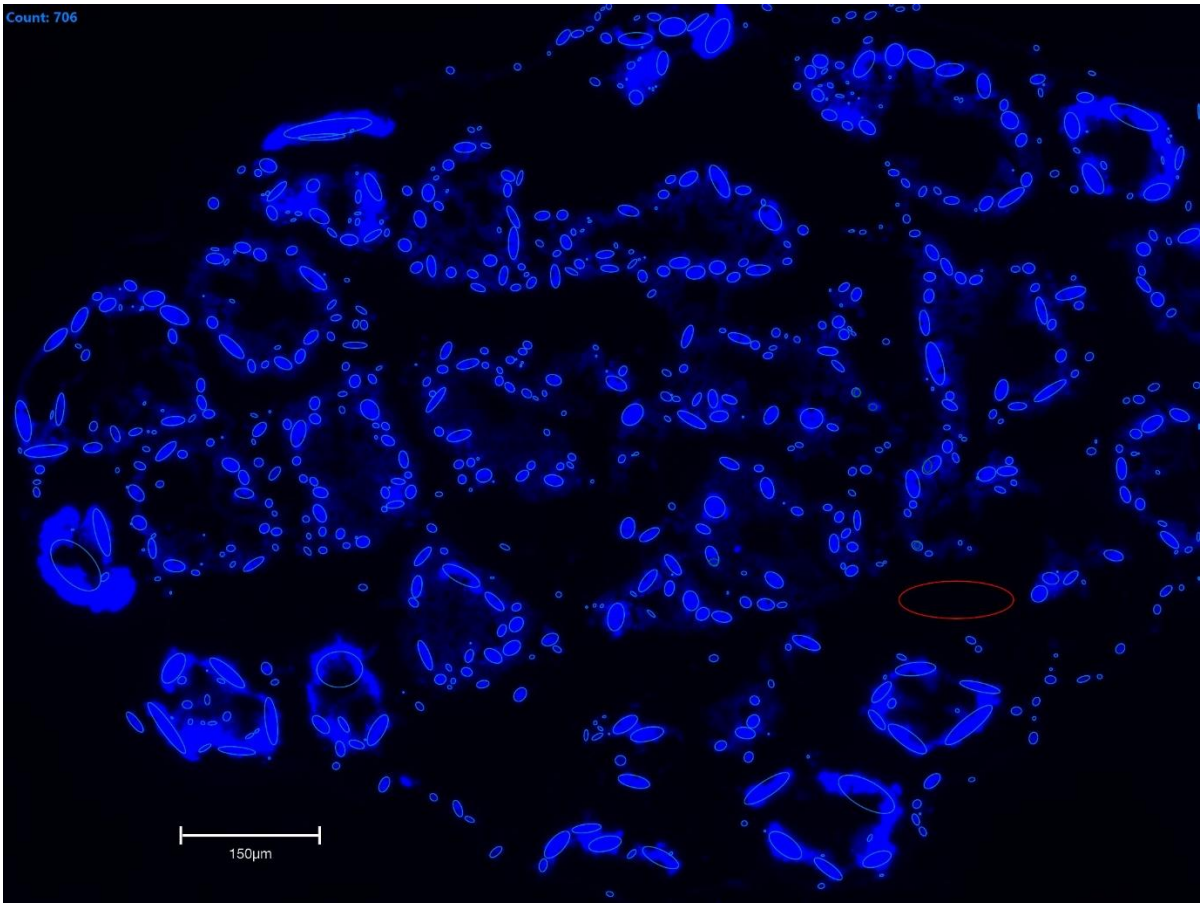
Hepatopancreas Cross-Sections Visualized Under an EVOS M5000 Microscope



Note. DAPI stained cell nucleus allow the EVOS M5000 to obtained cell counts. Retrieved from https://watch.liberty.edu/media/t/1_1yru397l.

