Spring 2019

Effects of Herbicides on Zebrafish Embryo Development and Viability

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EFFECTS OF HERBICIDES ON ZEBRAFISH EMBRYO DEVELOPMENT AND VIABILITY

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Kayla Ray King

May 2019

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Biology

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ABSTRACT

Environmental contaminants are chemicals of anthropogenic origin that are found in water, soil, and air, and are harmful to a wide variety of organisms (ORD US EPA, 2018-a). One common group of contaminants are herbicides. Though herbicides are used to control unwanted vegetation in agriculture, aquatic organisms and humans may be exposed to these herbicides through run off into streams and rivers, by drinking contaminated water, by consuming treated crops, by direct exposure, or through bioaccumulation. Thus the effect of these herbicides on animals needs further investigation. In this study, I sought to determine whether six different herbicides, which have had minimal testing in animal studies, have teratogenic effects. Thus, I exposed zebrafish embryos (from the blastoderm stage to 4 days post fertilization) to the herbicides, and assessed the effects of each herbicide on embryonic development. My results indicate that all herbicides tested, with the exception of nicosulfuron, led to some form of toxicity, cardiac dysfunction, or other developmental error. For example, exposure of zebrafish embryos to high concentrations of glufosinate-ammonium or thifensulforon-methyl resulted in some embryos exhibiting cardiac dysfunction. However, due to variation in the results at different concentrations, the LD50 (lethal dose 50%) of these herbicides could not be identified. Quizalofop-p-ethyl exposed embryos displayed cardiac dysfunction at the LD50. However, at concentrations slightly higher than the LD50, embryos exhibited a general toxicity that led to 100% mortality. Mecoprop treatment led to variability in mortality at different concentrations. However, within the suspected LD50, mecoprop treated embryos exhibited cardiac dysfunction, as well as a host of other abnormalities: shortened body axis, micropthalmia, curved spine, tail malformation, lack of motility, and abdominal edema. At a range of concentrations, hexazinone treatment resulted in cardiac dysfunction as well as defective pigment cell alignment, shortened body axis, micropthalmia, tail malformation, lack of motility, and abdominal edema. The results of this study provide evidence that one herbicide, nicosulfuron, appears to be safe for zebrafish embryonic development and survival. However, many of these herbicides have teratogenic effects that need to be explored further.

KEYWORDS: zebrafish, environmental toxicology, herbicides, heart inhibitors, morphological deformities, development

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ACKNOWLEDGEMENTS

I would like to thank the following people for their support during the course of my graduate studies. Dr. Udan for being my mentor and committee chair. Dr. Durham and Dr. Barnhart for being a part of my thesis committee and mentors during my graduate career. Jennifer Kaltz, undergraduate assistant, for helping with fish care. Additionally, I would like to thank Bruce Green, Tanner Hoog, and Blake Justis for keeping me sane during this stressful journey. I would also like to thank my family for listening to my griping for two years. Furthermore, I would like to thank Missouri State University for providing funding for this research. I would like to thank Christian Lawrence from Boston Children's Hospital for the donation of zebrafish to our lab, as well as Erik Sanders for guidance on what to do if we ran into troubles with zebrafish.

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INTRODUCTION

Environmental Contaminants

Due to a wide variety of anthropogenic influences, many lakes, rivers and streams throughout the world are polluted with chemicals that are harmful to humans and aquatic organisms. These environmental contaminants are found in water, soil, and air (Thompson & Darwish, 2019). Sources of environmental contaminants include: pharmaceutical use and improper disposal, agricultural runoff and waste treatments, industry runoff, inadequate septic tanks, and different forms of extraction such as mining (Thompson & Darwish, 2019). One common class of environmental contaminants are herbicides. Herbicides are used in agriculture to control unwanted plant flora (ORD US EPA, 2018-b). Although herbicides are good for agriculture, they pose a serious health risk for both humans and aquatic organisms.

Previously Tested Herbicides

One notable herbicide is atrazine, which is used to control broadleaf and grassy weeds in many different crops (Rosenfeld & Feng, 2011). Atrazine is one of the most commonly applied herbicides in the world; thus, it is particularly important since it is commonly found in bodies of water, including drinking water (Hayes et al., 2010). The mode of action of atrazine is by inhibiting photosynthesis through binding to D1 protein, which inhibits plastoquinine. Blocking plastoquinine interrupts photosynthetic electron transfer leading to disruption of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) synthesis (Cheremisinoff & Rosenfeld, 2011). In addition to its role in disrupting plant growth, atrazine is a well-known endocrine disruptor. For example, exposure of zebrafish embryos to

atrazine resulted in abnormal embryonic progesterone levels, alterations in gene expression associated with endocrine system development and function, followed by the fish developing into mature adults that exhibited reproductive dysfunction (Wirbisky et al., 2016). Careful analysis of atrazine-exposed zebrafish embryos also revealed numerous morphological deformities (circulatory system dysfunction, locomotion impairment, heart function abnormalities, generalized edema, and decreased eye and somite development) (Wiegand, Krause, Steinberg, & Pflugmacher, 2001). In another study, atrazine-exposed zebrafish embryos exhibited defects in sensory and motor neuron innervation, as well abnormal differentiation and alignment of muscle fibers (H. Wang et al., 2015). The ability for atrazine to function as an endocrine disruptor is not specific to embryos, as exposure of adult zebrafish to atrazine led to decreased follicle clearance needed for proper production of mature oocytes (D'Angelo & Freeman, 2017). Atrazine has been tested on another common model organism: the African clawed frog (*Xenopus laevis*). Male African clawed frog embryos exposed to atrazine until maturity were feminized (Hayes et al., 2010). The results from these laboratory studies suggest that atrazine does indeed act as an endocrine disruptor. However, future studies will need to be performed to determine if exposure to environmental sources of atrazine will have similar teratogenic effects in other types of organisms in aquatic ecosystems.

The most commonly used herbicide is glyphosate, which is marketed under the tradename Roundup (Krieger, Doull, & Vega, 2010). Glyphosate acts as a direct inhibitor of the enzyme enolpyruvylshikimate-3-phosphate synthase (EPSPS), which has the normal function of converting simple carbohydrates to important plant metabolites (Sparks, 2019). Glyphosate is used to control growth of broadleaf plants and grasses (Krieger et al., 2010). One concern of the herbicide is the potential effects it may pose on animals, especially since glyphosate is an

environmental contaminant that appears in a wide variety of locations including ground and surface water (Gunarathna, Gunawardana, Jayaweera, Manatunge, & Zoysa, 2018) and products consumed by humans (Cook, 2019). In fact, studies reveal that glyphosate can be commonly found in human urine samples (Parvez et al., 2018), including pregnant women in the U.S. (Niemann, Sieke, Pfeil, & Solecki, 2015; Parvez et al., 2018). Monsanto (acquired by Bayer in 2018), the producer of glyphosate, has come under recent fire because the company was sued for not properly advertising the cancer risk with glyphosate use (Jacobs, 2019). With the large prevalence of glyphosate use, it is important to understand the impacts of using this herbicide. Many studies have been conducted to investigate the impact on animals. In one study of zebrafish embryos, glyphosate exposure led to several morphological deformities including stalled development of epiboly and decreased size of body, head, and eyes (S. Zhang et al., 2017). Another study with glyphosate-exposed zebrafish embryos found larvae that exhibited decreased body length and ocular distance (distance between eyes), as well as decreased aggression and locomotion impairment (Bridi, Altenhofen, Gonzalez, Reolon, & Bonan, 2017). Glyphosate exposed zebrafish embryos also exhibited cardiotoxicity, with larval hearts exhibiting structural abnormalities of the atrium and ventricle, *situs inversus*, decreased heart rate, and irregular heart looping (Roy, Ochs, Zambrzycka, & Anderson, 2016). Another study, exposed *Xenopus laevis* oocytes to glyphosate, and found delayed maturation and morphogenesis of meiotic spindles (Slaby et al., 2019). So, similar to atrazine, it is clear that these herbicides may not directly target weeds, but may have extremely adverse effects on animals.

The chemical 2,3,5-triiodobenzoic acid (TIBA) was determined to quicken the flowering process of soybeans, but at high concentrations it behaved as a defoliant (Galston, 1947). U.S. military scientists investigated other chemicals that behaved as a defoliant, which led them to

formulate a mixture of two herbicides 2,4-Dichlorophenoxyacetic acid (2,4-D) and 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), which target broadleaf plants (OCSPP,OPP US EPA, 2019). These two herbicides made up the tactical herbicide called “agent orange”, which was used in various military campaigns to damage crops and vegetation in war zones. It was later banned due to the contamination with the 2,4,5-T synthesis side product called 2,3,7,8-Tetrachlorodibenzo-P-dioxin (referred to as TCDD or dioxin) (OCSPP,OPP US EPA, 2019). 2,4-D and 2,4,5-T both work by mimicking the hormone auxin, found specifically in plants (Dehnert, Karasov, & Wolman, 2019). However, the side product, dioxin, acts as a ligand to the aryl hydrocarbon receptor found in animal cells where it changes expression of select genes, and it may also have genotoxic effects (Dragan & Schrenk, 2000; Poellinger, 2000). Though 2,3,4-T has no reported teratogenic effects, 2,4-D and dioxin do have teratogenic effects. A study testing the presence of herbicides in umbilical cord blood of prenatal babies in China showed that 27% of the infants contained 2,4-D (Silver et al., 2019). At six weeks after birth the babies displayed slower auditory response and V-latencies at concentrations above 1.17 ng/mL (Silver et al., 2019). Zebrafish embryos exposed to 2,4-D, led to increased mortality and decreased hatching rate as well as pericardial sac extension (Li et al., 2017). In other studies, zebrafish embryos exposed to 2,4-D displayed extended pericardial sacs and tail deformities along with alteration in swim behavior (Gaaied, Oliveira, Domingues, & Banni, 2019) as well as increased mortality and neural defects causing reduced vision (Dehnert et al., 2019). Exposure of mice to dioxin resulted in a teratogenic effect of cleft lip and palate (Bryant, Schmid, Fenton, Buckalew, & Abbott, 2001). However, few studies have determined a clear role of dioxin as a teratogen in people (Center of Disease Control, 2017). Although several herbicides have been more thoroughly

tested, the adverse events on zebrafish embryos development mediated by other commonly used herbicides should be investigated.

