The Sublethal Response of *Cambarus sp*. to Acute Low Dose Herbicide Exposure Evidenced by a Change in GGT Levels and Apoptosis

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Abstract

Following application, herbicidal runoff can collect in freshwater ecosystems, briefly exposing non-target organisms. As biological indicators, crayfish serve as models to assess freshwater health. Chemically stressed crayfish form reactive oxygen species (ROS) which are neutralized by the glutathione pathway. As glutathione depletes, γ-glutamyl-transferase (GGT) upregulates to increase glutathione formation. High oxidative stress lowers glutathione levels and subsequent apoptosis occurs. For this acute study, crayfish were exposed to 5ppb and 50ppb of atrazine, glyphosate, and 2,4-D. Herbicide exposures were expected to induce higher GGT production, increased oxidative stress, and increased apoptosis. Instead, GGT and apoptosis predominately decreased relative to the control. This suggests that oxidative damage was mitigated because the crayfish were able to effectively neutralize ROS at 5 and 50ppb.

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Pesticide History and Applications

The nuisance of mosquitos at a summer barbeque, unsightly weeds in a beautifully cultivated flower bed, or even pathogenic bacteria causing mass disease are nothing new. Historians have uncovered ancient texts depicting swarms of locusts and worms devasting crops, causing famine, and orchestrating expansive death (Matthews, 2018). Some historically notable epidemics caused by pests include the Black Death and the Irish potato famine. Without an effective rodenticide, antibacterial, or insecticide, the bubonic plague, caused by the bacterium *Yersinia pestis,* rapidly spread through Europe and Western Asia in the blood of infected rats and fleas (Matthews, 2018; Duncan & Scott, 2005). The deadliest outbreak of bubonic plague occurred between 1346 and 1353 and was responsible for the deaths of over a third of the global population (Matthews, 2018; Benedictow, 2004).

In 1845, the fungus potato late blight (*Phytophthora infestans*) infected essential potato crops in Ireland (O'Neill, 2009; Andrivon, 1995). Since Ireland was without a fungicide at that time, potato late blight spread and devastated the potato harvest (Matthews, 2018). This was the catalyst to the Great Famine, or *An Gorta Mór,* meaning The Great Hunger in Irish (O'Neill, 2009). Between the deaths of over 1 million people, and the emigration of another 1 million, a third of Ireland's population was depleted between the years 1845 and 1852 as a direct result of the Great Famine (Matthews 2018; O'Neill, 2009). One hundred and seventy years later, the current population of Ireland is still below that of 1845 (Andrivon, 1995; Matthews, 2018).

Since the development and proper application of pesticides, disease and famine have been reduced (Matthews, 2018; United States Geological Survey, 2017). According to the

Environmental Protection Agency, a pesticide is any substance or mixture that is designed to prevent or destroy a pest (2021). These include, but are not limited to, insects, bacteria, plants, fungi, and rodents (U.S. Environmental Protection Agency, 2021). An example of one of the most successful developments and applications of a pesticide is the synthesis and use of dichloro-diphenyl-trichloro-ethane (DDT) in the fight against malaria.

Malaria, caused by five species of bacteria from the *Plasmodium* genus, is responsible for 627,000 deaths annually, and affects nearly half of the global population (World Health Organization, 2021). The bacteria breed within female *Anopheles* mosquitoes and are spread from one host to another through the mosquitos' need to feed on the blood of mammals (World Health Organization, 2021). Paul Muller, a German chemist who won the Nobel Prize in 1939 for his work with DDT, first recognized the chemical's insecticidal potential (Jarman & Ballschmiter; 2012, Kuswandi, 2017). Until its ban in 1970, DDT's insecticidal properties were used heavily to decrease infected mosquito populations in endemic countries with great success (Brenebaum, 2005). This same success was seen between 1952 and 1955 in Sarawak Borneo where a 1:4 DDT to water ratio was sprayed over an aquatic ecosystem (Smith, 2000). The infected mosquito population dropped from 35.6% to just 1.6% in 21 months (Smith, 2000). It was findings like this that gave the World Health Organization in 1955 the confidence to claim global eradication of malaria within 10-15 years (O'Shaughnessy, 2008; Mayo & Brady, 1955).

Due to this high success, insecticides and other pesticides are used heavily both in the United States and worldwide; evidenced by the estimated 1,000 million lbs. used by the United States and 6,000 million lbs. used globally in 2012 (Figure 1; Atwood & Paisley-Jones, 2017). Of these hundreds of millions of pounds of pesticides, herbicides account for the greatest percentage – about 57% in 2012 (Atwood & Paisley-Jones, 2017). Herbicides are designed to

specifically prevent or destroy unwanted weeds and plants and are used most often in agriculture – 91% of herbicides used in the United States in 2012 were applied to farms (Figure 2; Atwood & Paisley-Jones, 2017).

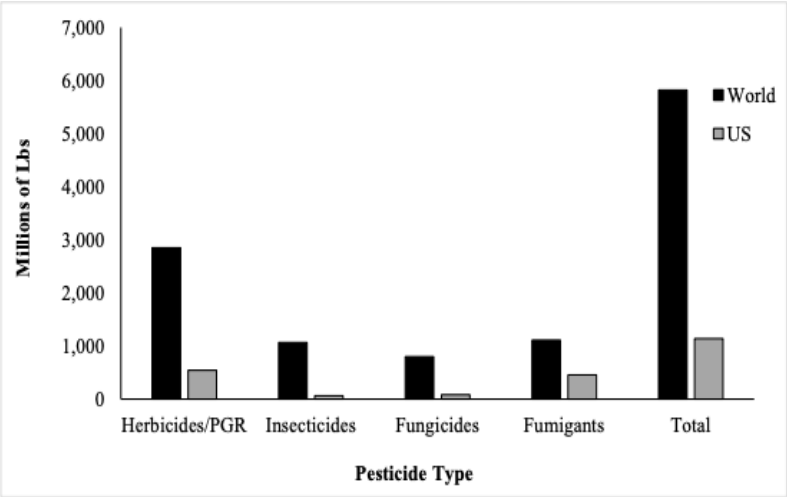

Figure 1. 2012 Comparison of World and United States total annual use of pesticides by type. Image from Atwood and Paisley-Jones, 2017. (https://www.epa.gov/sites/default/files/2017-01/documents/pesticides-industry-sales-usage-2016_0.pdf) Image in the public domain.

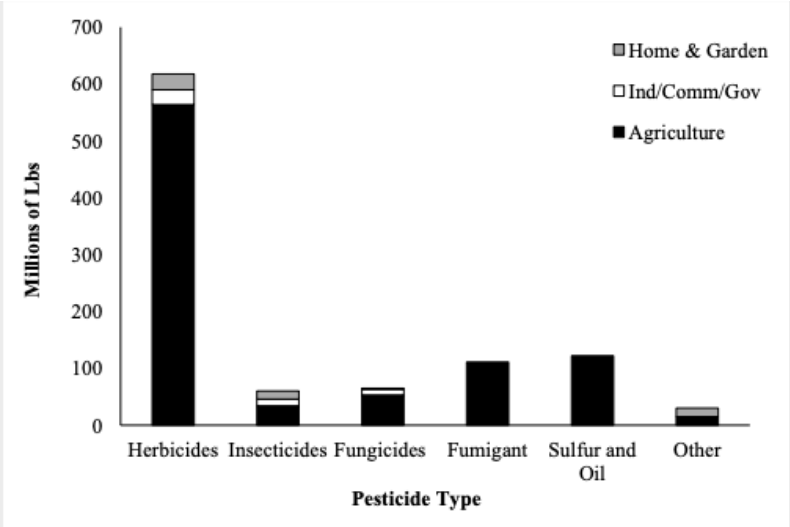

Figure 2. 2012 Allotment of pesticide type to each market sector. Image from Atwood and Paisley-Jones, 2017. (https://digitalcommons.liberty.edu/cgi/viewcontent.cgi?article=2150&context=honors) Image in the public domain.

Of the many types of chemical herbicides, three of the most common are atrazine, glyphosate, and 2,4-D. Within the United States, these three chemicals are used most frequently in the Mid-West but can be found anywhere in the contiguous states (Figure 3; Figure 4; Figure 5; United States Geological Survey, 2019).