Figure 8

The EVOS M5000 Selects and Counts DAPI Stained Cells



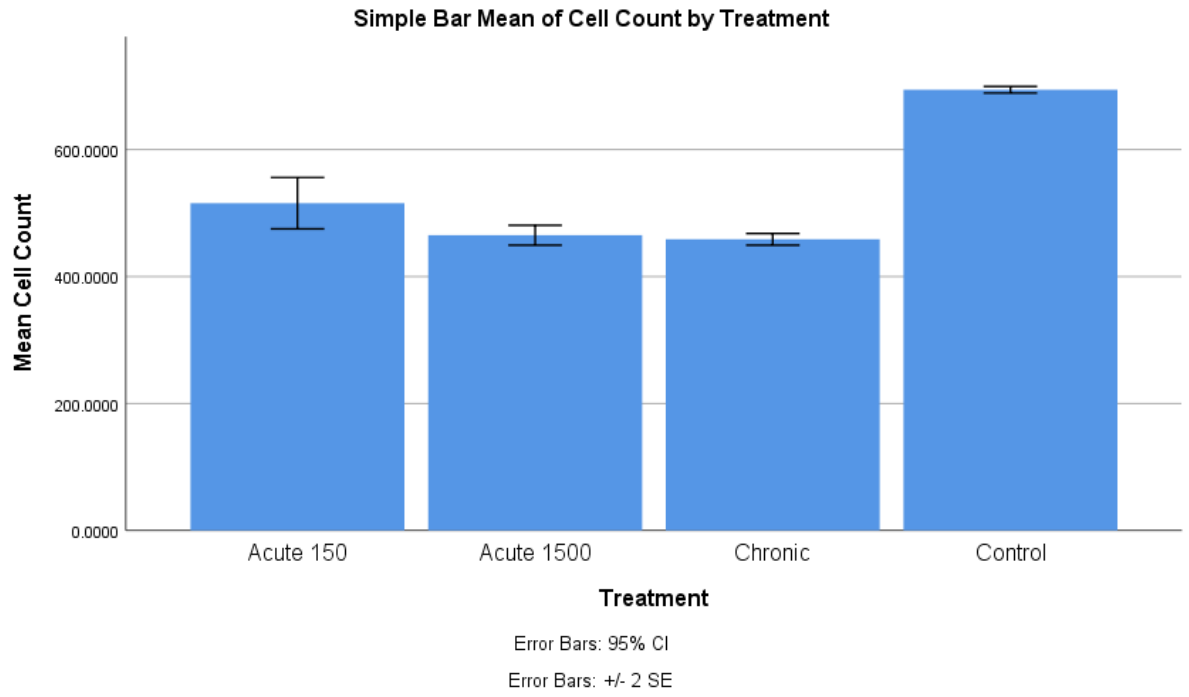
Note. Cells and the surrounding field were defined for the EVOS M5000 and cell counts were automatically generated. Retrieved from https://watch.liberty.edu/media/t/1_1yru397l.

Results

Results showed a significant difference between the apical hepatopancreas cell counts for the control group and each treatment group (F=53.53, DF=3,6, p<.001; Figure 9). The mean number of cells in the control group was $\bar{x}$=694, the 150 ppb treatment had a mean of $\bar{x}$=515, the 1500 ppb treatment mean was $\bar{x}$=465, and the chronic treatment mean was $\bar{x}$ =469.

Figure 9

Mean Cell Counts Per Treatment Groups Exposed to Combinations of Atrazine, Glyphosate, and 2,4-D



Note. The mean of the acute 150 ppb combination was $\bar{x}$=515, the 1500 ppb treatment mean was $\bar{x}$ =465, the chronic treatment mean was $\bar{x}$=469, and the control was $\bar{x}$=694.

A significant decline in cell counts was seen between the control and each of the three treatment groups (Table 2). However, none of the three treatment groups were significantly different from each other (Table 2).

Table 2

Bonferroni pairwise comparisons of cell counts between treatment groups

Multiple Comparisons

Dependent Variable: Mean Cell Count

Bonferroni

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	99.167% Confidence Interval	
					Lower Bound	Upper Bound
Acute 150	Acute 1500	50.51622575	17.89109575	.181	-49.5691031	150.6015546
	Chronic	56.92932099	20.00285315	.176	-54.9694784	168.8281204
	Control	-178.613536*	20.00285315	.001	-290.512336	-66.7147368
Acute 1500	Acute 150	-50.5162257	17.89109575	.181	-150.601555	49.56910307
	Chronic	6.413095238	20.00285315	1.000	-105.485704	118.3118946
	Control	-229.129762*	20.00285315	.000	-341.028561	-117.230963
Chronic	Acute 150	-56.9293210	20.00285315	.176	-168.828120	54.96947841
	Acute 1500	-6.41309524	20.00285315	1.000	-118.311895	105.4857042
	Control	-235.542857*	21.91202777	.000	-358.121850	-112.963864
Control	Acute 150	178.613536*	20.00285315	.001	66.71473676	290.5123356
	Acute 1500	229.129762*	20.00285315	.000	117.2309625	341.0285613
	Chronic	235.542857*	21.91202777	.000	112.9638640	358.1218503

Based on observed means.

The error term is Mean Square(Error) = 480.137.