Untested Herbicides

One of the untested herbicides examined in my study is mecoprop, which is commonly used to control broadleaf weeds (USEPA, 2007). Mecoprop goes under the common name Kilprop, Mecopar, Triester-II, Mecocomin-D, Triamine-II, and other mixtures of mecoprop with various herbicides (*Farm Chem. handbook.*, 2001). Mecoprop works by mimicking the plant hormone auxin, a similar mechanism to 2,4-D, which leads to abnormal growth of vegetation where the leaves grow downward (USEPA, 2007). Mecoprop is considered to have low toxicity to aquatic life; however, mecoprop has high solubility in water and does not bind to soil particles (SEPA, 2019). So, during the months before it is broken down in the environment naturally, aquatic organisms could be exposed to it. It has also been listed as a possible carcinogen by the International Agency for Research on Cancer (SEPA, 2019). In studies involving the treatment of aquatic organisms with mecoprop, it was found to be toxic (SEPA, 2019). However, in cases where mecoprop was either ingested or inhaled by humans, it caused a variety of acute symptoms. Low amounts of inhalation can lead to cough, nausea, dizziness, headache, and tingling sensation (SEPA, 2019). High amounts of inhalation can lead to spasms, weakness, polyneuritis, and unconsciousness (SEPA, 2019). When ingested, mecoprop can cause nausea, vomiting, abdominal pain, diarrhea, and effects similar to inhalation (SEPA, 2019). In adult rats, exposure to high concentrations of mecoprop led to morphological deformities in both the spleen and thymus along with changes in the number of blood lymphocytes and granulocytes (Moeller & Solecki, 1989). Another study found that mecoprop exposure led to decreased growth rate and

increased kidney weights in rats at high concentrations (Verschuuren, Kroes, & Den Tonkelaar, 1975). In rabbits, mecoprop exposure led to erythema, weight loss, high mortality, and histopathological changes in liver, kidneys, spleen, and thymus at high concentrations (Verschuuren et al., 1975).

Glufosinate-ammonium is another untested herbicide (in zebrafish) that I investigated in my research. Glufosinate-ammonium is used to control weeds in crops (Q. Zhang, Cui, Yue, Lu, & Zhao, 2019). Glufosinate-ammonium common names are Basta, Rely, Finale, Ignite, Challenge, and Liberty (Haschek, Rousseaux, & Wallig, 2013). Glufosinate-ammonium works by inhibiting synthesis of glutamine, which helps with nitrogen metabolism (Dekker & Duke, 1995). If ingested by humans, glufosinate-ammonium can lead to apnea, mental deterioration, convulsion, and cranial nerve palsy (Park, Kwak, Gil, Kim, & Hong, 2013). When male lizards, *Eremias argus*, were exposed to glufosinate-ammonium it led to morphological and reproductive dysfunction (L. Zhang et al., 2019). Some noted effects of exposure were changes in antioxidant enzyme activity, tissue distribution, steroid hormone levels, histopathological damage, decreased body mass, and related gene expression of sex steroid hormones (L. Zhang et al., 2019). The increased oxidative stress resulted in stress on the testes, which led to severe lesions, and it also changed plasma sex hormone levels and gene expression (L. Zhang et al., 2019). In newborn mice, neural stem cells exposed to glufosinate-ammonium showed impaired neural-glial cell differentiation and ependymal wall integrity (Feat-Vetel et al., 2018)

Hexazinone is another untested herbicide (in zebrafish), which is used to control annual, biennial, and perennial weeds (Pohanish, 2015). This herbicide blocks photosynthesis by inhibiting electron transport in photosystem II of chloroplast (Pohanish, 2015). In particular, this herbicide binds to D1 proteins in chloroplast thylakoid membranes, which leads to inhibition of

electron transport and CO_2 fixation (Pohanish, 2015). The common names for hexazinone are DPX 3674 and Velpar (LeBaron, McFarland, & Burnside, 2008). Hexazinone is considered safe for the environment with low toxicity to humans. The greatest risk is eye irritation (Epa & of Pesticide Programs, 2009). Ingestion leads to vomiting and nausea (Epa & of Pesticide Programs, 2009). Hexazinone has a moderate to long half-life, which would allow hexazinone to be carried to lakes and streams through ground water runoff (Bouchard & Lavy, 1985). One study investigated persistence of hexazinone in an aquatic setting and simulated aquatic environment, and the researchers detected high levels of hexazinone after a year (Mercurio, Mueller, Eaglesham, Flores, & Negri, 2015). Juvenile signal crayfish that were exposed to hexazinone displayed increased mortality (Velisek, Kouba, & Stara, 2013). Other studies were done on mammalian model organisms. Hexazinone was a severe irritant to the eye in albino rabbits, leading to mild to moderate corneal cloudiness, iritis, and conjunctivitis (Durkin & Bosch, 2005). Beagles fed hexazinone at 125 mg/kg per day showed decreased appetite, body mass, albumin/globulin levels, and small increase in liver weight (Durkin & Bosch, 2005).

Nicosulfuron is another one of our untested (in zebrafish) herbicides, which is used to control wide variety of weeds including annual and perennial in corn crops (Carles et al., 2018). Nicosulfuron works by inhibiting acetolactate synthase. Acetolactate synthase is an enzyme important for production of the amino acids valine, isoleucine, and leucine (Carles et al., 2018). Humans do not make this enzyme, though there is a gene with partial conservation. However, it is unlikely that it is functional in the same way, as humans cannot synthesize the essential amino acids of valine, isoleucine, and leucine. Nicosulfuron goes by the trade names of Accent, Challenger, Dasul, Lama, Milagro, Mistral, Motivel, Nisshin, and Sanson (Willoughby, 2001). Nicosulfuron has a high solubility in water and is often found in surface waters (Carles et al.,

2018). Along with high solubility, nicosulfuron has a degradation time of 70 days in when in water or in clay soil (Carles et al., 2018). In one study done of earthworms, it was noted that nicosulfuron leads to acetylcholinesterase induction and increased malondialdehyde concentrations (Hackenberger, Stjepanović, Lončarić, & Hackenberger, 2018). Both of these are indicators of oxidative stress (Hackenberger et al., 2018). In another study, testing nicosulfuron exposure in goldfish, researchers found that exposure led to changes in behavior related to burst swimming and grouping (Saglio, Bretaud, Rivot, & Olsén, 2003). Many studies done with nicosulfuron exposure resulted in little to no harmful effects on organisms. In an ingestion study done on beagle dogs, there was a slight decrease in neutrophils with no other symptoms (USEPA, 2005). In an ingestion study done on mice, the observed effects were decreased neutrophils, lymphocytes, monocytes, and eosinophils (USEPA, 2005). With nicosulfuron, there is a shortage of scientific literature on the effects of nicosulfuron on aquatic organisms.

Thifensulfuron-methyl is an untested herbicide, which is used to control unwanted grass and broad-leaved weeds (U.S. National Library of Medicine, 2019). Thifensulfuron-methyl works by inhibiting acetohydroxyacid synthase, an enzyme responsible for amino acid synthesis (Iwakami et al., 2017). Thifensulfuron-methyl goes by the trade names Treaty, Harass, Harmony, Thief, Veer, and Volta (Shaner, 2014). In ingestion studies done on rats exposed to thifensufuron-methyl, the main effects were decreased serum sodium levels and decreased body weight (National Institute of Heath, 2006). Ingestion in mice resulted in decreased body weight occurred (NIH, 2006). Similar studies in dogs resulted in increased thyroid/parathyroid to body weight ratios in females, and decreased body weight in males (NIH, 2006). There was little to no information on the effects of this herbicide on aquatic organisms.

Quizalofop-P-Ethyl is an untested herbicide under the common name Assure II, Pilot Super, Targa Super, Targa D+ (Kidd, 1991). Quizalofop-P-Ethyl is used to control annual and perennial grass weeds in crops (Kidd, 1991). Quizalofop-P-Ethyl works by blocking acetyl CoA carboxylase inhibitor this leads to a block in membrane synthesis (Kidd, 1991). The half-life of quizalofop-p-ethyl is 60 days in soil and water (Ahrens & Edwards, 1994). Exposure studies indicate that quizalofop-p-ethyl has low toxicity in mammals; however, in study done on bluegill sunfish it was highly toxic (Kidd, 1991). In one prolonged exposure study done on mice, researchers found changes in blood chemistry, increased liver weight, and malformations in liver tissue structure (ORD,NCEA,IRISD US EPA, 2019). In one reported case study, a farmer exposed to quizalopfop-p-ethyl exhibited obstructive cholestasis (Elefsiniotis, Liatsos, Stamelakis, & Moulakakis, 2007).

Though much information has been gained from toxicity studies in both mammalian and aquatic organisms, there is still a paucity of data regarding the teratogenic effects of these untested herbicides. Thus, additional studies need to be done on model organisms to determine whether these herbicides have teratogenic effects.

Zebrafish as a Model Organism

Zebrafish are a great model organism to use in many different areas of science including behavioral, regeneration, developmental, transgenics, toxicology, and reverse genetic studies (Meyers, 2018). Lessons learned from zebrafish research can be applicable to understanding human biology because they share 70% of their genes with humans, and they have similar molecular and cellular mechanisms that control development (Meyers, 2018). Zebrafish are part of the Cyprinidae family, which includes minnows and barbs (Meyers, 2018). They were first

recorded in literature in south Asia flora of fish, and can be found in a wide-variety of freshwater locations, including rivers, streams and rice paddies (Meyers, 2018). One of the primary reasons zebrafish are good model organisms is the rapid growth cycle during development. The zebrafish will be fully hatched and developed into a larvae by 96 hours post fertilization (Meyers, 2018). This allows for quick studies to be performed in a relatively short period. Additionally, zebrafish have external fertilization unlike other commonly used model organism, which means retrieval of embryos is a quick process that does not require surgery to remove from the mother (Meyers, 2018). Zebrafish embryos are transparent during development, which allows for easy viewing of development. It is easy to track any developmental abnormalities due to contaminant exposure. Therefore, zebrafish are useful model organisms because many chemicals can be tested at different concentrations relatively rapidly (Meyers, 2018). In addition, zebrafish are bred at the start of daylight (at any time of the year), and each fish can produce hundreds of eggs per week (Meyers, 2018).