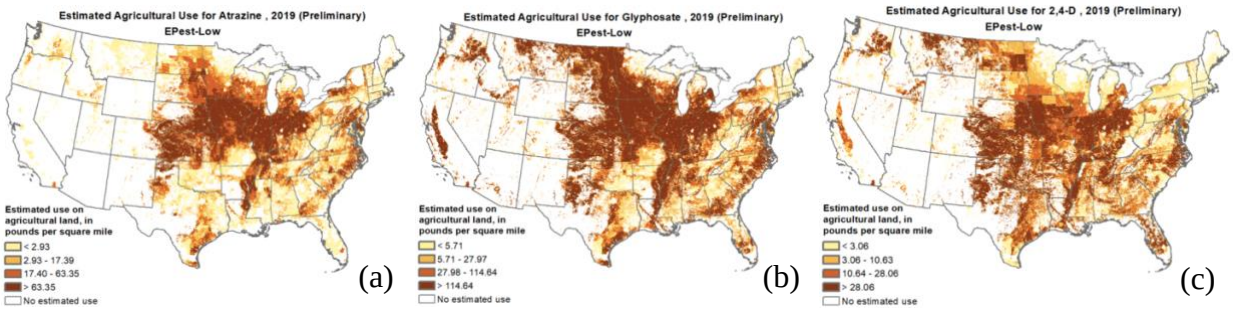

Figure 3. Map showing the estimated frequency distribution of atrazine (a), glyphosate (b) and 2,4-D (c) usage in the USA during 2019. Image from United States Geological Survey (2019). (https://water.usgs.gov/nawqa/pnsp/usage/maps/index.php) Image in the public domain.

Atrazine

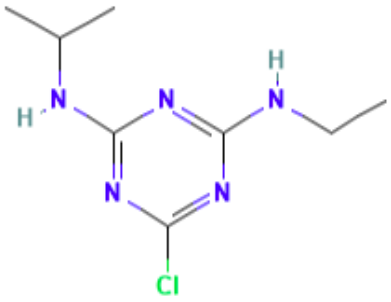

Figure 4. The chemical structure of atrazine. Image from PubChem and the National Center for Biotechnology Information (2022).

(https://pubchem.ncbi.nlm.nih.gov/compound/atrazine) Image in the public domain.

Introduced in the 1950s, atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is one of the most prevalent pesticides worldwide with an annual use of 70-90 million lbs. in the US alone (Figure 4; Graymore, Stagnitti & Allinson, 2001; Steinberg, Lorenz &

Spieser, 1995; Belluck, Benjamin & Dawson, 1991). Atrazine is used to deter the growth of broad-leaf vegetation and annual grasses in crops such as cereals, fruits, sugarcane, and most commonly corn and sorghum (Graymore et al., 2001; DeNoyelles, Kettle & Sinn, 1982). The chemical impacts target plants by actively interfering with their photosynthetic processes, specifically by blocking the electron transport chain within photosystem II (Garymore et al., 2001; DeNoyelles et al., 1982).

Atrazine is most effective on saturated fine soils and is usually applied after a season of heavy rains (Garymore et al., 2001). Because of this, the chemical is predominately used in European countries, Canada, and the United States (Garymore et al., 2001). However, because of atrazine's high affinity for water, high concentrations of the chemical and its metabolites are frequently found in natural water ecosystems from field run off (Garymore et al., 2001; DeNoyelles et al., 1982). This affinity explains why atrazine is the most frequently encountered pesticide pollutant in ground water, surface water, and precipitation (Hayes et al., 2011).

Once in nontarget ecosystems, atrazine will deteriorate in about 1.5 years, and does not bioaccumulate like other common pesticide, such as DDT (DeNoyelles et al., 1982; O'Shaughnessy, 2008). However, the metabolites of atrazine are of at least equal toxicity to atrazine itself, and are suspected human carcinogens (Belluck et al., 1991). Additionally, atrazine is found to be an endocrine disruptor, and can cause demasculinization in amphibians, reptiles, fish, and mammals, and complete feminization in amphibians, reptiles, and fish (Hayes et al., 2010, Hayes et al., 2011). Due to the health hazards associated with the herbicide, as well as its tendency to persist in freshwater ecosystems and soils with a stable pH, the chemical is banned in countries such as Germany and Sweden (Garymore et al., 2001; Steinberg et al., 1995).

Glyphosate

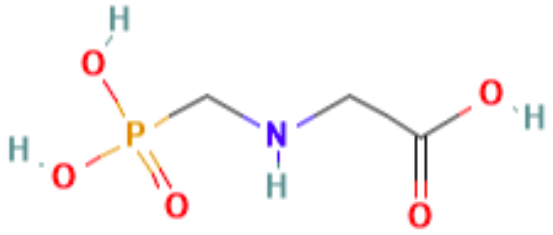

Figure 5. The chemical structure of glyphosate. Image from PubChem and the National Center for Biotechnology Information (2022). (https://pubchem.ncbi.nlm.nih.gov/compound/glyphosate) Image in the public domain.

The non-selective herbicide glyphosate (*N*-(phosphonomethyl)glycine) was first synthesized in 1950 by Henri Martin, a chemist in a Swiss pharmaceutical company (Figure 5; Duke, 2017; Szekacs & Darvas, 2012). However, it wasn't until 1971 that the compound's herbicidal qualities were realized by Monsanto Company based in St. Louis Missouri (Szekacs & Darvas, 2012). The chemical, patented and marketed as Roundup®, quickly became the most successful and most used herbicide in the world (Szekacs & Darvas; 2012, Duke, 2017; Borggaard & Gimsing, 2008). For application, glyphosate is sprayed on fields before planting of general grain crops or after planting of specialized glyphosate-resistant crops (Van Bruggen et al., 2018). Glyphosate is popular not only agricultural use, but also in non-specific crop applications such as along roads, near train tracks, and sprayed on waterways to control for aquatic plants and algae (Duke, 2017; Van Bruggen et al., 2018).

The herbicide functions by targeting the aromatic amino acid biosynthesis in plants (Szekacs & Davas, 2012). Specifically, glyphosate acting as a chelating agent, binds to the cofactor Mn and blocks the shikimate pathway in plant metabolism by inhibiting 5-enolpy-ruvyl-

shikimate-3-phosphate synthase (Huber & Johal, 2009; Duke & Cerderia, 2006). By affecting the plant's metabolism and by possessing phloem-mobile properties, glyphosate can slowly spread through and destroy the plant's meristematic tissues (Duke, 2017; Duke & Cerderia, 2006).

Even though the chemical is inactive in soil, like atrazine, glyphosate is also extremely water soluble due to its highly polar functional groups (Duke, 2017; Skark et al., 1998). Because of these physical characteristics, glyphosate is commonly washed off from its application site by precipitation or irrigation into non-target areas like freshwater ecosystems. Minute concentrations of glyphosate can be found in many sources of freshwater such as ground water, surface water and even drinking water (Skark et al., 1998). Once in the environment, glyphosate is slowly degraded by microorganisms into a metabolite called aminomethylphosphonate (AMPA) (Duke & Cerderia, 2006; Borggaard & Gimsing, 2008; Van Bruggen et al., 2018). The degradation process can be slowed further if glyphosate is absorbed into clay or organic matter. This can lead to an accumulation, and subsequently, a high concentration of glyphosate in the denser soils that can persist for over a year (Van Burggen et al., 2018).

AMPA and glyphosate are co-occurring and are distinguished by a low acute toxicity (Grandcoin, Piel & Baures, 2017; Van Burggen et al., 2018). The single greatest reason for the low acute toxicity of glyphosate is probably the absence a shikimate pathway in human and animal metabolism (Van Burggen et al., 2018). However, scientists are finding that glyphosate and its primary metabolite AMPA have serious negative effects from chronic exposure, even with lower concentrations. There are several human diseases that appear to be linked to chronic exposure to glyphosate including: Alzheimer's, ADHD, autism, reparatory diseases, kidney disease, liver damage, and cancers (Van Bruggen et al., 2018; Mesnage, Defarge, Spiroux de

Vendomois & Seralini, 2015). Similar diseases (cancers, liver disease, and kidney disease) were also found in laboratory rats exposed to an ultra-low concentration of glyphosate in their drinking water for a 2-year period (Mesnage, Renney, Seralini, Ward & Antoniou, 2016). In addition to disease, glyphosate and AMPA have been found to affect female fertility and increase miscarriages (Camacho & Mejia, 2017).

