10-19-2018

# Influence of Salinity, Sunlight, and Sediment on the Toxicity of Pesticides in Three Non-Target Organisms

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# INFLUENCE OF SALINITY, SUNLIGHT, AND SEDIMENT ON THE TOXICITY OF PESTICIDES IN THREE NON-TARGET ORGANISMS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Environmental Sciences

by
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B.S., Lycoming College, 2014
M.S., Louisiana State University, 2016
December 2018

## ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Kevin Armbrust, for all of his help, guidance, and encouragement throughout my Master's and PhD work. He has been such a great mentor since I started at LSU in 2014. I would also like to thank my graduate committee, Drs. Wei Xu, Ralph Portier, Kanchan Maiti, and Susanne Brander and my dean's representative Dr. Kristin Stair for all of their inspiration, support, and suggestions throughout my studies. My committee has been wonderful in helping me achieve my goals and success throughout my research, and I could never thank them enough. Thank you so much to Dr. Greg Lutz (and his students) for all of the help and access to as many crayfish as I needed throughout my research. A huge thank you to Dr. Mel Zimmerman, my undergraduate minor advisor from Lycoming College, for teaching me my ever-growing love for the environment, fish, and bugs as a "Zimmion." I would not have made it to graduate school without his teaching, his encouragement, and his continual support. The Chemistry Department at Lycoming College for was super influential and valuable in providing me with the background knowledge, education, and support I needed for graduate school: Drs. Holly Bendorf, Jeremy Ramsey, Chriss McDonald, and Charles Mahler; and Debbie Smith for continuing to check up on me after I graduated from Lycoming.

I would not have been able to make it through this degree without the support of my lab group, those who are here and have graduated: Dr. Parichehr Saranjampour, Jess Landry, Dr. Laura Basirico, Dr. Tammy Estes, Brenda Martinez, Xavier Poole, Emily Wall, and Brendan Marsh and the student workers who have come through the lab, especially Michael LaNasa and Maggie Knight. My past roommates, fellow graduate students, and best friends, Sarah Wood and Kara Callicott – thank you for always being there. They have all been such great colleagues

and friends. Charlotte St. Romain, Katie Grimes, Dr. Vince Wilson, and Christina Casey; thank you and I have enjoyed working with all of you throughout my assistantship and with recruiting. A huge thank you to Thaya Guedry from the Vet School's GeneLab for all of her help within my last few weeks to finish my PCR plates.

The support of my friends has been amazing the past 4½ years at LSU, especially Dacin Kemmerer for always being there no matter how busy she is and always editing my papers from 1,000 miles away. I cannot thank them enough for all of the support and encouragement. A huge thank you to Ryan Nolan, who has supported me and encouraged me throughout my Ph.D. journey. The biggest thank you, appreciation, and love to my family for supporting me and cheering me on for 8½ years of undergrad and grad school… thank you and I know how happy my mom and dad are that I am finally going to be graduating for the last time! I could not have achieved my goals of obtaining a Ph.D. without everyone who has helped guide and encourage me along the way.

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## ABSTRACT

Pesticides used in the United States must undergo registration by the United States Environmental Protection Agency (USEPA), after a multitude of analyses ranging from environmental fate to aquatic toxicological impacts to human risk exposure. Testing varies for each chemical, some requiring more testing than others. In many cases, environmental factors are restricted in the analysis of chemical behavior and organismal testing is limited to larvae. Many pesticides are formulated to breakdown in the environment by means of photolysis, hydrolysis, or oxidation, either to ensure low-persistence, limited transport, or to form the active ingredient (pro-pesticides). Environmental influences on chemical behaviors include salinity, sunlight, water, sediment, nutrients, etc. and can increase or decrease the half-life, persistence, formation and degradation of intermediate products, bioaccumulation, and toxicological effects to non-target organisms. Regulatory agencies often do not account for nontraditional aquatic toxicity testing, though research institutions will undergo extensive studies with nontraditional yet environmentally relevant scenarios. Two pesticides and one pesticide-degradation product were chosen to compare and assess the impacts sunlight, salinity, and sediment impose on the degradation, dissipation, and toxic response of three aquatic organisms (fathead minnows, inland silversides, and red swamp crayfish) in various environmental scenarios. Chemically, the pesticides appear to follow [similar] trends with external factors accounted for; toxicologically, no model or trend appears to remain consistent or observed across the chemicals analyzed. Therefore, the need for further investigations for potential impacts to aquatic organisms due to pesticide exposure is appropriate when managing registration processes.