Zebrafish are commonly used in pharmaceutical studies, primarily to test novel drugs that are meant for people. The benefit of drug-testing in zebrafish, as opposed to using cultured cells, is to observe an effect of a drug on a complex system (like a tissue or organ), and to determine whether unanticipated side-effects in off-target tissues/organs emerge (Strähle & Grabher, 2010). For example, exposure of zebrafish embryos to celastrol, a potential anti-obesity drug, was thought to target adipose tissues specifically, but instead it led to decreased hatching rates, no blood flow in trunk vessels, tail malformations, and severe pericardial edema (S. Wang, Liu, Wang, He, & Chen, 2011). In addition, zebrafish are used to test the effects of pharmaceutical drugs that act as environmental contaminants in aquatic ecosystems. Pharmaceuticals are one of the leading forms of environmental contamination (USGS, 2018),

and the ultimate source of these drugs in the environment comes from improper disposal of the drugs (flushing drugs down the toilet), or through excretion of the drug in urine or excrement (originating from humans or from livestock) (USGS, 2018). For drugs that are dissolved in sewage, most are not completely removed by wastewater treatment plants; and thus can be introduced back into aquatic ecosystems via the effluent (Xia, Zheng, & Zhou, 2017). This was shown in a study done by USGS that tested the effluent from different wastewater treatment plants (USGS, 2018). Some of the common drugs found in water are ibuprofen, acetaminophen, antibiotics, and other commonly subscribed pharmaceuticals (USGS, 2018). It has been commonly found that exposure to pharmaceuticals leads to behavioral alterations in fish (Corcoran, Winter, & Tyler, 2010). A study of commonly prescribed non-steroidal anti-inflammatory drugs (NSAIDS) on zebrafish embryo development found that ibuprofen and diclofenac lead to decreased hatching rate, decreased spontaneous movement, and decreased free swimming distance (Xia et al., 2017). In this same study, they found that neuron-related gene, neurog1, was down regulated with exposure to ibuprofen and diclofenac (Xia et al., 2017).

Although pharmaceutical drugs are a major concern, another developing concern is illicit drugs in aquatic ecosystems. For example, lakes and rivers in highly populated areas have detectable levels of cocaine, opioids, amphetamines, and cannabis (Zuccato et al., 2008). Further, the levels of these drugs are high enough to have physiological/developmental effects, as prolonged exposure of European eels to environmental levels of cocaine resulted in hyperactivity, severe skeletal muscle injury (Capaldo et al., 2018), and damage to the gills (Capaldo, Gay, & Laforgia, 2019). The results of these studies indicate that human medications could affect development of organisms just as effectively as other environmental contaminants. Though the exposure level can have effects on adults, embryos and larvae may be more sensitive

than the adults, as they do not have functional livers to detoxify the contaminant. So, scientists should carefully assess whether embryos exhibit teratogenic effects upon exposure to the contaminants.

Hypothesis and Rational for Study

Exposure of developing metazoans to environmental contaminants can have adverse effects on the health of an organism. This can lead to the formation of various types of disease and even death. I am particularly interested in the teratogenic effects that the environmental contaminants may have on embryos. Though my study focuses on the treatment of herbicides on zebrafish embryos, my study may also be relevant to effects that may occur in other aquatic organisms, as many share common environment, developmental signaling pathways, and mechanisms of morphogenesis.

To identify contaminants with previously unknown teratogenic activities, I first sought to identify contaminants that have not been tested in zebrafish. After an extensive literature review, and analysis of well-established zebrafish contaminant screens (such as the toxcast study) (ORD US EPA, 2019-c), I formed a list of many untested herbicides. The analysis of these herbicides and their effects on zebrafish embryonic development was the main premise of my research project. Based on previous studies of the exposure of other herbicides on developing zebrafish, I propose the following hypothesis: exposure of zebrafish embryos to various untested herbicides will cause teratogenic effects.

METHODS

Zebrafish Care

Adult TU line zebrafish were housed in two separate 20 gallon tanks holding a maximum of 45 zebrafish per tank, and on a 12hr:12hr light/dark cycle. Zebrafish are known to reproduce better if they have a regulated circadian rhythm, and at the start of light cycle fish will spawn.. The fish were fed the same amount two times a day at set times, and the amount of food given was modulated, depending on density of the fish (typically 0.01 grams per zebrafish). The main food source used was the Zeigler Zebrafish Diet (PN: 388765-101-686). To ensure water quality was maintained within optimal levels, 20% water change outs were done for each tank every day, and 80% water change outs were done every two months along with doing water quality checks. The water quality was evaluated weekly in the tanks, and standard test kits were used to determine pH (Ricca Chemical Company: 8882-1), ammonium (API: LR8600), nitrate (API: LR1800), and nitrite (API: 26). The system water used was distilled and purified water (ELGA: 6GN0026), which starts with a low conductivity (~0.5 micro-Siemens), and salts was added back to an optimal level for zebrafish ---0.25PPT instant ocean salts (Instant Ocean: 4905). If any variation of pH occurred in the system water, so that it was below a pH of 7, sodium bicarbonate (Fisher Chemical: S233-500) was added to bring the system water back to a range of pH 7-8. To maintain water quality within the tank, a power filter was used to remove particulate matter, and a biofilter was used to ensure growth/colonization of nitrifying bacteria. A bacteria supplement was occasionally administered to the tank, as per the manufacturer's instructions (Topfin: 5112330). Also, if ammonia levels exceeded their appropriate level of tolerance, above 0.25

mg/L, ammonia remover was administered to the tank (IPSD: 2728450). Zebrafish were handled as per the Institutional Animal Care and Use Committee (IACUC) protocol (18.003.0)

Mating

For mating, two approaches were used. In one approach, in-tank spawning trays were placed into the tank as soon as the light came on in the morning. These trays are designed to have a grating system on the top of the tray which provides an artificial shoreline for the zebrafish to use in the spawning process. When spawning occurs, fertilized eggs would fall through the grates where they collect in the tray. Mating would occur over a four-hour period for mating, and at the end of the time, the tray was removed and embryos were evaluated and selected at the blastoderm stage. In another approach, isolation tanks were used for breeding in order to increase productivity. For this, individual fish were selected in the main tank and placed in the isolation tank overnight. The isolation tank had a divider that separated males from the female. It also had a similar mesh bottom, as described above. The ratio for male to female is 2:1 respectively. The fish were put in the isolation tank overnight and in the morning, when light cycle began, a small water change out was done and divider was removed. Fish were also allowed to mate for four hours, and at the end of that period of time, embryos were evaluated, sorted, collected, and transferred (via pipet) into a 60 mm Petri dish (Falcon: 351008). The embryos selected were in the 4-8 cell stage, and then they were washed. To wash the embryos, egg water was prepared, as well as a 5% bleach solution in egg water. Egg water was made using the distilled and purified water with 1.5ml of instant ocean salts stock (4g instant ocean in 100mls ultrapurified water) and 1.5ml sodium bicarbonate stock (100grams in 1liter ultrapurified water) added. To make the bleach solution, 5% of bleach was added to egg water. For the first wash, the embryos were

placed in 5% bleach wash for 5 minutes. Then, the embryos were washed in egg water three times for 3 minutes each wash. The embryos were then ready to be used for experiments.

Determination of LD50

In the first time testing an herbicide, the LD50 (i.e. the concentration at which half of the embryos die) needed to be determined. Thus, a standard range of herbicide concentrations was used for the treated/experimental group (1000 µg/ml, 100 µg/ml, 10 µg/ml, 1 µg/ml diluted with egg water), and egg water was used for the control group. When diluting the herbicides, a stock solution was made freshly at the start of every experiment, and this stock is carefully diluted to obtain the lower concentrations. To set up the experiment, 24-well trays were setup with 2mL of the herbicide or egg water solution per well, and two blastoderm-stage embryos per well. A total of 8-12 embryos tested per each treatment group. Embryos were monitored every 24 hours, and the entire time of the experiment was 96 hours post fertilization (hpf), or 4 days post fertilization (dpf), without water change outs. At the end of the experiment, embryos were analyzed and imaged. For imaging, an Amscope camera (14MP) attached to an Amscope dissection microscope (SKU: SM-1TSX-L6W). To determine the lethal dose, each 4 dpf embryo (now a larvae) was evaluated for death. Death was determined based on whether the embryo did not continue developing (this usually appeared as a white ball of tissue that failed to continue developing past 1 dpf). In many cases, embryos were able to develop to a larval stage; however, they were considered "dead" if the heart fails to contract and move blood properly through the body, and the larva does not elicit a touch response (swims away from upon being touched). In addition to determining death, other information gleaned from the analysis was whether the zebrafish "hatched" from its chorion (egg shell/fertilization envelope). After the first test, an

approximate concentration of where the LD50 was established. Further tests, using more specific concentrations between the 1-1000 µg/ml range were performed to find the LD50. Once an approximate LD50 was found, any specific developmental abnormalities could be evaluated. Though this was predominantly using concentrations at or near the LD50, in some cases, developmental abnormalities could be analyzed away from the LD50. To evaluate the embryos for developmental abnormalities, various aspects were analyzed, impairment to heart function and morphology, motor development impairment (via touch test), and other abnormalities (more details in analysis section). After tests were run, embryos received an overdose of tricaine (MS222) in order to euthanize them. Test were performed as per IACUC approved animal protocols (IACUC: 18-003.0).

Analysis

To analyze health of the embryos, extent of development, and the formation of apparent abnormalities, I evaluated the embryos based on a previously published study (Yamashita et al., 2014). For this teratogenic analysis system, the embryos were evaluated for different morphological deformities listed in Table 1 below. Each trait was given a score of 0 to 1: 0 being no deformities, 0.5 moderate deformities, and 1 severe deformities. The final analysis system was cumulative including all the final scores of each of the 8 traits. Thus, a score of 8 indicates severe morphological deformities, while a score of zero indicate no deformities. The traits indicator in charts are as follows: Heart defects (H), Edema (E), Spine (S), Locomotion (L), Facial (F), Swim Bladder (SB), Hatched (HA), and Pigmentation and Apoptotic tissue (P). Embryos that failed to develop due to contaminant exposure and suspected general toxicity were not included in the scoring system. Thus, if a zebrafish embryo that showed severe spine

abnormality, slight pericardial sac extension, severe locomotion impairment, severe edema, complete absence of swim bladder, and no other noticeable abnormalities, the teratogenic analysis system would work as follows. Each one of the severe traits would receive a score of 1. So, severe spine abnormality, severe edema, severe locomotion impairment, and complete absence of swim bladder would each receive a score of 1. Any of the traits that had moderate issues would receive a score of 0.5. Hence slight pericardial sac extension, which is a heart implication, would receive a score of 0.5. Any of the traits, which had no noticeable abnormalities would be given a score of zero in this analysis system. Then after assigning scores for every trait, the totals would be added together so the calculation would be as follows S:1+E:1+L:1+H:0.5+SB:1+F:0+P:0+HA:0=4.5/8. Thus the final score for this embryo is 4.5 out of 8.