2,4-D

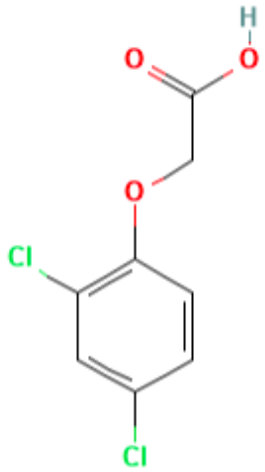

Figure 6. The chemical structure of 2,4-D. Image from PubChem and the National Center for Biotechnology Information (2022). (https://pubchem.ncbi.nlm.nih.gov/compound/2_4-Dichlorophenoxyacetic-acid) Image in the public domain.

2,4-Dichlorophenoyacetic acid (2,4-D) is the oldest synthetic herbicide (Figure 6; Song, 2013). 2,4-D was originally synthesized and applied in minute doses by physiologists investigating growth-regulating hormones in plants (Peterson, 1967). Two American physiologists working with growth-regulating hormones to develop seedless tomatoes, E. J. Kraus and John W. Mitchell, discovered that large doses of 2,4-D would cause physical abnormalities in plant tissues and structure (Peterson, 1967). These abnormalities could even kill the plant (Peterson, 1967). The herbicidal properties of 2,4-D were recognized in the early 1940s

and the chemical's popularity quickly grew to increase crop production in the United States during World War II (Song, 2013; Peterson, 1967). Presently, 2,4-D is used to control weeds in cereal crops, grasses, vegetables, soft fruits, cotton, and aquatic plants (Shaw, Willard & Bernard, 1955; Hammond, 2015).

Since it is a selective herbicide, 2,4-D works by hormone disruption – specifically the hormone auxin (Song, 2013). Like hormone regulation in humans, plant growth, metabolism, response, and physiology are controlled by naturally occurring chemicals called phytohormones (Grossmann, 2009). Phytohormones influence the plant by interacting with plant proteins called receptors (Grossmann, 2009). The phytohormone auxin is invaluable in plant growth and regulation (Grossmann, 2009). Since 2,4-D behaves as an auxin imitator, the herbicide can stimulate plant growth at very low doses (Grossmann, 2009). However, when applied at very high doses, 2,4-D results in an overdrive of plant growth which presents itself in stem twisting, leaf cupping and stunting, and general growth abnormalities (Song, 2013). According to the EPA, 2,4-D specifically kills broad leaf plants by causing uncontrolled cell division in vascular tissues by increasing cell wall plasticity, the biosynthesis of proteins, and the production of ethylene (Song, 2013; Gervais, Luukinen, Buhl & Stone, 2008). Eventually, this rapid growth results in stem curling, leaf desiccation, and plant death (Song, 2013).

As one of the most used pesticides around the world, 2,4-D is also subject to runoff by irrigation or precipitation. Because of the compound's chemical and physical properties such as high solubility, low molecular mass, and negative charge at a low pH, 2,4-D is one of the most mobile pesticides (Boivin et al., 2005). Moving through surface and ground water, 2,4-D is frequently detected in European and North American soils (Boivin et al., 2005). Once in the soil, sorption and degradation of 2,4-D by microorganisms primarily depends on soil composition,

specifically the amount of organic material present (Boivin et al., 2005; Dubus, Barriuso & Calvet, 2001). Sorption is the greatest factor affecting the transmission of pesticides, and 2,4-D has an extremely high sorption rate, which renders it difficult to be broken down into its metabolites, 2,4-dichlorophenol and 2,4-dichloroanisole (Barriuso & Calvet, 2001; Buerge, Pavlova, Bächli & Poiger, 2020; Boivin et al., 2005). 2,4-D is best metabolized by microorganisms in soil with a higher concentration of organic matter rather than at lower depths where less organic material is present (Barriuso & Calvet, 2001). Overall, the degradation of 2,4-D in the environment can take a few weeks to several months (Chinalia, Regali-Seleghin & Correa, 2007).

2,4-D is within the class of phenoxy compounds, which are potentially toxic to humans (Boivin et al., 2005). There have been several studies that suggest 2,4-D can negatively affect mammalian cells by mutations (Gollapudi, Charles, Linscombe, Day & Bus, 1999), neurologically (Bukowska & Hutnik, 2006), and hormonally (Rawlings, Cook & Waldbillig, 1998). While there is suspicion that 2,4-D is linked to an increase in cancers such as prostate cancer and non-Hodgkin's lymphoma, there is not sufficient evidence to fully accept this claim (Goodman, Loftus & Zu, 2015; Garabrant & Philbert, 2008).

Current Pesticide Impact

Pesticides are manufactured to combat a specific target organism or organisms. However, since the success of pesticides is partially due to their potency and strength, these chemicals can also cause significant damage to other non-target organisms, and even entire ecosystems (Hayes et al., 2011). The three common herbicides described above can all be washed into surrounding water ways after application by irrigation or precipitation (Figure 7). Once in the water, the herbicides can take several weeks to over a year to degrade, and in the meantime affect non-

target organisms. Because of this, the Environmental Protection Agency has placed limits on the concentrations of pesticides in water ways from field run-off. The limits of allowed atrazine, glyphosate, and 2,4-D in surface freshwater are 10 parts per billion (ppb), 30 parts per million (ppm), and 70 ppb, respectively (U.S. Environmental Protection Agency, 2020). However, the EPA determines these limits by averaging the results of water samples over 14 days. Realistically, there could be a much higher concentration for a short time during and immediately after the field run-off enters the waterway. This could potentially expose non-target organisms to acute, high concentrations of pesticides before the concentrations are diluted by water flow and degradation.

Figure 7. The runoff pathway of herbicides by precipitation entering freshwater ecosystems and affecting non-target organisms such as crayfish and other more pollution sensitive non-target organisms.

Acute and chronic toxicity effects of atrazine, glyphosate, and 2,4-D have been heavily studied through dietary exposure (Serra et al., 2021; Lim, et al., 2013), dermal exposure (Rafee, Sahid, Noor, Sulaiman & Othman, 2011), and environmental exposures (Chabera et al., 2018) including many studies concentrating on freshwater ecosystems and species (Benli et al., 2007;

Banaee et al., 2019; Benli et al., 2016; Yang, Lim & Song, 2021). However, little research has been allotted to understanding how the acute exposure to these herbicides can affect organisms at the sublethal level in mountain freshwater systems.

Cambarus sp. Crayfish

Crayfish are a valuable research subject because not only are they extremely abundant in central Virginia waterways, but they are also intimately connected to the increasing concentrations of pesticides in streams and rivers. Crayfish can be found under rocks and buried in the sediments on the bottom of streams and rivers. As generalist omnivores, crayfish will eat just about anything they encounter while sifting through the silt, sand, and organic matter that comprises the sediment lining (Cronin et al., 2002). Their diet primarily comprises of small insects, fish, mollusks, plants, and detritus (Cumberlidge, Hobbs & Lodge, 2015).

When pesticide run-off enters the waterways, the chemicals sink to the bottom of the waterway and accumulate in the sediment. As bottom feeders, crayfish are frequently encountering the mixtures of herbicides when downstream from field runoff entry points. The collection site used in this study was purposely selected because it is not downstream from any potential herbicidal pollutants. This resulted in a study population that had little to no baseline exposure to herbicides. Crayfish can also function as biological indicators of pollutants in freshwater systems and, therefore, serve as an important organism by which to assess the total health of the ecosystem (Alikhan, Bagatto & Zia, 1990; Kholodkevich et al., 2008). Using crayfish as a model, the impacts of pesticides on non-target organisms can be measured (Kholodkevich et al., 2008).

The crayfish investigated in this study were all from the *Cambarus* genus (Figure 8). *Cambarus* is part of the family *Cambaridae* – the largest crayfish family in the world with 423

recorded species (Cumberlidge, Hobbs & Lodge, 2015). Genus *Cambarus* is also one of the largest genera and has the greatest diversity of all crayfish genera (Buhay, Moni, Mann & Cradall, 2007). Species of *Cambarus* range from the Gulf of Mexico up into Canada, but most can be found in Appalachian streams and rivers in the Eastern United States (Hobbs & Barr, 1960). Virginia is home to 34 native species of crayfish and a few non-native species (Bzdyk, 2016).