# CHAPTER 1. INTRODUCTION

Pesticide use in agriculture dates back to ancient Greece and Rome with the application of biological and chemical agents to rid pests from crops. Advances in technology have led to more sophisticated chemicals to be used in agriculture, with an increase in usage which began with the synthesis of DDT in the 1940s (Crosby, 1998; Rathore et al., 2012). As the global demand for food increases, so does the need for high crop yields in designated agricultural lands. The use and the need for pesticides to enhance crop yields and health is at the global level, and therefore the registration of new pesticide formulations is occurring regularly. Over the last 10-15 years, more efficient pesticides have been developed due to technological advances in the agrochemical industry. New pesticides are continuing to be developed to sustain success against pesticide-resistant species and to decrease the risk of adverse impacts on non-target organisms by means of lower application rates or modifying formulations. The registration of a pesticide in the United States requires environmental testing of the pesticide formulation and active ingredients, and in many cases ecotoxicological analyses are included in testing. Many regulatory studies of chemicals, including but not limited to pesticides, are laboratory studies that do not account for external factors that occur in natural ecosystems such as sunlight, type of sediment, salinity, dissolved organics and/or solids, or nutrients and the impacts that these factors can potentially have on the behavior of chemicals.

## 1.1. Background research

The United States Environmental Protection Agency (US EPA) requires environmental fate assessments for pesticides proposed for registration for use on crops in the United States. The requirements for testing are chemical-dependent and not all pesticides require the same analyses for registration. Typical testing for pesticide registration includes toxicological

analyses on non-target organisms; freshwater aquatic species (fathead minnows, *Daphnia magna*, and rainbow trout), estuarine/marine species (mysid shrimp, inland silversides, and sheepshead minnows), sediment-dwelling species (eastern oysters), and terrestrial invertebrates (honey bees). These are among the most commonly used organisms in toxicological analyses (USEPA, 2002). For aquatic toxicity testing, many assays are acute (96 hr $LC_{50}$) analyses at the larval stage (0-7 days post-hatch). When chronic studies are completed, they consist of analyses ranging from 30-day flow-through systems to partial- or full life-cycle studies. Chronic assays are extensive and time consuming; therefore, they are not absolutely required for each pesticide undergoing registration, rather they are only conducted when a proposed pesticide has been shown to be resistant to degradation. A suite of environmental fate studies supporting pesticide registration include aqueous photolysis, hydrolysis, soil metabolism, and water-sediment degradation experiments that assess the persistence of a pesticide in a particular ecosystem (USEPAa).

EPA also requires reregistration of pesticides; an amendment in the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) in 1988 required an assessment of reregistration eligibly every 15 years to correct for missing environmental fate and toxicological data for pesticides that were registered prior to 1984 and to insure data requirements meet modern regulatory standards. Reregistration eligibility decision (EPA RED) documents are available through the EPA as a summary of the reports and testing the pesticides have undergone to maintain their registration for application in the United States (USEPAb).

Gaps in knowledge exist on how a pesticide may interact with an organism or within an ecosystem exist due to registration studies not accounting for environmental parameters that may be important to the behavior of a particular chemical in a particular environment. All pesticides

undergo field-testing and laboratory testing prior to registration, however the behavior of the pesticide may vary dependent upon field characteristics (i.e. soil type, nutrient input, water and soil pH, etc.). In additional to local variability, current regulation does not account for important variables that may be important to a particular chemical in a particular location. For example, chemicals can exhibit dramatically different behavior in aqueous systems as a function of salinity, however this is not considered in current studies. Additionally, while the potential of a chemical to degrade photochemically is a required study, the potential for it to be phototoxic to aquatic organisms is not assessed in any current registration requirement.

## 1.2. Research objectives

The purpose of this dissertation was to use environmental factors including sunlight, salinity, and sediment in addition to toxicological analyses to account for differences in organismal responses with and without said factors to pesticide exposure.

The fungicide dicloran, the herbicide benzobicyclon, and the degradation product (4-hydroxychlorothalonil) of the fungicide chlorothalonil were chosen for chemical analysis. Dicloran has previously been shown to photolyze and the distribution of its photodegradation products were impacted by salinity, therefore dicloran is used as a potential model pesticide for chemical behavior and toxicological responses by aquatic organisms (Vebrosky et al., 2018). Hydroxychlorothalonil is the primary soil degradation product of the fungicide chlorothalonil, and it has been reported to photodegrade by similar mechanisms and through a comparable proposed degradation pathway as dicloran (Armbrust, 2001). Therefore, the potential for similarities in organismal response to dicloran and hydroxychlorothalonil exposure exists. Benzobicyclon is a recently registered rice herbicide for use in California and is anticipated to be registered in Louisiana. It has been reported to hydrolyze quickly to form the active ingredient

benzobicyclon hydrolysate, and benzobicyclon hydrolysate has shown to photodegrade. The risk for aquatic species such as crayfish to be exposed to benzobicyclon [hydrolysate] is high, as crayfish thrive in Louisiana rice fields (Williams et al., 2016; 2017; 2018). Photodegradation in aqueous medias including distilled water, artificial seawater (ASW; 0.5, 1.2, or 2.5%), and/or rice water were considered in the analysis of the pesticides. The introduction of sediment as an influencing factor to photodegradation rates, potential dissipation, and resuspension of the pesticides was also considered.