Chemicals

The chemicals tested were purchased from Sigma-Aldrich in St. Louis, Missouri. Herbicides were purchased in there purified form. The herbicides purchased were mecoprop (36147-100MG LOT# BCBV9245, BCBV9245, SZBE010XV, BCBV9245), nicosulfuron (34210-100MG LOT#SZBE279XV, SZBE279XV), glufosinate-ammonium (45520-100MG LOT#BCBT2199), Thifensulfuron-methyl (46028-100MG LOT#SZBD339XV), quizalofop-P-ethyl (34074-100MG LOT#BCBT6249), and Hexazinone (36129-100MG LOT#BCBT6090). For the heart inhibitor, amlodipine besylate (A2353 LOT: ZFR8F-BH) from TCI in Portland, Oregon was used. For the herbicides, I started with a stock solution of 10000 µg/ml and then diluted to each test concentration. To make the stock solution, 0.01grams of herbicides was used per milliliters of egg water. The tricaine/MS222 anesthetic was purchased from Western

Chemical Inc in Scottsdale, Arizona(lot: 101719). For tricaine, 150mg of tricaine was used in 1liter of ultrapurified water.

Table 1. Morphological traits investigated in study

Morphological trait	Trait description
Heart defects **(H)**	The heart was evaluated for blood flow, heart contraction, and pericardial sac extension.
Edema **(E)**	Checked for general body swelling in zebrafish embryo. This indicates low blood flow.
Spine (notochord and neural tube) **(S)**	Checked for different spinal abnormalities including shortened body length and spinal curvatures.
Locomotion **(L)**	Did poke test to determine if movement or reflexes had been effected.
Facial **(F)**	Checked jaw, eyes, otic vesicle, and general facial shape.
Swim bladder **(SB)**	Determined if swim bladder had expanded..
Pigmentation and apoptotic tissue **(P)**	Looked for correct pigmentation and whitened tissue.
Hatching rate **(HA)**	Determine if zebrafish has hatched from coriander.

RESULTS

Summary of Herbicide Treatment Methodology

To identify herbicides that exhibit teratogenic effects on zebrafish development, I exposed zebrafish embryos to six herbicides (nicosulforon, glufosinate-ammonium, quizalofop-p-ethyl, mecoprop, thifensulfuron-methyl, and hexazinone). These herbicides were selected because they have had minimal testing in various developing model organisms, and no reported testing in zebrafish embryos. As previously mentioned, I first sought to determine the LD50 in order to identify the appropriate concentration that causes an effect, but without causing general toxicity. For the herbicides that caused a defect, some exhibited identifiable LD50s, while others were difficult to pinpoint. This will be described in more detail below. Of the herbicides that caused a defect, at or near their LD50, cardiac failure and various defects associated with cardiac failure was observed. Additionally, treatment with a subset of these herbicides resulted in unique teratogenic effects to other tissues. In some treatments, abdominal edema occurred, in others shorter larvae formed, in other cases a myriad of other defects were observed. To best portray these data, the results of each individual herbicide treatment will be described below in Table 2.

Nicosulfuron Treatment Results in No Defects in Development

In the attempt to determine the LD50 of nicosulforon, blastoderm-stage zebrafish embryos were exposed to a range of nicosulfuron concentrations (1000 µg/ml, 500 µg/ml, 100 µg/ml and 10 µg/ml) through the duration of development (from 0 dpf to 4 dpf). At the end of exposure, extent of survival/mortality was assessed during their larval stage (4 dpf). These results are shown in Table 3 and Table 4. Nicosulfuron exposure resulted in little to no

deformities when embryos were exposed to high concentrations (1000 µg/mL, 500 µg/ml, 100 µg/ml, 10 µg/mL) (Figure 1). High concentrations (1000 µg/ml) in nicosulfuron lead to saturated conditions; therefore, higher concentrations were not tested. At saturated conditions, no increased mortality was noticed; thus, no LD50 could be established (Table 3 and Table 4). Some larvae displayed absence of swim bladder in the presence of 1000 µg/ml nicosulfuron, although not all larvae displayed absence of swim bladder. This could be due to some embryos having a diminished heart contraction (which can prevent the swim bladder from inflating) or due to some larvae that have a slight developmental delay (at the start of inflation of the swim bladder), which occurs due to a natural variation. Additionally, one or two larvae showed slight pericardial sac extension as seen in Figure 1C. Although, with these, no noticeable effect on heart contraction was observed. There was no decrease in hatching rate or increase in mortality, and no noticeable differences in locomotion. Overall these larvae typically displayed between 0-1 in the teratogenic analysis system, with an average score of 0.35 (Table 5). However, there was a wide variance with some larvae with higher, and others with lower, values.

Glufosinate-Ammonium Treatment Exhibited Mild Cardiac Dysfunction at High Concentrations

Glufosinate-ammonium exposed embryos exhibited variable outcomes at various concentrations (1000 µg/mL, 900 µg/ml, 500 µg/ml, 100 µg/ml, 10 µg/mL), depending on the treatment trial as seen in Table 6 and Table 7. It was difficult to determine the LD50 because 100% lethality was observed at concentrations that range from 100 µg/ml and 900 µg/ml. However, in other trials, 33% survival and 58% survival did occur with the 100 µg/ml and 1000 µg/ml treatment groups, respectively. For the surviving larvae for the 100 µg/ml and 1000 µg/ml

treatment groups, some larvae exhibited cardiac defects which were characterized by a slight pericardial sac extension (Figure 2), as well as a weakened heart contraction. All hatched larvae displayed normal locomotion, suggesting that blood flow was sufficient enough for locomotion. Though treatment at the low concentration (10 µg/ml) resulted in a low survival (67%), the surviving larvae did not display any defects in morphology or cardiac function. Since an LD50 concentration could not be determined, larvae from the 1000 µg/ml treatment group were used in the teratogenic analysis system, and an average score of 0.93 was determined (Table 8).

Quizalofop-P-Ethyl Treatment Exhibited Cardiac Dysfunction at the LD50, and General Toxicity at High Concentrations

Quizalofop-P-ethyl treatment caused mortality of all of the embryos above a threshold-level concentration (at or above 100 µg/ml) (Table 9 and Table 10). This mortality was characterized by embryos acquiring a white mass of dead apoptotic cells that failed to develop (data not shown). Interestingly, concentrations between 90 µg/ml and 99 µg/ml resulted in an average ~ 46% survival; with 95 µg/ml being the likely LD50 concentration. Of this group, some larvae displayed decreased heart contraction which was comorbid with other phenotypes associated with decreased blood flow—extended pericardial sac, and absence of a swim bladder (Figure 3). Concentrations below 90 µg/ml resulted in much higher survival rates, and no defects in cardiac function. Thus, embryos treated at the likely LD50 (95 µg/ml) were used in the teratogenic analysis system, and the resultant larvae were rated as a 1-2 with an average score of 1.43 (Table 11). Anything under 90 µg/ml or above 100 µg/ml exhibited no defects in development, or no survival; thus, they were not considered in the analysis scale.

Thifensulfuron-Methyl Treatment Caused Mild Cardiac Dysfunction at High Concentrations

Similar to glufosinate-ammonium treatment, treatment with thifensulfuron-methyl resulted in a variation in survival at different concentrations which prevented a clear determination of the LD50 (Table 12 and Table 13). However, concentrations $\leq$ 100 µg/ml resulted in no defects in morphology, heart function or motility. For concentrations $\geq$ 200 µg/ml, some zebrafish larvae displayed reduced heart contraction, as well as other phenotypes associated with decreased blood flow (absence of swim bladder and slight pericardial sac extension) (Figure 4). Since no LD50 was determined, the highest concentration was used for the teratogenic analysis system, and the treated embryos were given a score of 2.3 (mild teratogenic effect) (Table 14).

Mecoprop Treatment Caused Cardiac Dysfunction, Potential Renal Dysfunction, Defects in Morphology, and General Toxicity at High Concentrations

Mecoprop treatments resulted in a variation in the percent mortality at different concentrations; however, exposure to high concentrations ($\geq$ 590 µg/ml) led to complete mortality of the embryos, suggesting that exposure to these concentrations result in general toxicity. Lower concentrations (less than 500 µg/ml) led to high survival, and no morphological deformities. The LD50 likely resides between 500 µg/ml and 600 µg/ml, and within this range, survivors displayed a range of morphological defects as seen in Table 15 and Table 16. For example, many of these larvae exhibited impaired heart contraction, as well as defects associated with reduced blood flow (pericardial sac extension, and failure for the swim bladder to inflate). However, an additional amount of deformities were observed in slightly fewer of the embryos:

shortened body axis, microphthalmia (small eyes), tail malformation, apoptotic tissues (dead white cells), lack of motion, and a severe abdominal edema (which may be associated with renal failure) (Figure 5). To describe the tail malformation in more detail, the posterior portion of the tails were either curved dorsally or ventrally, with the majority displaying ventral curvatures (Figure 5E-G). To describe the abdominal edema in more detail, treated larvae also displayed tissue swelling in the intestinal/yolk sac region. This type of edema tends to be associated with kidneys that fail to properly develop, suggesting that mecoprop treatment could lead to renal failure. For defects characterized by an alteration in organ/organism size (shortened body axis, micropthalamia), it remains to be determined the root cause; however, it may have to do the lowered amount of proliferation that occurs in these tissues, which will need to be evaluated at another time.

It should be noted that the embryos exhibited variability in severity of the defect, even at the same concentration. This resulted in some zebrafish larvae displaying moderate defects, while others exhibited severe defects (Figure 5B-D). This was not surprising, as the role of the LD50 is to find the concentration in which half of the embryos/larvae survive and half die, indicating that variability in phenotypes are to be expected. Overall, embryos exposed to concentrations less than 500 µg/ml typically had a zero on the teratogenic analysis scale. Embryos exposed to 500-600 µg/ml fell between 4-5. In Table 17, the exact breakdown of analysis for embryos at 560 µg/ml is shown with the average score being 5.35 on the teratogenic analysis scale.