Crayfish sexually reproduce between June and October (Tack, 1941). However, anecdotal evidence has found females in berry as early as late April and early May. Since the mating period is dependent on both hormonal control and largely on external stimuli, mainly water temperature, the general mating season can fluctuate depending on the year (Yazicioglu, Reynolds & Kozák, 2016). When in berry, the female will attach the eggs to her abdomen and swimmerets and carry them with her until they hatch (Tack, 1941). After several weeks, the eggs hatch producing juvenile crayfish that look identical to the adults, just much smaller. The juvenile crayfish are attached to the mother by a telson thread (Longshaw & Stebbing, 2016). For the first two life stages, the young crayfish still rely on the remaining egg yolk for nutrition (Longshaw & Stebbing, 2016). After the juveniles detach, they go through a series of molts and rapid growth until they reach their adult size and sexual maturity (Longshaw & Stebbing, 2016).

Figure 8. Live crayfish from the *Cambarus* genus on ice before dissection.

Oxidative Stress in Crayfish

When crayfish are exposed to toxic stressors, herbicides for example, reactive oxygen species are produced during the breakdown of the toxins (Banaee et al., 2019). Reactive oxygen species (ROS) are toxic to the crayfish and are neutralized on the surface of the hepatopancreas to prevent oxidative damage (Banaee et al., 2019). An important enzyme that is activated in the neutralization pathway of reactive oxygen species is Glutathione (GSH) (Banaee et al., 2019). When GSH reacts with the ROS, GSH is converted into glutathione disulfide (GSSH). GSSH is removed from the cell and reformed through a series of reactions by the enzyme γ-glutamyl-transferase (GGT) (Figure 9). The components of GSSH recombine to form new GSH which is pumped back into the cell to neutralize more ROS resulting from oxidative stress (Banaee et al., 2019).

GGT is an apical membrane protein, meaning it can be found on the surface of liver cells, and its function is to cleave peptide bonds to break down extracellular glutathione into amino acids for cell use (Whitfield, 2008). When there is a large increase in ROS accumulation due to increased toxicity, GSH responses are overwhelmed and the overall levels decrease within hepatopancreases cells (Banaee et al, 2019). This leads to compensation by an increase in GGT

levels (Koening & Seneff, 2015). A previous study (Banaee et al., 2019) observed an increase in GGT levels when crayfish were exposed to glyphosate (2019). These cellular responses to toxicity can lead to compounding harm, apoptosis, and injury to critical organs which can result in potential organ failure and death (Hanigan, 2014).

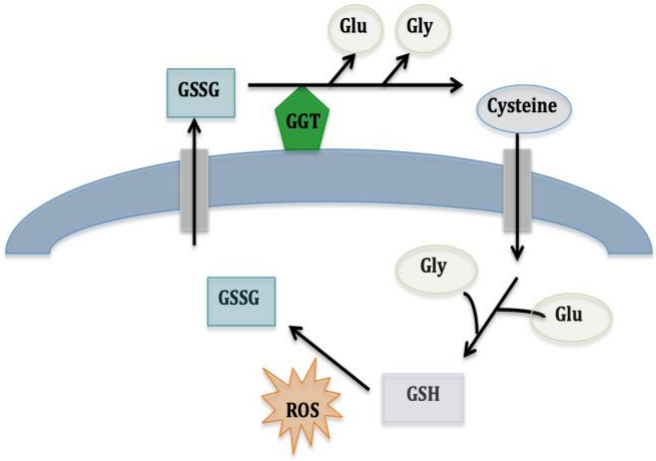

Figure 9. The neutralization pathway for reactive oxygen species. Note the presence and role of GGT in the neutralization pathway.

In addition to abnormal GGT levels expressed on the cells, apoptosis (programmed cell death) is also an indicator of toxin stress at the cellular level. There are two kinds of apoptosis: intrinsic apoptosis (mitochondria mediated pathway) and extrinsic cell death (death receptor pathway) (Reed, 2000). Extrinsic apoptosis is initiated by changes in the cell's surrounding environment, such as cytotoxins, that trigger the binding of a death ligand to a death receptor on the cell's surface (Creative Diagnostics, n.d.). The death domain (DD) then carries the initiation signal from the cell's surface to intercellular signal pathways (Creative Diagnostics, n.d.; Elumalai et al., 2012). Once the intercellular pathways have been signaled, the cell undergoes apoptosis in several steps. First, the cell shrinks and the DNA within the mitochondria condenses and fragments (Reed, 2000). Next, the cytoskeleton collapses, and the cell undergoes

plasma membrane blebbing (Reed, 2000). Finally, the cell breaks down into apoptotic bodies which are cleared away by phagocytosis (Reed, 2000).

Since extrinsic apoptosis can be triggered by environmental toxins in crustaceans, Annexin V and Propidium Iodide (PI) were used to quantify cells involved in apoptosis (Menze, 2010). To signal the onset of apoptosis, the phospholipid phosphatidylserine flips from the inner leaflet to the outer leaflet of the cell membrane. FITC Annexin V can detect early stages of apoptosis by binding directly to phosphatidylserine (Affymetrix, 2016). A later signal for apoptosis occurs when the cytoskeleton weakens and begins to bleb. The weakened membrane allows fluorochrome PI to infiltrate the nucleus of the cell, bind to, and label the fragmented DNA (Riccardi & Nicoletti, 2006). By using Annexin V and PI in conjunction, a broader range of cells undergoing apoptosis can be detected and a more definitive picture of how oxidative stress affects hepatopancreas cells can be obtained.

Objectives

Previous studies, including some conducted by undergraduate students at Liberty University, have found that three of the most common herbicides used in central Virginia, atrazine, glyphosate, and 2,4-D, have negative growth pattern effects on crayfish (Chandler et al., 2017; Benli et al., 2016). Additionally, Benli et al. observed organ damage effects from oxidative distress when crayfish were chronically exposed to environmentally relevant concentrations of 2,4-D (2016).

The objective of this research was to broaden the understanding of how herbicides can potentially affect non-target organisms in freshwater ecosystems through acute exposure of crayfish to environmentally relevant concentrations of herbicides atrazine, glyphosate, and 2,4-D. Three possible responses to the introduced herbicides and the increased herbicide

concentrations were: the increase of GGT and apoptosis levels (Figure 10), the decrease of GGT and apoptosis (Figure 11), or the crayfish could have shown metabolic resistance to the chemical change and displayed GGT and apoptosis levels that were equal to those of the control (Figure 12).

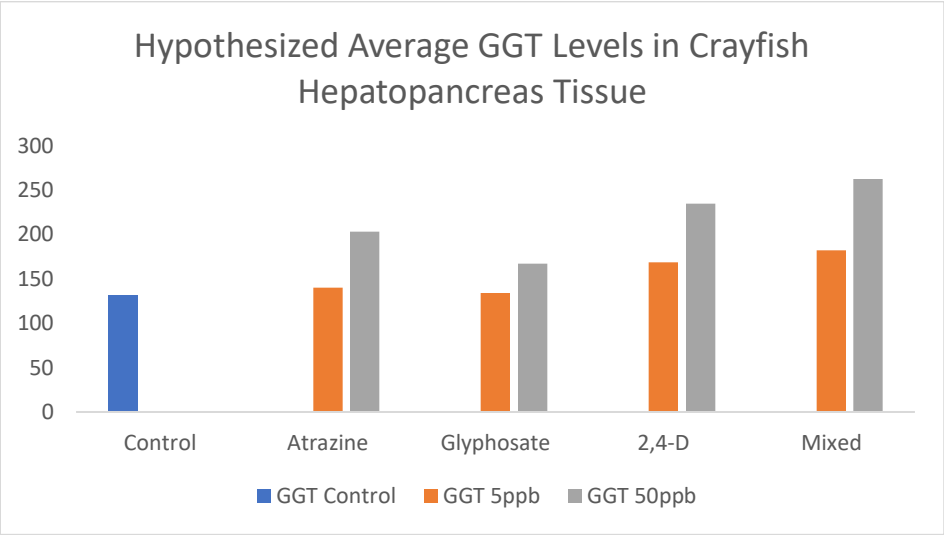

Figure 10. Potential metabolic response to treatment and increased concentration of herbicides. This example graph represents the expected increase response by GGT in the crayfish when exposed to the herbicides.