The use of red swamp crayfish (*Procambarus clarkii*) as a toxicity testing species is appropriate because it is an ecologically valuable species but also economically valuable for regions such as south Louisiana. While crayfish are not a typical species used in regulatory toxicity testing, research studies have previously used them on a case-by-case basis (Muncy et al., 1963). Standard toxicity assay species commonly used in regulatory studies include fathead minnows (*Pimphales promelas*) and inland silversides (*Menidia beryllina*). Fathead minnows are a freshwater fish, while inland silversides are a euryhaline fish species that can tolerate salinities from 0.0-3.0% therefore they are a model species to account for salinity as an influencing factor in their response to pesticide exposure.

Juvenile organisms were chosen as the age of test species due to their minimal pigmentation and that they are considered a sensitive population (Mohammed, 2013). Phototoxicity may occur as a result of multiple mechanisms including internal degradation of the chemical after uptake by the organism due to light penetration through the lightly pigmented test species (Speiser et al., 2014). Many juvenile populations are found in shallow-water areas, where light attenuation is less than in waters deeper than 2 meters and the potential for UV-light to activate chemicals or impact organisms exists (Brito et al., 2013; Vinagre et al., 2018).

While salinity and sunlight have are known to impact the degradation of a chemical, the question remains about the potential for photodegradation to be directly correlated to the potential for phototoxicity of the chemical to aquatic organisms. The following statements suggest overarching the hypotheses for this dissertation:

1. Sunlight will enhance the toxicity of dicloran to three non-target aquatic organisms.
2. Salinity will enhance the phototoxicity of dicloran to inland silversides.
3. Dicloran is a model pesticide for chemical and toxicological responses for degradation and toxic responses with salinity and sunlight enhancements; 4-hydroxychlorothalonil and benzobicyclon [hydrolysate] undergo photolysis and therefore can possess phototoxic results, both lethal and sublethal, to aquatic organisms.

In order to examine these hypotheses, three non-target aquatic organisms (fathead minnows, inland silversides, and red swamp crayfish) were exposed to dicloran with and without the presence of artificial sunlight to adopt a standard model of dicloran and its phototoxic potential; three salinities were chosen for the analysis with inland silversides to monitor any potential variation in response as a result of salinity. The model of dicloran as a phototoxic pesticide was applied to inland silversides exposed to 4-hydroxychlorothalonil and red swamp crayfish exposed to benzobicyclon [hydrolysate], as both pesticides have previously been reported in literature to degrade via photolysis (Armbrust, 2001; Williams et al., 2018). Specific objectives and hypotheses that guided this work were as follows:

Objective 1: Determine the distribution of dicloran in simulated shallow water (water-sediment) systems under the influence of light and salinity.

$H_0$: Dicloran will persist longer in water-sediment systems than water only systems, with the sediment acting as a reservoir for dicloran.

$H_1$: Dicloran will distribute into sediment at higher levels in seawater systems than in freshwater systems, as observed for other organic molecules.

Objective 2: Determine the potential for dicloran to be phototoxic to a regulatory freshwater fish species.

$H_0$: Dicloran will be phototoxic to fathead minnows as its photodegradation products are similar to other compounds that have elicited phototoxicity.

$H_1$: Surviving organisms will show sublethal impacts resulting from phototoxicity.

Objective 3: Determine the potential for dicloran to be phototoxic to an estuarine fish species.

$H_0$: Dicloran will be phototoxic to inland silversides as its photodegradation products are similar to other compounds that have elicited phototoxicity.

$H_1$: Dicloran will be more phototoxic at higher salinities.

$H_2$: Surviving organisms will show sublethal impacts resulting from phototoxicity.

Objective 4: Determine the potential for a compound with a similar degradation pathway, 4-hydroxychlorothalonil (4-OH-CHT), to be phototoxic to an estuarine fish species.

$H_0$: 4-OH-CHT will be phototoxic to inland silversides as it has a similar degradation pathway to dicloran.

$H_1$: Surviving organisms will show sublethal impacts resulting from phototoxicity.