Hexazinone Treatment Caused Cardiac Dysfunction, Potential Renal Dysfunction, Defects in Morphology, and General Toxicity at High Concentrations

The LD50 for Hexazinone could not be determined since there was much variation within the data when comparing treated embryos/larvae at the same and/or at different concentrations (Table 18 and Table 19). Exposure of embryos to concentrations between 10 µg/mL to 600 µg/mL typically resulted in the presence of various developmental deformities and embryos surviving. Whereas exposure to the highest concentration (1000 µg/ml) resulted in complete mortality that was characterized by embryos not developing and acquiring a whitened/dead tissue appearance. At lower concentrations (350 µg/ml or less), the embryos exhibited range of defects (from moderate to severe). In the group of embryos exhibiting moderate defects, a modest impairment in cardiac function (weak contractions, slight pericardial extension), and presence of a slight abdominal edema was observed (Figure 6). Embryos that had severe deformities exhibited body axis shortening, a largely extended pericardial sac, abdominal edema, and dead tissue (white apoptotic tissue) (Figure 6B and D). At higher concentrations (350 µg/ml-600 µg/ml), similar phenotypes were observed, with some additional defects: severe cardiac function defects (large pericardial sac extension, lack of swim bladder, lack of heart function), severe abdominal edema, misalignment of melanocytes, apoptotic tissue, body axis shortening, inhibited locomotion, microphthalmia (small eyes), and severe tail deformities (Figure 7). Comparing these phenotypes to those in mecoprop-treated embryos, some defects were similar and some were unique. One similarity is the severe abdominal edema and pericardial sac extension, which could be indicative of either renal failure and/or cardiac dysfunction (Hankes et al., 2013). Also, tail deformities were present; however, these deformities were characterized by both left/right and dorsal/ventral curvatures, The tail curvatures may

explain the altered locomotion, with fish moving in circles instead in straight line as seen in Figure 7D-E. A defect that was unique to hexazinone-treated embryos was a pigmentation issue (Figure 7D-E). Specifically, the normal role of melanocytes are to align themselves into stripes, each extending along the anterior-posterior axis, with several stripes forming at different positions along the dorsal-ventral axis (Figure 7A). In control embryos, melanocytes align normally along the ventral midline; however, in hexazinone-treated embryos, the melanocytes fail to properly align. In regards to the overall teratogenicity of hexazinone, embryos exposed to levels of hexazinone under 350 µg/ml were typically rated as a 1.5 on the teratogenic analysis scale, and embryos exposed to 350 µg/ml to 600 µg/ml were typically rated as a 4-6.5 (both when deformities were present). Table 20 shows analysis breakdown for embryos exposed to 500 µg/ml, which resulted in an average score of 4.9. Anything higher than 600 µg/ml typically did not develop and was not considered for our teratogenic analysis system.

Comparing the Cardiac Defects Induced by Herbicide Treatment with the Amlodipine Cardiac Inhibitor

Considering that all herbicide treatments that had a teratogenic effect also had heart failure, I wanted to assess what specific development defects are caused by a direct impairment to heart contraction. Thus, zebrafish embryos were treated with amlodipine, a calcium-channel blocker, which is a drug that prevents heart contraction. I found that amlodipine treatment completely stopped heart contraction in zebrafish embryos, but showed few other abnormalities. Zebrafish were still surviving with no heart contraction since they do not need heart contraction to survive during the embryonic stage (Bakkers, 2011). In all zebrafish embryos, where heart contraction was impaired, there was extended pericardial sac present as seen in Figure 8B-C. In a

majority of zebrafish embryos exposed to amlodipine absence of swim bladder was seen but not in all (Figure 8C). Additionally, in Figure 8C general abdominal edema not seen in most embryos exposed to amlodipine. These results provide evidence that other abnormalities seen in zebrafish embryos exposed to herbicides have to be stemming from something other than heart dysfunction. It also verifies that the extended pericardial sac and absence of swim bladder is a good indicator of cardiac dysfunction in zebrafish embryos. The values for LD50 are shown in Table 21 and Table 22.

Table 2. Summary of results for each herbicide

Herbicide	Summary of results
Nicosulfuron	• No LD50 was seen since even at saturation no mortality was seen. • No defects in development even at high concentrations.
Glufosinate-ammonium	• LD50 was not able to be determined. • Exhibited mild cardiac defects at high concentrations.
Quizalofop-P-ethyl	• LD50 was around 95 µg/ml. • At concentrations above 100 µg/ml acute toxicity occurred. • At LD50, defects indicative of cardiac dysfunction.
Thifensulfuron-methyl	• LD50 could not be established due to variation within data. • Mild cardiac dysfunction at high concentrations.
Mecoprop	• LD50 could not pinpointed due to variation within data. • Severe defects seen that indicate cardiac dysfunction or potential renal failure at high concentrations. • Acute toxicity at concentrations greater than 590 µg/ml.
Hexazinone	• LD50 could not pinpointed due to variation within data. • Severe defects seen that indicate cardiac dysfunction or potential renal failure at high concentrations. • General toxicity seen at concentrations greater than 600 µg/ml.

Table 3. Survival data for different concentrations of nicosulfuron

Concentrations (µg/ml)	# Alive H	# Alive NH	# Dead H	# Dead NH	Total
0	26	0	0	6	32
0	11	0	0	1	12
0	7	0	0	3	10
10	12	0	0	0	12
100	24	0	0	8	32
100	12	0	0	0	12
500	7	1	0	2	10
900	0	0	1	9	10
1000	9	0	0	3	12

* H-Hatched NH-Not Hatched

Table 4. Survival percentage for different concentrations of nicosulfuron

Concentrations (µg/ml)	% Alive H	% Alive NH	% Dead H	% Dead NH
0	81%	0%	0%	19%
0	92%	0%	0%	8%
0	70%	0%	0%	30%
10	100%	0%	0%	0%
100	75%	0%	0%	25%
100	100%	0%	0%	0%
500	70%	10%	0%	20%
900	0%	0%	10%	90%
1000	75%	0%	0%	25%

* H-Hatched NH-Not Hatched

Table 5. Teratogen analysis breakdown for 1000 µg/ml nicosulfuron

Embryo #	H	S	E	L	F	SB	P	HA	Total score
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0.5	0	0	0	0	1	0	0	1.5
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	1	0	0	1
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	1	0	0	1
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
Total	0.5	0	0	0	0	3	0	0	3.5
Mean	0.05	0	0	0	0	0.3	0	0	0.35

Table 6. Survival data for different concentrations of glufosinate-ammonium

Concentration (µg/ml)	# Alive H	# Alive NH	# Dead H	# Dead NH	Total
0	6	0	0	6	12
0	10	0	0	2	12
10	4	0	0	8	12
100	4	0	0	8	12
100	0	0	0	12	12
250	0	0	0	12	12
500	0	0	0	12	12
900	0	0	0	12	12
1000	7	1	0	4	12

* H-Hatched NH-Not Hatched

Table 7. Survival percentage for different concentrations of glufosinate-ammonium

Concentration (µg/ml)	% Alive H	% Alive NH	% Dead H	% Dead NH
0	50%	0%	0%	50%
0	83%	0%	0%	17%
10	33%	0%	0%	67%
100	33%	0%	0%	67%
100	0%	0%	0%	100%
250	0%	0%	0%	100%
500	0%	0%	0%	100%
900	0%	0%	0%	100%
1000	58%	8%	0%	33%

* H-Hatched NH-Not Hatched

Table 8. Teratogen analysis breakdown for 1000 µg/ml glufosinate-ammonium

Embryo #	H	S	E	L	F	SB	P	HA	Total score
1	0.5	0	0	0	0	0.5	0	0	1
2	0	0	0	0	0	0.5	0	0	0.5
3	0	0	0	0	0	0.5	0	0	0.5
4	0.5	0	1	0	0	1	0	0	2.5
5	0.5	0	0	0	0	0.5	0	0	1
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	1	1
8	0.5	0	0	0	0	0.5	0	0	1
Total	2	0	1	0	0	3.5	0	1	7.5
Mean	0.25	0	0.13	0	0	0.44	0	0.125	0.9375

Table 9. Survival data for different concentrations of quizalofop-P-ethyl

Concentrations (µg/ml)	# Alive H	# Alive NH	# Dead H	# Dead NH	Total
0	11	0	0	1	12
0	11	0	0	1	12
0	11	0	0	1	12
30	9	0	0	3	12
50	9	0	0	3	12
90	6	0	0	6	12
90	2	0	0	10	12
95	7	0	0	5	12
99	7	0	0	5	12
100	0	0	0	12	12
500	0	0	0	12	12
1000	0	0	0	12	12

* H-hatched NH-Not Hatched

Table 10. Survival percentage for different concentrations of quizalofop-P-ethyl

Concentrations (µg/ml)	% Alive H	% Alive NH	% Dead H	% Dead NH
0	92%	0%	0%	8%
0	92%	0%	0%	8%
0	92%	0%	0%	8%
30	75%	0%	0%	25%
50	75%	0%	0%	25%
90	50%	0%	0%	50%
90	17%	0%	0%	83%
95	58%	0%	0%	42%
99	58%	0%	0%	42%
100	0%	0%	0%	100%
500	0%	0%	0%	100%
1000	0%	0%	0%	100%

*H-Hatched NH-Not Hatched

Table 11. Teratogen analysis breakdown for 95 µg/ml quizalofop-P-ethyl

Embryo #	H	S	E	L	F	SB	P	HA	Total score
1	0	0	0	0	0	0	0	0	0
2	1	0	0	0	0	1	0	0	2
3	0	0	0	0	0	1	0	0	1
4	1	0	0	0	0	1	0	0	2
5	1	1	0	0	0	1	0	0	3
6	0	0	0	0	0	1	0	0	1
7	1	0	0	0	0	0	0	0	1
Total	4	1	0	0	0	5	0	0	10
Mean	0.6	0.14	0	0	0	0.7	0	0	1.43

Table 12. Survival data for different concentrations of thifensulfuron-methyl

Concentration (µg/ml)	# Alive H	# Alive NH	# Dead H	# Dead NH	Total
0	11	0	0	1	12
0	5	0	0	7	12
0	10	0	0	2	12
10	4	0	0	8	12
10	11	0	0	1	12
100	6	0	0	6	12
100	10	0	0	2	12
200	10	0	0	2	12
200	8	0	0	4	12
500	4	0	0	8	12
750	9	0	0	3	12
900	8	0	0	4	12
1000	12	0	0	0	12
1000	3	0	0	9	12

* H-Hatched NH-Not Hatched

Table 13. Survival percentage for different concentrations of thifensulfuron-methyl

Concentration (µg/ml)	% Alive H	% Alive NH	% Dead H	% Dead NH
0	92%	0%	0%	8%
0	42%	0%	0%	58%
0	83%	0%	0%	17%
10	33%	0%	0%	67%
10	92%	0%	0%	8%
100	50%	0%	0%	50%
100	83%	0%	0%	17%
200	83%	0%	0%	17%
200	67%	0%	0%	33%
500	33%	0%	0%	67%
750	75%	0%	0%	25%
900	67%	0%	0%	33%
1000	100%	0%	0%	0%
1000	25%	0%	0%	75%