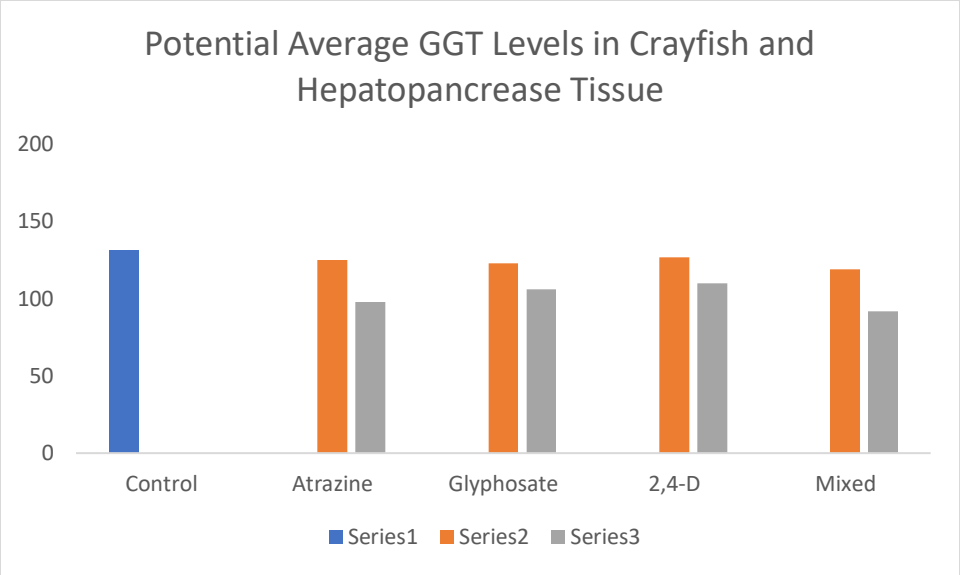

Figure 11. Potential metabolic response to treatment and increased concentration of herbicides as seen by a decrease in GGT response.

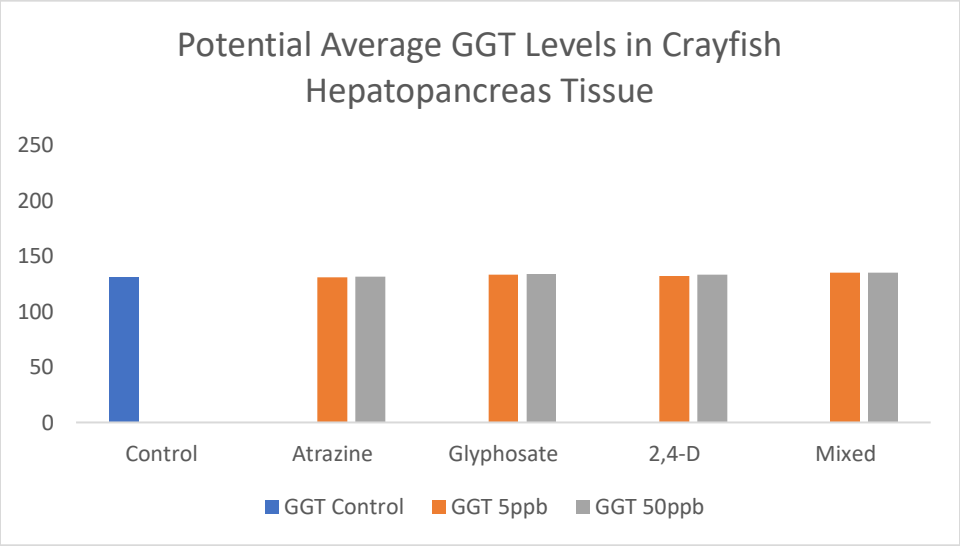

Figure 12. Potential metabolic response to treatment and increased concentration of herbicides. This example graph of potential results represents a metabolic resistance of the crayfish to low levels of herbicides.

A sublethal stress response, evidenced by increased detoxification needs and therefore increased GGT compensation coupled with increased apoptosis, was expected (Figure 10).

Methods

Crayfish Collection and Maintenance

The study subjects were collected through a kick-sein technique (Williams, Brewer, & Ellersieck 2014) from a local freshwater stream (approximately 37.337404, -79.151011) in Campbell County Virginia between July and September 2021. The stream was selected because it is rural and well removed from any source of anthropogenic pesticides or other pollutants. The kick seine method was selected because it is easy to use, and one of the fastest techniques when collecting a large sample of crayfish (Williams et al., 2014). While in the field, the collected crayfish were individually stored in Whirl-Pak® collection bags until returned to the lab. Once in the lab, the crayfish were kept in individual 15L aquaria and fed one shrimp pellet, daily, until treatment. Each tank was glass bottomed and filled with 15L of cycled water that had been prepared weeks in advance. Other than a sponge aeration system that was run constantly, the tanks were devoid of rocks, algae, and other objects.

Treatment

Crayfish were individually placed in aquaria and allowed to acclimate for 24 hours prior to treatment. An environmentally relevant concentration, 5 parts per billion (ppb) or 50 ppb, of atrazine, glyphosate, and 2,4-D were individually added to treatment tanks. To prepare the atrazine and 2,4-D treatments, the herbicides were dissolved in acetone. The glyphosate treatment was prepared by adding the herbicide in deionized water. A combination treatment was prepared by combining 5 ppb of the atrazine, glyphosate, and 2,4-D treatment concentrations for a total concentration of 15 ppb pesticide (or 150 ppb when concentrations of 50 ppb were tested). The control was prepared by filling a control tank with cycled water free of any herbicide contamination.

For treatment, five collected crayfish of estimated similar sizes were selected and placed in separate treatment aquaria regardless of size or sex. The treatment crayfish were subjected to the atrazine, glyphosate, 2,4-D, and combination treatments for 48 hours while the control was left in the unaltered freshwater for the same period of time under the same conditions. After 48 hours, all crayfish were taken out of the tanks with small aquarium nets and transferred, on ice, for dissection.

Dissection

Prior to dissection, the blotted wet mass, total length, carapace length, and sex of the crayfish were determined and recorded. All crayfish were sacrificed by severing the ventral nerve cord, dissected, and the hepatopancreases were removed. This specific organ was selected because, as stated above, the hepatopancreas is the site of reactive oxygen species neutralization and, subsequently, GGT production (Banaee et al. 2019). Once harvested, the hepatopancreas tissues were weighed and recorded. A portion of each hepatopancreas was set aside in cryostat media for future analyzation of apoptosis and organ degradation with DAPI and an EVOSM5000 microscope.

Cell Preparation

The remaining hepatopancreas tissue from each crayfish was prepared for analysis of GGT levels and apoptosis by flow cytometry. As preparation, the cells were suspended in 10x volume ice-cold PBS based on the used mass of each tissue. The tissues were homogenized in a cold homogenizer and the suspended cells were then transferred to Eppendorf tubes. These mixtures were centrifuged for 3 minutes at 300 times gravity (300 g) and the supernatants were removed and discarded. Two samples were prepared from each crayfish so that separate analyses of GGT production and apoptosis were completed.

Preparation for GGT Analysis

For GGT quantification, the primary antibody was diluted to a 10μg/mL working solution with 2.6μL of antibody and 197.4μL of ice-cold PBS. One sample from each crayfish were stained with 1 mL of the 10 mg/mL dilution per gram of the used weight. A volume of 9x the used mass of PBS was used to increase the sample volume to the initial amount. The stained cells were incubated for 30 minutes at 4°C in the dark. After incubation, the cells were washed 3 times by centrifugation at 400 g for 5 minutes and resuspended in ice-cold PBS, to bring the samples back to initial volume, between each successive washing. To dilute the second antibody, a 1:2000 dilution was applied, and the cells were stained with 1 mL of the second antibody. The samples were again returned to incubate in the refrigerator for 30 minutes in the dark at 4°C. Following the second incubation, the cells were washed one time by centrifuge for 5 minutes at 400 g and then resuspended in 1 mL PBS. The suspended cells were kept in the dark on ice until analysis.