Objective 5: Determine the potential for dicloran to be phototoxic to a freshwater benthic aquatic invertebrate (crayfish).

$H_0$: Similar to the above, dicloran will be phototoxic to crayfish.

$H_1$: Dicloran will be less phototoxic to crayfish in comparison to fathead minnows.

$H_2$: Dicloran will show sublethal impacts in gene expression resulting from phototoxicity.

Objective 6: To use this system to assess the potential of a new pesticide, benzobicyclon [hydrolysate] (BZB, BH), to be phototoxic to crayfish.

$H_0$: BZB/BH will be phototoxic to crayfish as it does photodegrade.

$H_1$: BZB/BH will show sublethal impacts in gene expression resulting from phototoxicity.

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# CHAPTER 2. LITERATURE REVIEW: CHEMICAL PROCESSES, PESTICIDES, AND ORGANISMS

## 2.1. Pesticide use

Pesticides include many classes of chemicals and are composed of three major groups comprising herbicides, fungicides, and insecticides, that are used to regulate the spread and persistence of deemed pests (Aktar et al, 2009). Pests can include insects, fungal diseases, viruses, weed species, pathogens, as well as organisms present in locations they do not belong, such as outbreaks of zebra mussels (*Dreissena polymorpha*) in the Great Lakes (Fernandez-Cornejo et al., 2014). The use of chemical and synthetic pesticides has increased since the introduction of DDT (dichlorodiphenyltrichloroethane) in the 1940s, securing food supplies for the increasing growth in global population. Prior to DDT, pesticide use was often biological or by application of metals such as arsenic. Early writing by Homer describes the use of sulfur to control pests in ancient Greece, and of hellebore (*Veratrum alba*) used by the ancient Romans (Crosby, 1998). Pesticides benefit humans by means of crop health and yield by decreasing the risk of crop loss and harm due to pests and diseases, but they have unfortunately harmed terrestrial and especially aquatic ecosystems as a side effect (Rathore et al., 2012).

The increased use of pesticides has caused direct and indirect impacts to human health and biodiversity. Pesticides have the ability to bioaccumulate, biomagnify, or bioconcentrate throughout ecosystems. Dependent upon its mode of action, pesticides can cause great harm to non-target organisms as a result. Climate change will continue to have impacts on the productivity of agricultural commodities. Increasing temperatures, salinity changes, saltwater intrusion, and land loss or coastal erosion will impact agricultural lands, water sources, water salinity and pH, the amount of available freshwater for use on crops, and the tolerance of crops grown in regions based upon temperatures and underlying conditions. With environmental

variations in ecosystems, the use of pesticides has the potential to continue to increase crop health and high yields to maintain a sustainable future (Rathore et al., 2012).

## 2.2. Light influences on chemical behavior

Chemical compounds have the potential to absorb energy from ultraviolet (UV) light and visible light dependent upon their physicochemical properties. Often referred to as photodegradation or photolysis, this process is the degradation of a chemical as a result of exposure to UV-light. Chemicals transform from their stable, ground-state to an unstable, excited-state when the molecules absorb photons of light and proceed to break down as a result of the energy transfer (Hage et al., 2011). This can be in combination with other degradation processes like hydrolysis or oxidation (Crosby, 1998; Rathore et al., 2012). Hydroxyl radicals (•OH) in water can react with chemicals that enter surface waters; hydroxyl radicals are considered an oxidant and can be generated by a photochemical reaction. The hydroxyl radicals act as scavengers to dissolved organic matter and ions in water; indirect photolysis and photodegradation may occur and generate •OH, which may further hydrolyze or photolyze. Most oxidants are generated as a result of sunlight exposure (Crosby, 1972). Hydroxyl radicals form photochemically and often react with organic pollutants, but can also react with inorganics (Crosby, 1998).

In some cases, chemicals such as pesticides are designed to degrade by processes such as hydrolysis and photolysis to form their active ingredient. These pesticides are referred to as "pro-pesticides." These "active" ingredients may not be stable in their applied form and degrade to form the stable biologically desired active ingredient.

UV radiation occurs between 290-400 nm, and is sorted into the classifications, UVA, UVB, and UVC (Figure 2.1) (CA LightWorks). UVB is considered the more energetic

classification and more strongly absorbed (290-320 nm) (Larson et al., 1988). Photodegradation occurs as a result of both direct and indirect photolysis. Direct photolysis occurs as a result of chemical absorption of light energy at wavelengths greater than 290-300 nm (USEPA, 2008a). Indirect photolysis occurs in a few forms; energy transfer is a common source for indirect photolysis to degrade pesticides, for example the transfer of light energy can result in the oxidation of the compound. Photolysis typically occurs as a result of a combination of both direct and indirect factors (Crosby, 1998; Rathore et al., 2012; USEPA, 1998a, b).