* H-Hatched NH-Not Hatched

Table 14. Teratogen analysis breakdown for 1000 µg/ml thifensulfuron-methyl

Embryo #	H	S	E	L	F	SB	P	HA	Total score
1	0	0	0	0	0	1	0	0	1
2	1	0	0.5	0	0	1	0	0	2.5
3	1	0	0	0	0	1	0	0	2
4	1	0	1	1	0	1	1	0	5
5	0	0	0	0	0	1	0	0	1
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	1	0	0	1
9	1	0	0	0	0	1	0	0	2
10	0	0	0	0	0	0	0	0	0
Total	4	0	1.5	1	0	7	1	0	14.5
Mean	0.4	0	0.15	0.1	0	0.7	0.1	0	1.45

Table 15. Survival data for different concentrations of mecoprop

Concentration (µg/ml)	# Alive H	# Alive NH	# Dead H	# Dead NH	Total
0	86	0	0	82	168
5	7	0	0	3	10
10	13	0	0	9	22
50	8	0	0	8	16
100	10	0	0	42	52
150	9	0	0	15	24
200	11	0	0	9	20
500	12	0	0	10	22
530	0	0	0	12	12
540	7	0	0	5	12
550	6	0	0	4	10
560	15	0	0	19	34
570	7	0	0	3	10
580	7	0	0	7	14
590	0	0	0	12	12
600	0	0	0	24	24
700	0	0	0	12	12
800	0	0	0	12	12
900	0	0	0	20	20
1000	4	0	0	64	68

* H-Hatched NH-Not Hatched

Table 16. Survival percentage for different concentrations of mecoprop

Concentration (µg/ml)	% Alive H	% Alive NH	% Dead H	% Dead NH
0	51%	0%	0%	49%
5	70%	0%	0%	30%
10	59%	0%	0%	41%
50	50%	0%	0%	50%
100	24%	0%	0%	81%
150	38%	0%	0%	63%
200	55%	0%	0%	45%
500	55%	0%	0%	45%
530	0%	0%	0%	100%
540	58%	0%	0%	42%
550	60%	0%	0%	40%
560	44%	0%	0%	56%
570	70%	0%	0%	30%
580	50%	0%	0%	50%
590	0%	0%	0%	100%
600	0%	0%	0%	100%
700	0%	0%	0%	100%
800	0%	0%	0%	100%
900	0%	0%	0%	100%
1000	6%	0%	0%	94%

*H-Hatched NH-Not Hatched

Table 17. Teratogen analysis breakdown for 560 µg/ml mecoprop

Embryo #	H	S	E	L	F	SB	P	HA	Total score
1	1	0	1	1	0	1	1	1	6
2	1	0	1	1	1	1	1	0	6
3	1	1	1	1	1	1	0	0	6
4	1	0	1	1	1	1	1	0	6
5	1	1	1	0	1	1	1	0	6
6	1	0	0.5	0.5	0	0	0.5	0	2.5
7	1	1	1	1	1	1	1	1	8
8	1	0	1	0.5	0	1	0	0	3.5
9	1	1	1	0.5	0.5	1	0	0	5
10	1	0	1	0.5	0	1	1	0	4.5
Total	10	4	9.5	7	5.5	9	6.5	2	53.5
Mean	1	0.4	1	0.7	0.6	0.9	0.65	0.2	5.35

Table 18. Survival data for different concentrations of hexazinone

Concentration (µg/ml)	# Alive H	# Alive NH	# Dead H	# Dead NH	Total
0	86	0	0	18	104
10	20	0	0	4	24
25	11	0	0	1	12
50	9	0	0	3	12
90	9	0	0	3	12
100	17	0	0	7	24
150	12	0	0	0	12
200	18	0	0	6	24
300	7	0	0	5	12
350	9	0	0	1	10
380	4	0	0	8	12
400	4	0	0	8	12
480	9	0	0	3	12
500	18	0	0	6	24
520	10	0	0	2	12
540	12	0	0	10	22
550	5	0	0	7	12
560	13	0	0	15	28
580	4	0	0	8	12
600	4	0	0	20	24
620	5	0	0	7	12
1000	0	0	0	12	12

* H-Hatched NH-Not Hatched

Table 19. Survival percentage for different concentrations of hexazinone

Concentration (µg/ml)	% Alive H	% Alive NH	% Dead H	% Dead NH
0	83%	0%	0%	17%
10	83%	0%	0%	17%
25	92%	0%	0%	8%
50	75%	0%	0%	25%
90	75%	0%	0%	25%
100	71%	0%	0%	29%
150	100%	0%	0%	0%
200	75%	0%	0%	25%
300	58%	0%	0%	42%
350	90%	0%	0%	10%
380	33%	0%	0%	67%
400	33%	0%	0%	67%
480	75%	0%	0%	25%
500	75%	0%	0%	25%
520	83%	0%	0%	17%
540	55%	0%	0%	45%
550	42%	0%	0%	58%
560	46%	0%	0%	54%
580	33%	0%	0%	67%
600	17%	0%	0%	83%
620	42%	0%	0%	58%
1000	0%	0%	0%	100%

* H-Hatched NH-Not Hatched

Table 20. Teratogen analysis breakdown for 500 µg/ml hexazinone

Embryo #	H	S	E	L	F	SB	P	HA	Total score
1	1	1	1	1	0.5	1	1	0	6.5
2	1	1	1	1	0.5	1	0.5	0	6
3	1	1	0.5	1	0.5	1	1	0	6
4	1	1	1	0.5	0.5	1	1	0	6
5	1	0	1	1	0	1	1	1	6
6	1	1	1	1	0	1	1	0	6
7	1	0	1	0.5	0	0	1	0	3.5
8	1	0	1	0.5	0	1	0	0	3.5
9	1	0	0.5	0.5	0	1	0	0	3
10	1	0	0	0.5	0	1	0	0	2.5
Total	10	5	8	7.5	2	9	6.5	1	49
Mean	1	0.5	0.8	0.8	0.2	0.9	0.7	0.1	4.9

Table 21. Survival data for different concentrations of amlodipine

Concentration (µg/ml)	# Alive H	# Alive NH	# Dead H	# Dead NH	Total
0	11	0	0	1	12
0	11	0	0	1	12
0	9	0	0	3	12
15	8	0	0	4	12
20	8	0	0	4	12
25	11	0	0	1	12
30	11	0	0	1	12
35	7	0	0	5	12
40	10	0	0	2	12
45	6	0	0	6	12

***** H-Hatched NH-Not Hatched

Table 22. Survival percentage for different concentrations of amlodipine

Concentration (µg/ml)	% Alive H	% Alive NH	% Dead H	% Dead NH
0	92%	0%	0%	8%
0	92%	0%	0%	8%
0	75%	0%	0%	25%
15	67%	0%	0%	33%
20	67%	0%	0%	33%
25	92%	0%	0%	8%
30	92%	0%	0%	8%
35	58%	0%	0%	42%
40	83%	0%	0%	17%
45	50%	0%	0%	50%

* H-Hatched NH-Not Hatched

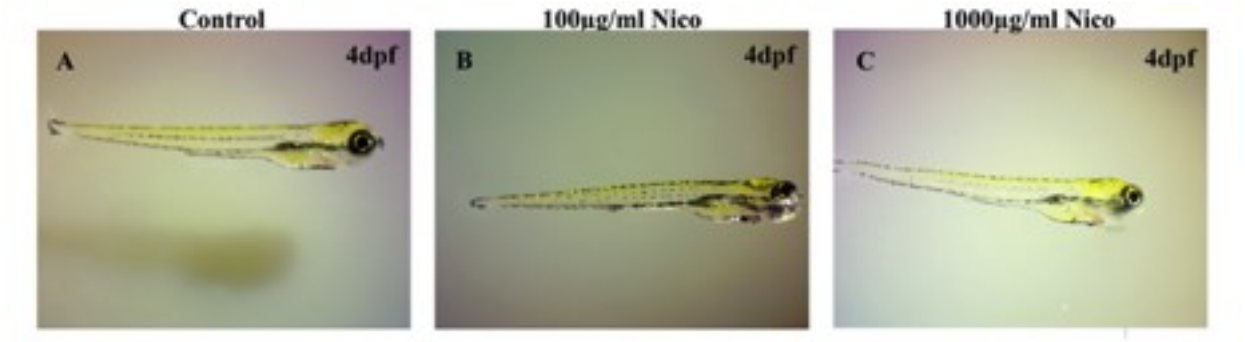

Figure 1. Phenotypes of zebrafish embryos exposed to nicosulfuron. All embryos are at 4 days post fertilization and were imaged at 20X magnification. A: represents embryos exposed to egg water control. B: represents embryo exposed to 100 µg/ml nicosulfuron in egg water. Notice normal development of embryo. C: represents embryo exposed to 1000 µg/ml nicosulfuron in egg water. Notice the slight extended pericardial sac and absence of swim bladder.

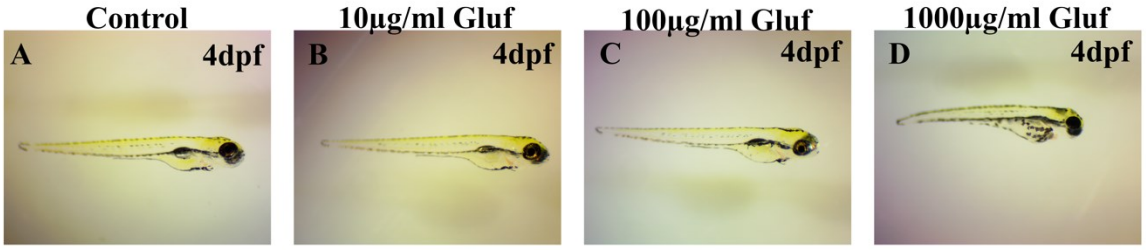

Figure 2. Phenotypes of zebrafish embryos exposed to glufosinate-ammonium. All embryos are at 4 days post fertilization and were imaged at 20X magnification. A: represents egg water control and normal development. B: represents embryo exposed to 10 µg/ml glufosinate-ammonium notice normal development; no morphological deformities. C: represents embryo exposed to 100 µg/ml glufosinate ammonium notice slight extension of pericardial sac. D: represents embryo exposed to 1000 µg/ml glufosinate ammonium notice extension of pericardial sac and absence of swim bladder.