Preparation for Apoptosis Analysis

Concurrent with GGT analysis preparation, the other crayfish hepatopancreas samples were prepared for apoptosis analysis according to modified protocols by Molecular Probes® (2011, 2006). The annexin binding buffer was diluted to 1x by 100mL of the buffer and 400mL of PBS for each sample. A 100mg/mL working solution of PI was also prepared by diluting 5mL of PI stock with 45mL of the 1x buffer. After washing the samples 3 times at 300g, the cells were resuspended in 100μL of the 1x buffer. After resuspension, 5μL of Annexin V and 1μL of the 100mg/mL PI working solution were added to each sample. The cells were then set to incubate in the dark at 4°C for 15 minutes. After incubation, 400μL of the 1x buffer were added to each sample and the samples were immediately analyzed with the flow cytometer.

Flow Cytometry

To measure potential sublethal effects on hepatopancreas cells through GGT quantification, the antibody tags were visualized with fluorochrome dye FITC at a wavelength of 495-519nm in green visible light. To investigate potential apoptosis effects of pesticide toxins, annexin V-stained cells were quantified by FITC at 495-519nm wavelengths. Finally, to visualize and quantify the propidium iodide-stained cells, a common phycoerythrin, PE, was applied at 566-574nm in the red wavelengths.

Analysis

During flow cytometric analysis, gating was done freehand based on the areas of the scatter plot with the highest concentrations of fluorescing cells. From the gated fluorescence, the average fluorescence of each sample was compared to the corresponding body conditions (total length/crayfish blotted wet mass/hepatopancreas blotted wet mass) of the crayfish. The samples were separated by treatment group. The mean average fluorescence/body conditions of each treatment sample and its standard error (SE) were then compared to the corresponding control through two sample *t*-tests (assuming equal variances) and one-way ANOVAs on Excel.

Results

Fluorescence level outputs for GGT and apoptosis were collected and recorded as histograms (Figure 14, Figure 15, and Figure 16). Higher fluorescence is characteristic of higher levels of GGT on the cells' surface (Hammond, 1990). Similarly, for Annexin V and PI, outputs showing higher levels of fluorescence are indicative of higher amounts of apoptosis within the cell samples (Henry, Hollville, & Martin 2013).

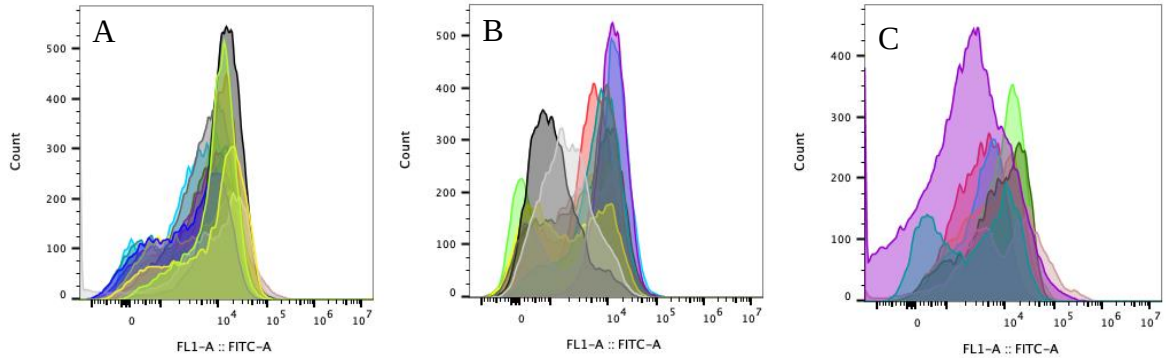

Figure 14. Histograms showing levels of GGT for control (A) and mixed treatments at 5 ppb (B) and 50 ppb (C). The horizontal axis shows the amount of fluorescence expressed while the vertical axis represents the number of cells displaying the corresponding fluorescence.

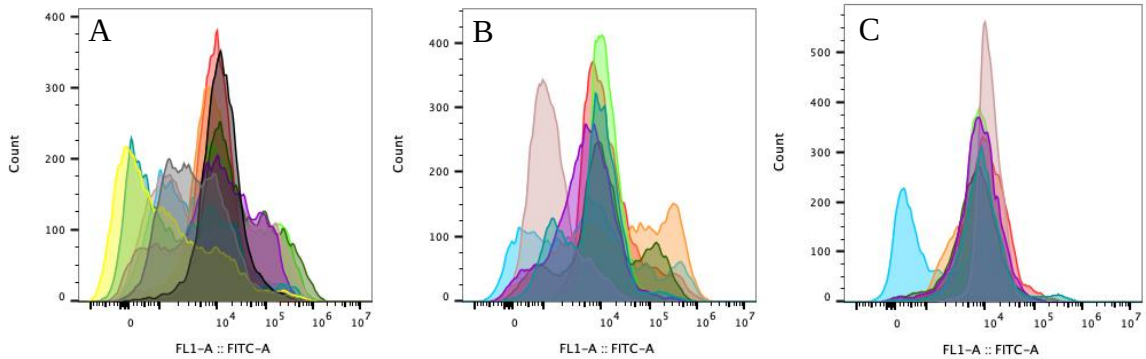

Figure 15. Histograms showing quantification of apoptosis by Annexin V at a control (A) and mixed treatments of 5 ppb (B) and 50 ppb (C). The horizontal axis shows the amount of fluorescence expressed while the vertical axis represents the number of cells displaying the corresponding fluorescence.

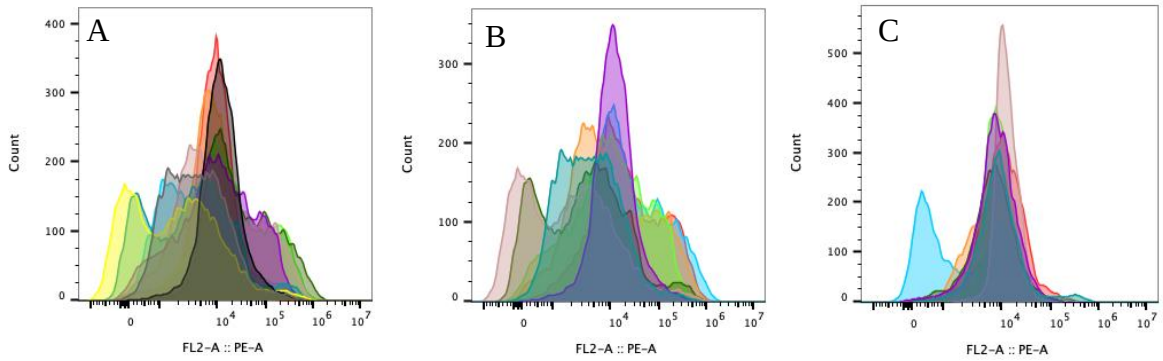

Figure 16. Histograms showing quantification of apoptosis by Propidium Iodide (PI) at a control (A) and mixed treatments of 5 ppb (B) and 50 ppb (C). The horizontal axis shows the amount of fluorescence expressed while the vertical axis represents the number of cells displaying the corresponding fluorescence.

No significant differences were found for any of the 5 ppb or 50 ppb treatment group mean average fluorescence/body conditions compared to the control mean average fluorescence/body conditions for the GGT analysis (Table 1). However, as seen on the bar chart, there was a decrease from the control GGT levels to those of the 5 ppb treatment groups (Figure 17). This decrease was more pronounced when the herbicide concentration was increased to 50 ppb as evidenced by the shift towards lower p-values when comparing the control to each 50 ppb treatment group.