*. The mean difference is significant at the .00833 level.

Note. Six pairwise comparisons were made and the experimentwise error rate was set at 0.05. Three comparisons compared the control to each treatment group (Acute 150, Acute 1500, and Chronic), and three comparisons were made comparing the acute and chronic treatments.

Discussion

Significance of Cell Counts

Although a significant decline in hepatopancreas cell counts was seen between the control and each of the treatment groups, none of the treatment groups were significantly different from each other. This indicates that neither concentration nor length of exposure may significantly impact cell counts, but that crayfish experience similar sublethal affects to both acute and chronic pesticide exposure at these concentrations. If this remains true in freshwater

systems, even low concentration and short-term bursts of herbicides mixtures entering freshwater systems could cause similar sublethal damage to high bursts.

If a significant number of hepatopancreas cells are dying after crayfish are exposed to the herbicides, it is possible that the decrease in enzymatic activity and protein concentrations seen by Banaee et al. (2020) are due to the decrease of cells. The decrease in the hepatopancreas cell counts shows that the crayfish are responding to the environmental stress created by the chemical combinations, but it is unknown if these sublethal effects are temporary with the possibility of recovery.

Environmental Relevance of Treatments

Although the concentrations used in this study were slightly higher than the concentrations seen in some surface water studies (K. R. Solomon et al., 1996), the concentrations of individual herbicides used in this study are comparable to the values described by others, such as Wu et al. (2010), where many concentrations of atrazine were found to be over 50 ppb in freshwater samples. The acute 150 ppb treatment in this study had individual herbicide concentrations at levels below either EPA approved MCLs or experimentally found concentrations in drinking water (U.S. Environmental Protection Agency, 2018; Wu et al., 2010). The 500 ppb concentrations of atrazine, glyphosate, and 2,4-D composing the 1500 ppb treatment are higher than many values seen in the environment, but they are well beneath EPA acute benchmarks for aquatic organisms (U.S. Environmental Protection Agency, 2020).

The ecological significance of the effects seen in the treatment groups of this study is that ecosystems could be impacted by lower chemical doses than those currently recorded in freshwater systems or accepted by government standards. This is supported by other studies,

which have found significant sublethal impacts with chemical concentrations below governmental standards (Hayes & Hansen, 2017); government MCLs for drinking water are 3 ppb for atrazine, 70 ppb of glyphosate, and 70 ppb for 2,4-D (ATSDR, 2003; U.S. Environmental Protection Agency, 2018). Doses of atrazine under 3 ppb have been shown to impact microbial communities (Britt et al., 2020). Loss of smell was observed in crayfish after an 80 ppb exposure to atrazine, potentially inhibiting their ability to find a mate (Belanger et al., 2017). 2,4-D was also shown to inhibit chemoreception in crayfish, affecting their ability to gather food (Browne & Moore, 2014). The compromised ability to find a mate and food could be a factor leading to the population declines described by Reynolds et al. (2013). Glyphosate can also cause a range of sublethal effects, including increased glucose and triglyceride counts, which could be correlated with hepatopancreatic damage (Banaee et al., 2019). Hepatopancreas enzymes showed significant decrease after 21-day exposure to glyphosate at 80 ppb (Banaee et al., 2020), and combinations of glyphosate and other chemicals used in the study (chlorpyrifos) showed a greater impact on the crayfish than glyphosate alone (Banaee et al., 2020), indicating that low dose combinations of chemicals may cause greater sublethal impact than individual concentrations.

Direction for Further Studies

Future studies should repeat the experiment above with a larger sample size to validate the results of this study. Then, the minimum combination concentrations at which significant hepatopancreas cell degradation occurs should be determined in order to lay a baseline for safe chemical concentrations in freshwater ecosystems. Studies comparing crayfish exposed to a single herbicide with crayfish exposed to combination treatments could be run to specify the

significance of combination doses. Recovery of the crayfish hepatopancreas could also be investigated by returning crayfish to a toxin free environment and observing cell counts. In summary, further research should be done to set guidelines for environmental concentrations of herbicides and investigate the extent of *in vivo* freshwater ecosystem disruption due to pesticide introduction occurring in the environment. Best management practices should then be utilized to maintain chemical concentrations below these levels.

Conclusion

Due to the prevalence and variety of herbicides used in agricultural practices and sublethal effects seen in crayfish, the effects of herbicide combinations should be considered in ecological assessments. If crayfish, as indicator organisms, are impacted by the chemical bursts created in this study, ecological disruption due to chemical runoff could be taking place throughout freshwater ecosystems.

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