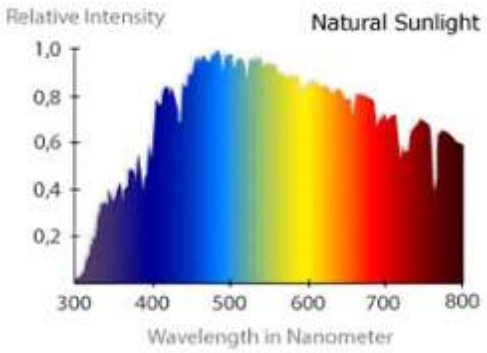



Figure 2.1. Spectrum of natural sunlight (300-800 nm).

Pesticides such as pentachlorophenol (PCP), 3,4-dichloroaniline (DCA), thiobencarb, and nitrofen have previously been reported to undergo photolysis by a variety of pathways including photonucleophilic substitution and photoreduction. Photonucleophilic substitution occurs as a result of a nucleophile, an electron rich species (example: hydroxide, amine, or cyanide), attacking a light-activated aromatic ring in its excited state (Crosby, 1998). The photodegradation of PCP has been reported in distilled water as a result of photonucleophilic substitution which displaces the chlorine substituent for a hydroxide on the ring structure (Wong et al., 1981). Nitrofen (2,4-dichlorophenyl p-nitrophenyl ether) is an herbicide reported to photodegrade as a result of photonucleophilic displacement of the nitro group by a hydroxide (Nakagawa et al., 1974). Thiobencarb, on the other hand, does not directly absorb UV-light;

instead, when exposed to sunlight, it is oxidized and degrades as a result of indirect photolysis (Crosby, 1998).

Anthropogenic chemicals, such as pesticides, are not the only types of compounds/materials that can undergo photolysis. In aquatic systems, DOM (dissolved organic matter) and DOP (dissolved organic phosphorus) also can absorb the energy from UV-light and undergo photolysis. As DOM photodegrades, it breaks down into smaller organic constituents. These degradation products are commonly more bioavailable to aquatic organisms than the DOM itself. The degradation products may also behave similar to •OH and act as scavengers to other compounds in the waters. While photolysis typically degrades organic pollutants such as pesticides in receiving waters into less toxic compounds, in some cases more toxic degradation products may form as a result of photodegradation (Reddy et al., 2008; USEPA, 2008b).

### 2.3. Salinity influences on chemical behavior

Seawater is composed of various anions and cations that interact with chemicals and organisms that inhabit saline waters. By definition, [surface] seawater consists of 11 major ions: chloride ($Cl^-$), sulfate ($SO_4^-$), sodium ($Na^+$), potassium ($K^+$), magnesium ($Mg^{++}$), calcium ($Ca^{++}$), strontium ($Sr^{++}$), fluorine ($F^-$), bromine ($Br^-$), bicarbonate ($HCO_3^-$), and boric acid ($B(OH)_3$) (Stumm et al., 1981). These ions can interact with chemicals that enter water and influence degradation, degradation product formation, and overall behavior.

Salinity has been reported to impact bioaccumulation factors (BAF, log $K_{ow}$), half-lives ($t_{1/2}$), degradation rate, degradation product formation, overall degradation, and composition of chemicals (Crosby, 1998; Saranjampour et al., 2017; Vebrosky et al., 2018). $K_{ow}$ values, or the water-octanol partition coefficient, are the ratio of chemical that partitions between water and 1-octanol, which is a representation of fatty tissue as you would find in an organism. Salinity has

been shown to influence the bioavailability of chemicals, metals, and ions in waters (Crosby, 1998). Both temperature and salinity have previously been reported to impact the solubility of chemicals such as polycyclic aromatic hydrocarbons (PAHs), however there does not appear to be uniformity to this rule even within a chemical class. Solubilities of PAHs such as anthracene have been shown to be insensitive to minor changes in salinity but highly sensitive to changes in temperature while the solubility of 1,2-benzanthracene on the other hand appeared to be influenced by small variations in salinity; and solubility and $K_{ow}$ values of PASHs such as dibenzothiophene are also impacted by salinity changes (Saranjampour, 2017; Whitehouse, 1984).