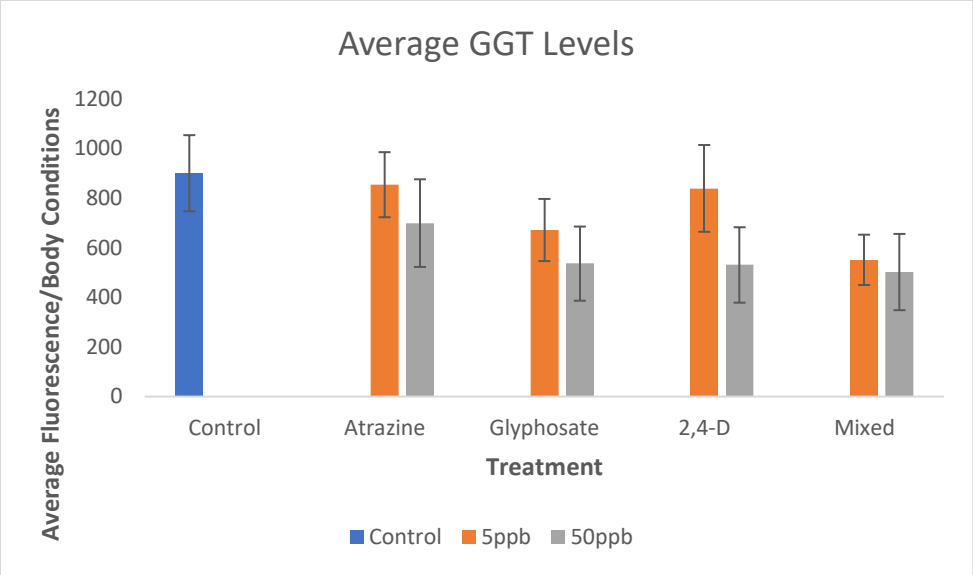

Figure 17. Comparison of mean average fluorescence/body conditions for GGT between the control and each treatment group at both 5 ppb and 50 ppb herbicide concentrations. The error bars represent SE.

Table 1.

Results of one-way ANOVAs and two-sample t-tests (assuming equal variance) between the average GGT levels of treatment groups and controls at 5 ppb and 50 ppb concentrations.

Test	Treatment	Concentration	T or F stat	P Value	d.f.
ANOVA	Control vs treatments	5ppb	0.9847647	0.42239401	4,63
t-test	ATZ	5ppb	0.20792824	0.41834507	30
t-test	GLY	5ppb	1.03565834	0.15431902	30
t-test	2,4-D	5ppb	0.25537906	0.40008657	30
t-test	Mix	5ppb (15ppb)	1.63295248	0.05646622	30
ANOVA	Control vs treatments	50ppb	1.2397254	0.30737502	4,46
t-test	ATZ	50ppb	0.7507637	0.22976809	26
t-test	GLY	50ppb	1.39215933	0.08783427	26
t-test	2,4-D	50ppb	1.33812182	0.0964507	25
t-test	Mix	50ppb (150ppb)	1.51643169	0.07073776	26

No significant differences were found for any of the 5 ppb or the 50 ppb Annexin V and PI treatment groups mean average fluoresce/body conditions compared to the Annexin V and PI control mean average fluorescence/body conditions (Table 2 and Table 3). Unlike the GGT levels which decreased for all treatment groups at all concentrations, the 5 ppb treatments of atrazine, 2,4-D, and mixed had a slight, albeit not significant, increase in apoptosis levels, measured by both Annexin V and PI, over the control. However, when the treatment concentrations were increased to 50 ppb, a decrease in apoptosis and a trend towards a significant difference relative to the control was observed for all herbicides (Figure 18).

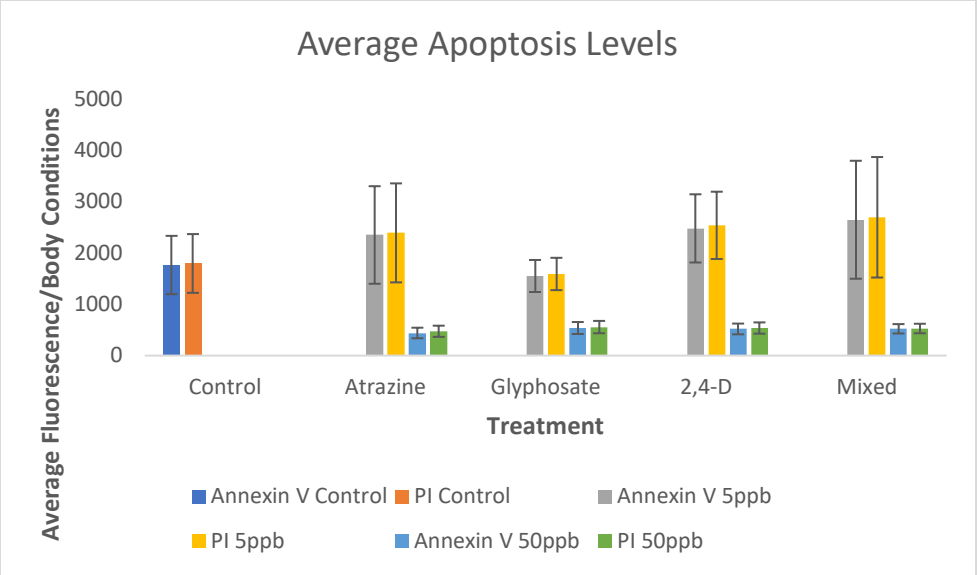

Figure 18. Comparison of mean average fluorescence/body conditions for Annexin V and PI between the control and each treatment group at both 5 ppb and 50 ppb herbicide concentrations. The error bars represent SE.

Table 2.

Results of one-way ANOVAs and two-sample t-tests (assuming equal variance) between the average apoptosis levels, measured by Annexin V, of treatment groups and controls at 5 ppb and 50 ppb concentrations.

Test	Treatment	Concentration	T or F stat	P Value	d.f.
ANOVA	Control vs. treatment	5ppb	0.38152665	0.82059736	4,43
t-test	ATZ	5ppb	-0.5600327	0.29055774	8
t-test	GLY	5ppb	0.25413216	0.40087682	8
t-test	2,4-D	5ppb	-0.7645791	0.22632296	8
t-test	Mix	5ppb (15ppb)	-0.7762632	0.22292976	8
ANOVA	Control vs. treatment	50ppb	2.214385	0.08373749	4,42
t-test	ATZ	50ppb	1.6241322	0.05929507	8
t-test	GLY	50ppb	1.50362356	0.07344958	8
t-test	2,4-D	50ppb	1.42728057	0.08410023	7
t-test	Mix	50ppb (150ppb)	1.52483626	0.07077391	8

Table 3.

Results of one-way ANOVAs and two-sample t-tests (assuming equal variance) between the average apoptosis levels, measured by PI, of treatment groups and controls at 5 ppb and 50 ppb concentrations.

Test	Treatment	Concentration	T or F stat	P Value	d.f.
ANOVA	Control vs. treatment	5ppb	0.38690578	0.81680695	4,43
t-test	ATZ	5ppb	-0.5643638	0.28910787	8
t-test	GLY	5ppb	0.24183402	0.40557437	8
t-test	2,4-D	5ppb	-0.7937669	0.21790483	8
t-test	Mix	5ppb (15ppb)	-0.7804388	0.22172467	8
ANOVA	Control vs. treatment	50ppb	2.21450093	0.0837243	4,42
t-test	ATZ	50ppb	1.60711954	0.06114342	8
t-test	GLY	50ppb	1.50652032	0.07307939	8
t-test	2,4-D	50ppb	1.42977999	0.08374498	7
t-test	Mix	50ppb (150ppb)	1.54372964	0.06845869	8

Discussion

The objectives of this study were to determine if and how crayfish are affected by acute exposure to low doses of common herbicides atrazine, glyphosate, and 2,4-D. It was expected that the addition and increase of herbicide concentrations would result in an increase in GGT levels paired with an increase in apoptosis due to unsustainable oxidative stress (Koening & Seneff, 2015; Benli et al., 2016). Contrary to expectations, both GGT and apoptosis levels predominately decreased in treatment groups compared to control groups.

While a decrease in GGT in response to pesticide pollution is not unheard of, a study conducted by Hatami, Banaee, and Haghi found that hemolymph GGT levels were depressed in common carp exposed to insecticides, it would be assumed that apoptosis levels would increase as a response to the interruption in the neutralization pathway and subsequent oxidative damage (2019; Hanigan, 2014; Awali et al., 2019). However, at 50 ppb, apoptosis levels were decreased for all treatment groups compared to the control groups. Even though the decrease was not significant, the p-values were strongly shifted towards significance when the herbicide concentrations were increased from 5ppb to 50 ppb (Figure 19 & Figure 20).