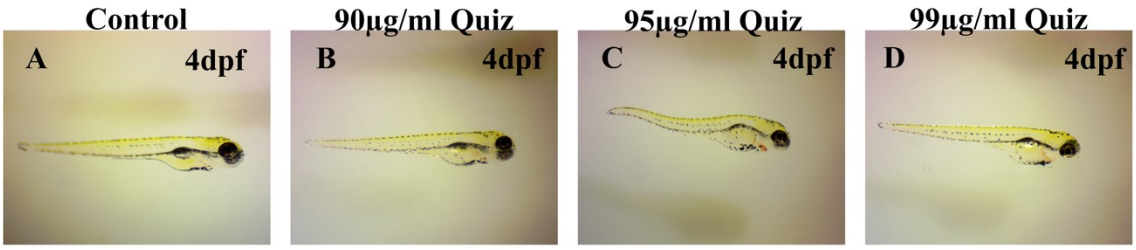

Figure 3. Phenotypes of zebrafish embryos exposed to quizalofop-P-ethyl. All embryos were imaged at 20X magnification and images were taken at 4 days post fertilization. A: represents egg water control embryo with normal development. B: represents embryo exposed to 90 µg/ml quizalofop-p-ethyl in egg water. Notice swim bladder not fully expanded and slight extension of pericardial sac. C: represents embryo in 95 µg/ml quizalafop-p-ethyl in egg water. Notice slight spinal curvature, slightly extended pericardial sac, and absence of swim bladder. D: represents embryo in 99 µg/ml quizalafop-p-ethyl in egg water. Notice absence of swim bladder and slightly extended pericardial sac.

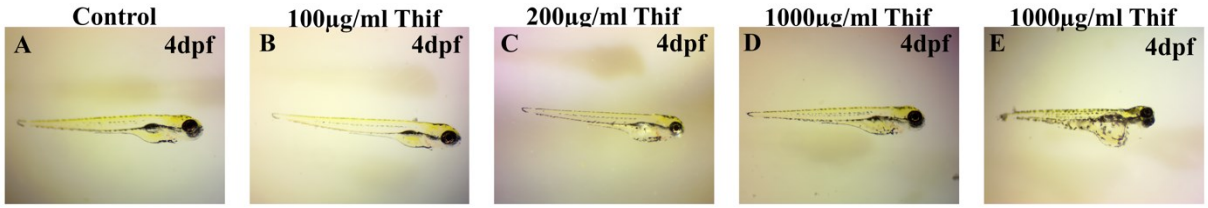

Figure 4. Phenotypes of zebrafish embryos exposed to thifensulfuron-methyl. All images were taken at 22.5X magnification and all embryos were imaged at 4days post fertilization. A: represents an egg water control with normal development. B: represents an embryo in 100 µg/ml thifensulfuron-methyl and egg water with normal development. C: represents an embryo in 200 µg/ml thifensulfuron-methyl and egg water. Notice the extended pericardial sac, white apoptotic tissue, absence of swim bladder, and slight edema. D: represents an embryo in 1000 µg/ml thifensulfuron-methyl and egg water. Notice the extended pericardial sac and absence of swim bladder. This represents mild form of deformities at this concentrations. E: represents an embryo in 1000 µg/ml thifensulfuron-methyl and egg water. Notice the severe extended pericardial sac, severe edema, and absence of swim bladder. This represents severe form of deformities at this concentrations.

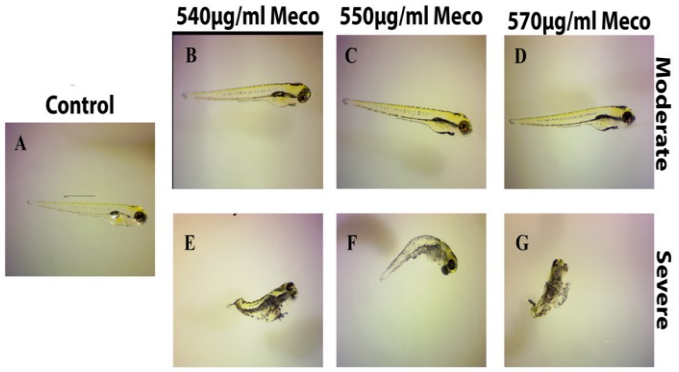

Figure 5. Phenotypes of zebrafish embryos exposed to mecoprop. All pictures were taken at 20X magnification and all embryos were imaged at 4 days post fertilization. Figure is meant to show the difference between the severities of phenotypes in different embryos at the same concentration. A: represents zebrafish embryo in egg water control. B: represents zebrafish embryo in 540 µg/ml mecoprop and egg water with moderate deformities. Notice slight extension of pericardial sac and absence of swim bladder. C: represents zebrafish embryo in 550µg/ml mecoprop and egg water with moderate deformities. Notice the slightly extended pericardial sac and absence of swim bladder. D: represents zebrafish embryo in 570 µg/ml mecoprop with moderate deformities. Notice the absence of swim bladder and slightly extended pericardial sac. E: represents zebrafish embryo in 540 µg/ml mecoprop and egg water with severe deformities. Notice the severe edema, extended pericardial sac, tail malformation, spine deformity, shortened anterior-posterior axis, and discoloration. F: represents zebrafish embryo exposed to 550 µg/ml mecoprop and egg water with severe deformities. Notice the severe edema, extended pericardial sac, spine deformity, and discoloration. G: represents zebrafish embryo in 570 µg/ml mecoprop and water with severe deformities. Notice severe edema, extended pericardial sac, discoloration, tail malformation, and spine deformity.

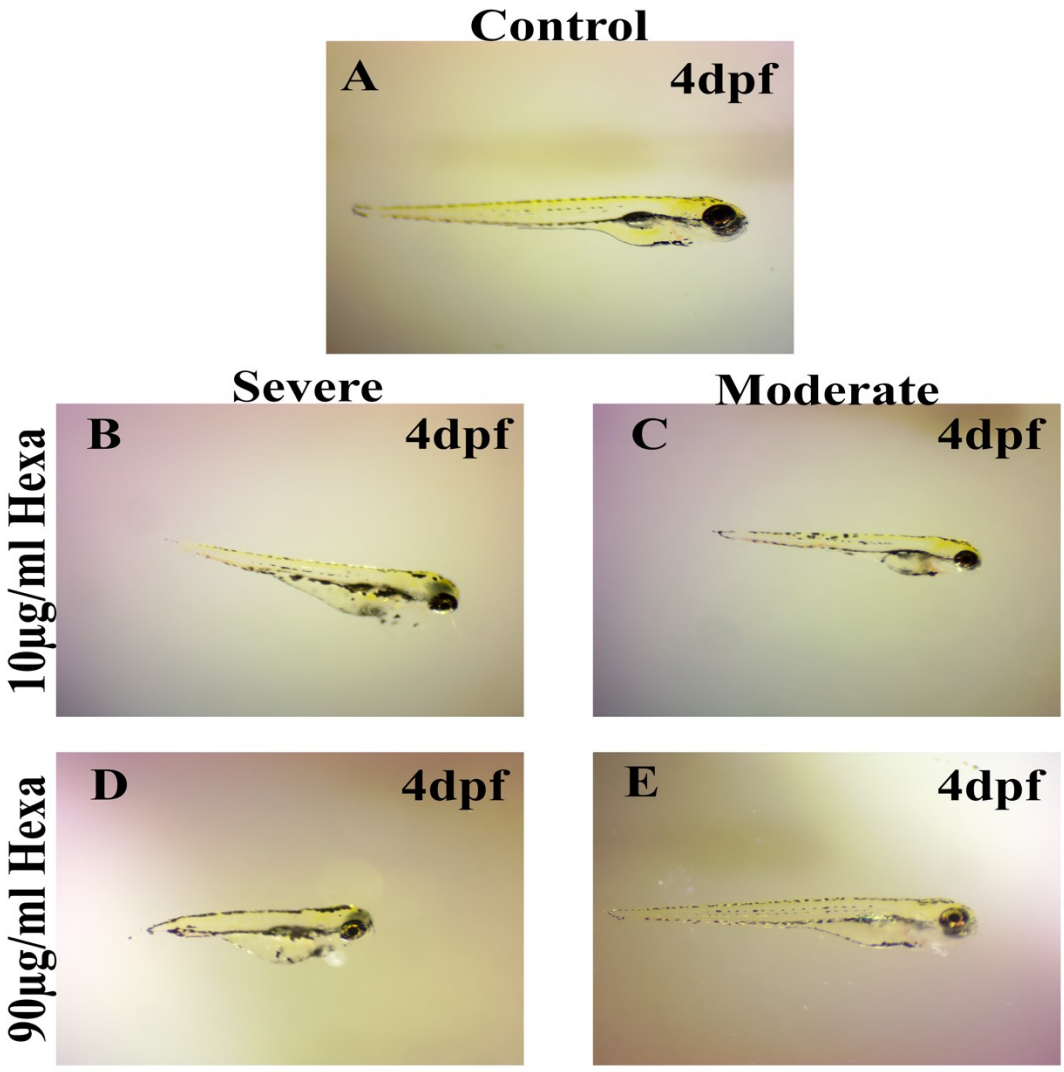

Figure 6. Phenotypes of zebrafish embryos exposed to low concentrations of hexazinone. All embryos were imaged at 2X zoom and were imaged on 4 days post fertilization. The purpose of this figure is to show the difference in levels of deformities within the same concentration of hexazinone. A: represents egg water control embryo and normal development. B: represents severe deformities in zebrafish embryo exposed to 10 µg/ml hexazinone and egg water. Notice the extended pericardial sac, absence of swim bladder, and body edema. C: represents moderate deformities in zebrafish embryos exposed to 10 µg/ml. Notice the slight body edema and absence of swim bladder. D: represents zebrafish embryo exposed to 90 µg/ml hexazinone and water with severe deformities. Notice the discoloration, shortened body length, slight tail malformation, severe body edema, and extended pericardial sac. E: represents zebrafish embryo exposed to 90 µg/ml hexazinone with moderate deformities. Notice the absence of swim bladder and slightly extended pericardial sac.