Previously mentioned pesticides that are known to be influenced by sunlight may also be impacted by salinity. PCP, 4-hydroxychlorothalonil, and dicloran have all previously been reported to have salinity-dependent behaviors. While salinity did not appear to impact the degradation rate of dicloran at 60 $W/m^2$ light intensity, the distribution of intermediate photoproducts (2-chloro-1,4-benzoquinone – CBQ, and 1,4-benzoquinone – BQ) were impacted by salinity; in 32 ppt (3.2%) seawater, CBQ was measured at twice the concentration than in distilled water (Vebrosky et al., 2018). In freshwater, PCP has a half-life of 0.9 hr in distilled water and 2.3 hr in seawater (Miille et al., 1983; Wong et al., 1981). The photodegradation DCA did not result in differences between distilled water and seawater, with half-lives of 17.2 and 17.3 hr respectively, but variation in the distribution of photoproducts was observed (Miller et al., 1979).

The insecticide diazinon was monitored for its photodegradation in filtered river water and seawater, and it was reported to degrade faster in river water (43 days) compared to seawater (47 days). Dimethoate is significantly impacted by salinity with a reported half-life in river

water of 29 days and 74 days in seawater, possibly a result of indirect photolysis. Natural waters contain humic acids and other organic pollutants that interact with pesticides and can increase or decrease degradation rates (Hall et al., 1995; Lartiges et al., 1995).

### 2.4. Sediment influences on chemical behavior

Sediment type and properties vary vastly dependent upon region, season, climate, and external influences. Soils consist of different volumes of matter (i.e. sand, silt, clay, organic materials), water, and air; this composition defines if it is organic or mineral soil dependent upon the composition (Crosby, 1998). Common properties and characteristics of sediments include % sand, % silt, % clay, % moisture, pH, bulk density, mineral content, and aerobic or anaerobic characteristics. These physical, chemical, biological, and geological characteristics of sediments have the potential to influence chemical behavior (Reddy et al., 2008).

Chemicals in sediments can degrade via similar influences and processes as described in section 2.2. Oxidation in sediments is a common degradation process in oxygenated soils and sediments; microbially mediated degradation is also a common degradation process in sediments. Reduction, or the gain of electrons, occurs in sediments when anaerobic conditions favor these processes (Crosby, 1998). Photodegradation on soil and sediment surfaces is a possible although it is difficult to measure in the laboratory. Light attenuation is high within the first 0.2 mm of the soil/sediment surface, therefore photodegradation within soils is much lower than in surface waters or at the sediment-water interface (Reddy et al., 2008).

The sediment-water interface is regulated by microbial and chemical processes circulating chemicals or elements between sediment and water. Nitrogen, oxygen, iron, sulfur, manganese, and carbon are a few of the elements that greatly influence the chemical fluctuation and cycling between water and sediment; the influence of pH, redox potential, and microbial and

biological activities also play a role. Abiotic reactions such as hydrolysis and photolysis commonly occur at the sediment-water interface, impacting chemical behavior. Bioturbation as a result of macroinvertebrate activity mix and interact with chemicals that are potentially found in the sediment-water interfaces (Crosby, 1998; Reddy et al., 2008).

Some pesticides are applied directly to sediments, while others reach sediment as a result of runoff, spray drift, or atmospheric deposition. Chemicals, including pesticides, move through soils or sediment by mechanisms including dissolution, adsorption, and as vapor. The potential for mobility once in sediment varies on the physicochemical properties of chemicals; chemicals can leach, or they can move after they absorb to particles in the sediments due to sediment transport (Crosby, 1998; USEPA, 2008c). The interactions of pesticides in sediments and their potential to be transported throughout the waters is highly variable between specific pesticides yet important to understand. Pesticides that bind to sediments are unlikely to be transported via waters, but the potential for sediment transport is a risk. Benthic species influence and are affected by the fate of pesticides in sediments as a result of disturbances of the sediment or possible ingestion by a detritivore. The degradation of a pesticide once in sediments or soils may be microbially mediated, photochemically mediated, a result of reduction or oxidation, or it may persist (Pionke et al., 1973; USEPA, 2008b).

### 2.5. Phototoxicity

When chemicals absorb photons of light, the energy transfer causes the compound to enter the excited state and organisms in contact with the activated chemical may show phototoxic responses upon exposure. Phototoxicity can be defined as, "the result of direct cellular damage following exposure to UV radiation (or visible light) in the presence of a [phototoxic] substance" (Maibach et al., 2014). Many chemicals appear nontoxic without sunlight, but when sunlight is

introduced into the experimental design, the result is phototoxicity and often death for aquatic organisms (Crosby, 1998). The uptake of a UV-activated chemical by an organism can initiate cellular damage, such as DNA damage (Larson et al., 1988).