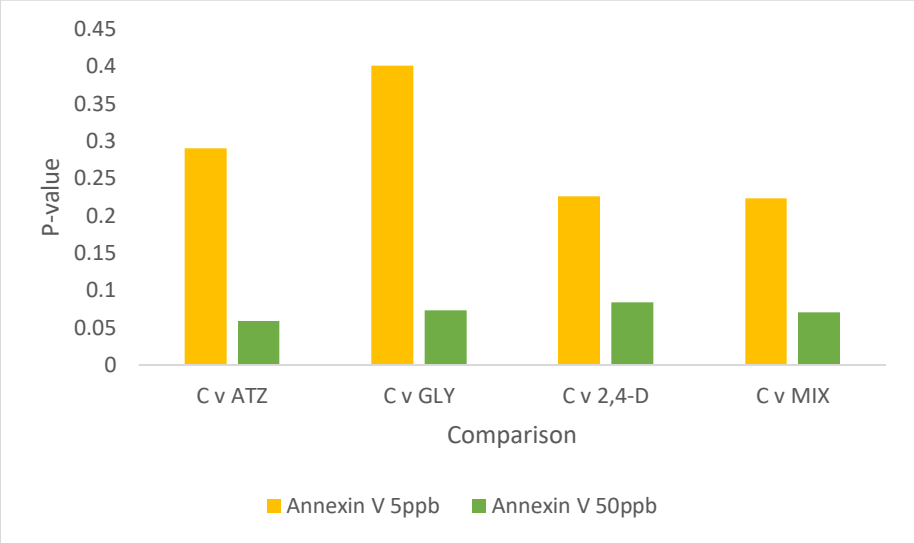

Figure 19. Comparison of P values from apoptosis (Annexin V) analyses between the control and each treatment group at 5 ppb and 50 ppb herbicide concentrations.

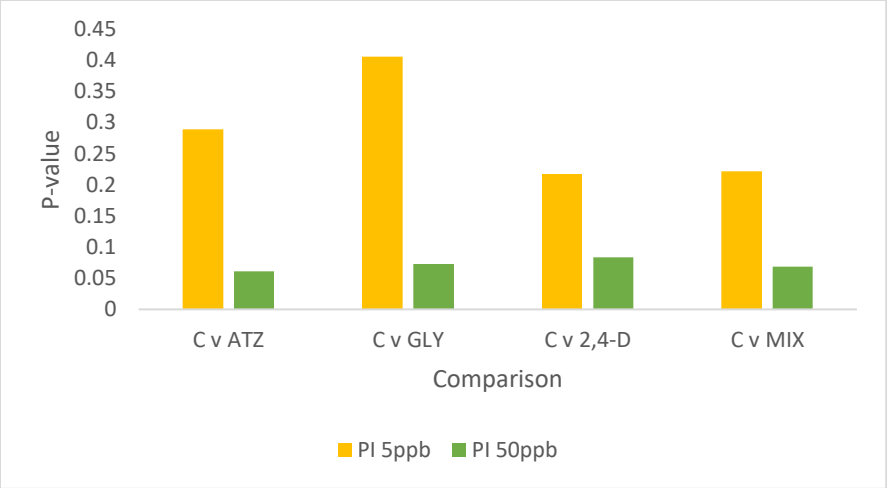

Figure 20. Comparison of P values from apoptosis (PI) analyses between the control and each treatment group at 5 ppb and 50 ppb herbicide concentrations.

A possible explanation for a coupled decrease in GGT and apoptosis with increased herbicide concentrations is the ROS reacting directly with the herbicides and being neutralized while by-passing the glutathione pathway partially or completely (Balci et al., 2009). Direct neutralization by the herbicides would require less GSH to be recycled and therefore less GGT activation and involvement. Overall, there would not be an increase in oxidative damage, even though GGT levels are decreasing. There is little research on the potential of this reaction, however a study conducted by Balci et al. found that hydroxyl radicals (a ROS) would react with and degrade atrazine in an aqueous medium (2009). Future work should be centered on uncovering the possibility of these reactions within the hepatopancreas of the crayfish.

Another possible explanation for a decrease in GGT levels and a decrease in apoptosis could be related to the activation and function of cytochrome P450 (CYP450) in the hepatopancreas. In the first phase of herbicide oxidation, the CYP450 enzyme is activated to neutralize the compounds (Awali et al., 2019). The products of this reaction then act as substrates for the second phase of detoxification – the glutathione pathway (Awali et al., 2019,

Belanger et al., 2022). By GSH and GGT, the glutathione pathway converts the herbicidal substrates to polar molecules which are excreted from the body (Awali, 2019; Brzezicki et al., 2003). Since CYP450 itself is transcriptionally activated by increased expression of the *CYP1A1* gene, the herbicides may be affecting the first phase of the detoxification pathway through hepatopancreas DNA and *CYP1A1* gene expression. This interruption could hinder the breakdown of the herbicides into substrates for the second phase of detoxification by the glutathione pathway (Belanger et al., 2022).

This effect was not directly measurable by our methods. However, if the reactions by the CYP450 enzyme were disrupted, the activity of the glutathione pathway could be decreased (Awali, 2019). The lack of substrate for the glutathione pathway may account for the drop in GGT levels at higher concentrations of herbicides from the control because with less substrate entering the glutathione pathway, less GGT is required for neutralization.

Numerous human and mammalian cell studies have consistently found that ROS have a major impact on cell proliferation and the cell cycle (Nicco et al., 2005; Verbon, Post & Boonstra, 2012; Burdon, 1995; Suh et al., 1999). In normal cells, consistent low concentrations of ROS or ephemeral high concentrations of ROS can stimulate cell proliferation (Nicco et al., 2005; Burdon, 1995). This means that when mammalian cells are exposed to a brief increase in ROS – cyclins, cyclin dependent kinases (CDK), and cell growth factors are activated. Activation of these compounds turns on cell cycle and presents an exponential increase in cell number accompanied by rapid tissue growth (Conlon & Raff, 1999).

While no studies showing how ROS specifically affect the cell cycles of hepatopancreas tissues of crustaceans are known to the writer, studies have observed the presence and roles of cyclins in crustaceans for cell growth and proliferation (Wang et al., 2013; Qui & Yamano, 2005;

Qui & Liu, 2009). Cyclin B, a recognized regulator in meiosis and mitosis of eukaryotes, and its subunit Cdc2 kinase have been identified in crustaceans (Qui & Liu, 2009). Cyclin B and Cdc2 kinase together comprise the M-phase-promoting factor (MPF) which has been heavily studied in oocyte maturation and was found to activate cell progression through proliferation (Qui & Liu, 2009; Qui & Yamano, 2005; Shui et al., 2016).

Since the same cell cycle activating proteins are present across eukaryotes, it can be reasonably suggested that the presence of ROS would have a similar effect on the cell cycle of crustaceans as it does on mammals. Like in mammalian cells, if the consistent presence of low concentrations of ROS or transient high concentrations of ROS result in cyclin activation and cell proliferation, the apparent decrease in apoptosis from the control groups to the 50 ppb treatment groups may be explained (Nicco et al., 2005; Burdon, 1995). Apoptosis levels may not actually be changing from the control to the 50 ppb treatment groups. Instead, the ratio of living cells to those undergoing the apoptosis pathway may be increasing because of the rapid cell proliferation induced by the brief spike in cellular ROS. Essentially, the increase in proliferation could dilute a constant quantity of apoptotic cells, from control to treatment, which would decrease the likelihood of encountering an apoptotic cell in cell counts such as the one mentioned here (Burdon, 1995). This could cause the results to appear as though apoptosis levels decreased from the control to the 50 ppb treatments, when the levels may have stayed relatively consistent between the two groups.

Future work should focus on better understanding how these and other herbicides affect the hepatopancreas at the genetic and metabolic levels. This may more directly reveal where and how the detoxification pathways of the crayfish are being affected by herbicides and other chemical pollutants and deepen the understanding of how these compounds affect non target

organisms. Developing a more accurate and complete knowledge of these effects will be invaluable in determining the best policies for these chemicals based on their impacts.

Even though the mechanisms are not fully understood, the results of this study suggest that the herbicides do have an impact on the hepatopancreas cells and tissue of the crayfish. This impact, however, does not seem to be negatively affecting the organisms because of the decrease in apoptosis in the treatment groups compared to the control. The crayfish were able to effectively neutralize the ROS formed by the herbicides and mitigate any oxidative damage. This supports the long-standing knowledge that crayfish, and other crustaceans, are tolerant of and adaptable to pollutants and environmental stressors (Marina, Tabche & García, 1997; van der Velde, Rajagopal & Kelleher, 1998).

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