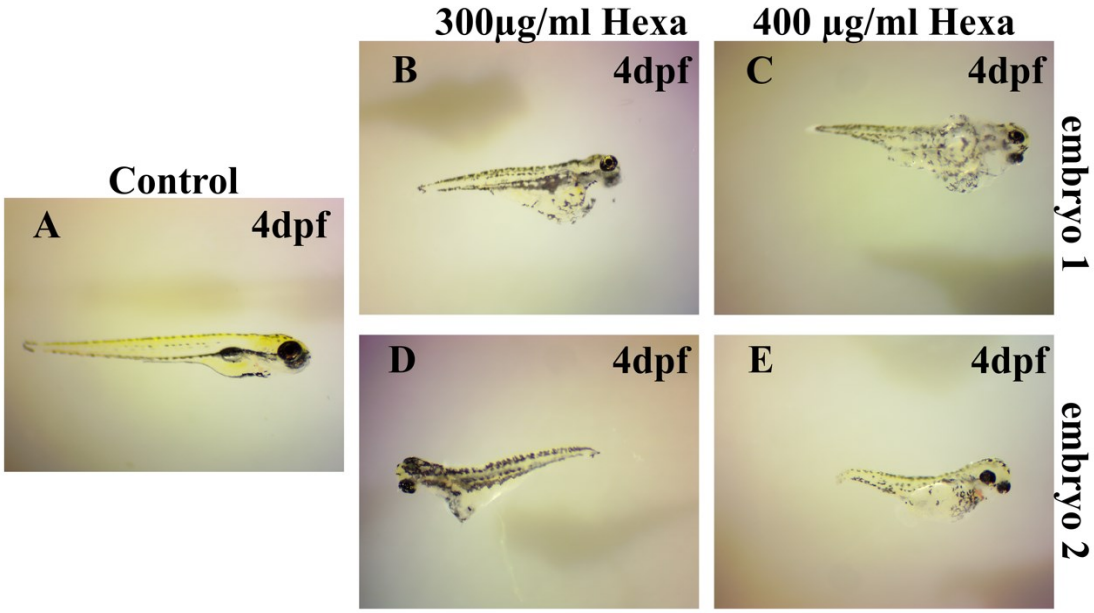

Figure 7. Phenotypes of zebrafish embryos exposed to high concentrations of hexazinone. All zebrafish embryos were imaged at 20X magnification on 4 days post fertilization. A: represents an egg water control with normal development. B: represents zebrafish embryo in 300 µg/ml hexazinone and egg water. Notice the severe body edema, severe extended pericardial sac, microphthalmia, and discoloration. C: represents zebrafish embryo in 400 µg/ml hexazinone and water. Notice severe body edema, discoloration, absence of swim bladder, microphthalmia, apoptotic tissue, and extended pericardial sac. D: represents zebrafish embryo exposed to 300 µg/ml hexazinone and egg water. Notice the spine abnormality, microphthalmia, severe edema, absence of swim bladder, absence of pericardial sac, and discoloration. E: represents zebrafish embryo exposed to 400 µg/ml hexazinone and egg water. Notice the absence of swim bladder, spine deformity, microphthalmia, severe body edema, pericardial sac extension, and discoloration.

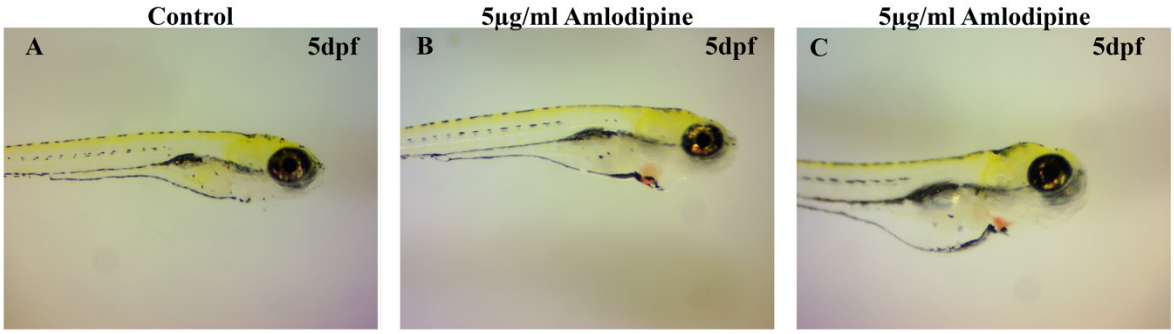

Figure 8. Phenotypes of zebrafish embryos exposed to amlodipine. All zebrafish embryos were measured at 5dpf and at 45X magnification. A: represents egg water control with normal development. B: represents zebrafish embryo exposed to 5 µg/ml amlodipine. Notice lack of heart contraction, absence of swim bladder, and extended pericardial sac. C: represents zebrafish embryo exposed to 5 µg/ml amlodipine. Notice lack of heart contraction, swim bladder is present, extended pericardial sac, and general abdominal edema.

DISCUSSION

Our results indicate that all of the herbicides tested, with the exception of nicosulfuron, led to some form of toxicity or developmental defect. Nicosulfuron is the only herbicide that showed little to no effects at saturation (1000 µg/ml). Thus, based on results from this study, it cannot be determined to be a teratogen. At high concentrations, glufosinate-ammonium treated embryos were able to hatch and to continue developing, but the embryos exhibited cardiac dysfunction. Cardiac dysfunction was determined by observing the quality of heart contractions, as well as the presence of phenotypes observed when the embryonic heart stops beating (failure for swim bladder to inflate, pericardial sac extension, and in some cases abdominal edema). Since heart function is one of a few parameters used to determine mortality, impaired heart function likely caused the increased mortality. Treatment with thifensulfuron-methyl led to cardiac dysfunction at concentrations greater than 200 µg/ml; however variation in the cardiac dysfunction phenotype and mortality made it difficult to determine the LD50. Treatment with quizalofop-p-ethyl (at or near the LD50 of 95 µg/ml) also exhibited cardiac dysfunction, but concentrations greater than or equal to 100 µg/ml increased mortality and decreased hatching rate. This mortality differed from glufosinate-ammonia and thifensulfuron-methyl treatments because the embryonic tissues were apoptotic, and as a result the embryos failed to develop. Thus, a high concentration of quizalofop-p-ethyl was toxic to cells. In summary, four of the six tested herbicides resulted in cardiac dysfunction and/or general toxicity, with no other abnormalities.

Similar to thifensulfuron-methyl, quizalafop-p-ethyl and glufosinate-ammonia, treatment with mecoprop and hexazinone resulted in cardiac dysfunction and increased mortality at high

concentrations. However, mecoprop and hexazinone treatment also led to additional developmental abnormalities not explained by cardiac dysfunction or general toxicity. Though a precise LD50 was not determined, the additional defects observed upon mecoprop treatment was rather diverse including abdominal edema, shortened body axis, spine deformity, tail malformation, micropthalmia, apoptotic tissues and discoloration. Mecoprop mimics the plant hormone auxin, which neither humans or zebrafish express (produce). Therefore, a teratogenic effect would not be expected. Perhaps, the chemical can inhibit another molecule, such as an animal hormone, that causes defects in zebrafish embryo. More studies will need to be done to determine the mechanism of mecoprop on zebrafish embryos. Hexazinone treatment also led to additional developmental abnormalities, some that were similar to mecoprop treatment and some that were new. Similar abnormalities observed were body axis shortening, micropthalmia, and tail deformity. Defects unique to hexazinone treatment were an edema that was located throughout the embryo (and not just in the abdominal region), and the failure for melanocytes to properly align in a ventral stripe. These unique phenotypes suggest that hexazinone may be targeting different biochemical pathways/molecules than mecoprop. Thus, further study will need to be done to find the molecular targets of mecoprop and hexazinone. Hexazinone works by binding to D1 proteins during photosynthesis, which are primary acceptors of the electrons in photosystem II, and a blast search does not reveal homology to any other genes, such as those of the electron transport of mitochondria. Thus, based on this mechanism this herbicide should have no effect on animal cells, however, the potential mechanism in animal cells needs to be investigated.

As far as determining the LD50, we have an established a potential LD50 for one herbicide, quizalofop-p-ethyl. Nicosulfuron has no LD50 due to lack of mortality within the

range of concentrations used in my study. Treatments with other herbicides resulted in different concentrations being the LD50, even when comparing different concentrations of the same herbicide. One explanation for the observed variability could be due to the degradation of herbicides over time. This was observed when treatments at later dates resulted in fewer defects, as compared to earlier dates. Another explanation for the variability could be due to the usage of different lots of the same chemical. This was observed with mecoprop treatments. However, there may be other explanations as well: genetic variation present amongst the embryos, and slight inconsistencies in weighing and pipeting the chemicals, More studies would need to be done in the future using consistent lots and within a shorter period of time to minimize this variance.

A common phenotype observed with thifensulforon-methyl, mecoprop, and hexazinone, was the abdominal edema, a generalized edema and/or a pericardial sac edema. A study done by Hanke et al., suggests that these observed phenotypes in zebrafish are comorbid with renal failure (Hanke et al., 2013). One of the primary and leading indicators of renal failure is generalized edema (Hanke et al., 2013). This edema is caused by collection of fluid in several cavities of the body. Another indicator of renal failure is extended pericardial sac (Hanke et al., 2013). However, both of these signs (general edema and extended pericardial sac) can also be caused by cardiac dysfunction due to increase in hydrostatic pressure. (Hanke et al., 2013). In my study, treating zebrafish embryos with amlodipine (a calcium-channel blocker that prevents heart contraction) resulted in a lack of heart contraction, pericardial sac extension and absence of the swim bladder. However, the treated embryos did not show any signs of generalized edema, suggesting that the abdominal or general edema observed upon thifensulforon-methyl, mecoprop or hexazinone treatment may be due to renal failure. In future studies, extent of kidney function

will be determined by injecting fluorescent dextran into the vasculature of treated zebrafish larvae, followed by confocal imaging to monitor how much of the dextran leaks out through the kidneys.

In my study, all herbicides that showed effects seemed to exhibit cardiac failure in some form. This in large part is why I tested amlodipine in the study, so that a baseline phenotype for cardiac failure could be established. It is not uncommon for teratogens to cause heart defects due to sensitive nature of heart development (Sarmah & Marrs, 2016). There are many types of agents, including environmental, pharmacological, and infectious, that are known cardiotoxic agents. Some examples include ion or calcium inhibitors, which lead to impaired cardiac function. Other cardiotoxic agents cause congenital malformations such as alcohol or thalidomide (Brown, Samsa, Qian, & Liu, 2016). In five of the six treatments, embryonic hearts did develop; however, they exhibited reduced or absent heart contractions. Thus, it is likely that the herbicides do not impair cardiac formation, but they may impair the further differentiation/morphogenesis of the heart and/or heart contraction itself. More studies will need to be done to evaluate how the herbicides cause cardiac dysfunction.

In summary, the results of my thesis reveal that a majority of these six herbicides, which are designed to precisely target plant cells, also have affects in animals by impairing cardiac function and development in zebrafish embryos/larvae. Further, two of the six herbicides have additional teratogenic effects in other tissues/organs not caused by cardiac dysfunction. So, it is clear that these herbicides are indeed teratogens, and they could potentially be harmful to aquatic organisms. Whether the concentrations that causes these teratogenic effects are similar to those found in the environment is not known at this moment. However, even if environmental levels are much lower than those tested in this study, it is unclear what roles bioaccumulation and

biomagnification may have on the production of eggs with higher concentration of herbicides than those found in the water. Thus, it is still valuable to continue evaluating the teratogenic effects of these herbicides, particularly since these six herbicides are currently in use.

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