Phototoxicity may be a result of multiple circumstances including, (1) the activation of the chemical due to energy transfer after absorption of photons of light causing cellular or DNA damage to organisms, (2) juvenile, transparent, or low-pigmented organisms may uptake the chemical and light has the potential to penetrate through their "skin" or outer barrier, if the chemical passes through the organisms' defense lines (i.e. gills) and enters the blood stream or organs, the chemical may photodegrade within the organism itself, (3) absorption of photons by the chemical causes the chemical to break down through a photodegradation pathway generating intermediate degradation products that have the potential to be more toxic and harmful to organisms than the parent compound. Not all chemical compounds that photodegrade are phototoxic, but the potential for phototoxicity exists as a result of the photodegrading compounds. The result of bioaccumulation of an activated chemical by an aquatic organism is referred to as photosensitization, this is a result of photoenhanced toxicity (Barron et al., 2001; Barron, 2007; Crosby, 1998; Larson et al., 1988; Lee, 2003; Pelletier et al., 1997).

Extensive studies have been completed on the potential phototoxic responses of aquatic organisms, specifically marine organisms, exposed to oil and oil constituents in the presence of sunlight. Polycyclic aromatic hydrocarbons (PAHs) are a class of organic chemicals generally associated with oils; while PAHs encompass a large class of chemicals, a few commonly known PAHs include benzo[a]pyrene, benz[a]anthracine, and benzo[a]fluorene (Crosby, 1998; Harvey, 1991; Lyons et al., 2002). PAHs are known to be persistent, especially in marine environments, lipophilic, are often toxic, carcinogenic, and genotoxic to organisms, and have shown to

bioaccumulate (Alloy et al., 2011; Daskalakis et al., 1995; McDonville et al., 2018; Newsted et al., 1987; Pelletier et al., 1997; Yu, 2002).

Many laboratory-designed [photo]toxicological studies use fluorescent light and do not consider UV-light in the experiments, although it has been reported that PAH phototoxicity can increase up to 1000-fold when UV-light is taken into consideration (Barron et al., 2001; Pelletier et al., 2001). PAH phototoxicity to aquatic organisms is likely a result of cellular damage to the respiratory system, such as oxidative stress (McDonald et al., 2002; Oris et al., 1985). Environmental elements such as humic acids have been shown to influence the potential for phototoxicity of PAHs to aquatic organisms; the presence of humic acids appear to decrease the phototoxic response of fluoranthene to fathead minnows (*Pimephales promelas*) by decreasing bioacculuation of the chemical (Weinstein et al., 1999).

Barron *et al.* determined a risk for increased phototoxicity to organisms inhabiting Prince William Sound and the Gulf of Alaska following the Exxon Valdez oil spill, despite the increased latitude for the location of the spill. While light intensity, including UV intensity, decreases with an increase in latitude, the potential for photoenhanced toxicity exists at these higher latitudes, which results in aquatic organisms to be at risk for the effects of exposure to UV-activated oils. Larvae are most susceptible, as they are a sensitive population, and juveniles are also more at risk than adults due to lacking pigment as a result of immaturity, inability to move (larvae and juveniles lack the ability to migrate from an oiled area), and larvae and juveniles typically inhabit shallow regions which oil may accumulate in and light penetration is greater in shallow regions opposed to deeper waters (Barron et al., 2001). Other studies conducted by Barron *et al* used larval Pacific herring (*Clupea pallasii*) and juvenile pink salmon (*Oncorhynchus gorbuscha*), economically valuable marine species, to analyze the effects of

exposure to weathered crude oil from the Exxon Valdez spill. Exposure to oil and UV-light resulted in greater than 90% mortality at 87 µg/L dose for 4 days for Pacific herring (Barron et al., 2003). The pink salmon used in the study were highly pigmented and showed no indicated of increased mortality after exposure to oil and UV-light. This suggests pigmentation may act as a barrier to light penetration and lesser pigmented juvenile fishes and invertebrates may be more at risk for photoenhanced toxicities (Barron et al., 2005). Most PAH phototoxicity investigations use marine organisms, therefore salinity is an influencing factor; Barron *et al.* used sediment as an influence on bioaccumulation to assess the potential for PAH phototoxicity. While benthic organisms typically have less UV-exposure due to their habitat, the risk for activated chemicals (PAHs) exists. It was determined that benthic organisms are less susceptible to phototoxicity, despite PAHs previously measured in marine sediments (Barron et al., 2007; Hemmer et al., 2010a, b; Wade et al., 2008).

Pelletier *et al* selected three PAHs, anthracene, fluoranthene, and pyrene, to analyze the potential phototoxicity to juvenile and larvae mysid shrimp (*Mysidopsis bahia*) and dwarf surf clam (*Mulinia lateralis*). Increased toxicities were observed for both species exposed to the PAHs and UV-light, with all concentrations of PAHs below the solubility limit; previous analyses have shown toxicities at levels above the water solubilities and therefore when UV-light is considered for the laboratory studies, environmentally relevant concentrations appear to cause phototoxic responses (Pelletier et al., 1997).

Latitude plays an important role in the potential for phototoxicity due to the intensity of light varying from lower to higher latitudes. There is an increase in light intensity in lower latitudes compared to higher latitudes, and therefore there is a higher intensity of UV-light in these regions. Alloy *et al* reported the impacts of Deepwater Horizon oil on Mahi-mahi

(*Coryphaena hippurus*), an important sport fish found at latitudes at or below 30°. In comparison to the previously mentioned studies by Barron *et al* in using Exxon Valdez oil, and the spill location in the Gulf of Alaska and Prince William Sound, organisms in the Gulf of Mexico where the Deepwater Horizon spill occurred would be exposed to higher intensities of UV-light due to the latitudinal locations. Hatching rate ($EC_{50}$) of Mahi-mahi embryos was used as a measurement of phototoxic response, and a decrease in hatching was observed in all treatments of UV and PAH exposure (Alloy et al., 2011).

Pesticides have also shown similar phototoxic potentials as PAHs to aquatic organisms but there is less available published data on pesticide phototoxicity compared to PAHs. Irradiated dicloran has been reported to influence the heartbeat rate and relative gene expression (mRNA) of defensin, heat shock protein, and calmodrin to cardiomyocyte cultures from eastern oysters (*Crassostrea virginica*) (Xu et al., 2018).

### 2.6. Fungicides

Fungicides are a specialized group of pesticides that target fungal diseases. Many classes of fungicides with various modes of action exist, including: alkylenebis(dithiocarbamate)s, anilinopyrimidines, antibiotics, aromatic hydrocarbon derivatives, azoles, benzimidazoles, carboxamides, dicarboximides, dimethyldithiocarbamates, dinitrophenol, guanidines, morpholines, organophosphorus, phenoxyquinoline, phenylamines, phenyl carbamates, pyrimidines, and *N*-trihalomathylthio. Carboxamide fungicides, such as flutolanil, are a systemic class of fungicides that inhibit succinate dehydrogenase by interfering with fungal respiration (Roberts et al., 1998B).

Two commonly used fungicides were chosen for analysis in this dissertation, dicloran and chlorothalonil, which were both first registered in the 1960's in the United States.

### 2.6.1. Dicloran

Dicloran, 2,6-dichloro-4-nitroaniline (Figure 2.2), is a substituted aniline fungicide, currently manufactured under the trade name Botran 5F® by Gowan Company, LLC (Gowan). It is registered for use on crops such as grapes, lettuce, celery, sweet potatoes, and tomatoes throughout the southern and western United States and Hawaii via chemigation, sprinkler irrigation, areal spray, and dip tanks to inhibit fungal spore germination of species such as *Botrytis spp., Montilinia spp., Rhizopus spp.,* and *Sclerotinia spp*. (USEPA, 2006).

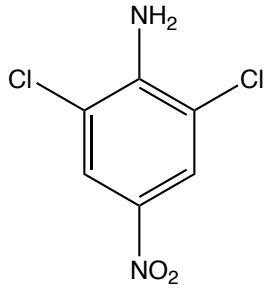



Figure 2.2. The chemical structure of dicloran.

Crops such as sweet potatoes are at risk for fungal diseases like soft rot (*Rhizopus stolonifer*), which can cause >50% post-harvest crop loss. For example, in Louisiana dicloran is commonly applied by the post-harvest dip tank application method to sweet potatoes; post-harvest fungicide application can also be applied to sweet potatoes by waterfall or spray. The roots of sweet potatoes are dipped in a recirculating system containing dicloran after the crop has been harvested and washed to prevent post-harvest fungal diseases from causing decay to the crop. Fungal species, particularly *R. stolonifera*, can potentially enter the potato after injury, such as bruising that commonly occurs during the packing process; therefore, the use of fungicides like dicloran is to prevent further damage or decay to the sweet potatoes and limit crop loss (Edmunds et al., 2003).

Dicloran is applied as a wettable powder, dust, or liquid flowable. Its maximum application rate is 4 lb ai/acre/year (pounds of active ingredient per acre per year) for all crops

with the exception of celery and fennel, with a maximum application rate of 5 lb ai/acre/year, and potatoes which have a 7.5 lb ai/acre/year maximum application rate (USEPA, 2006). The estimated use of dicloran in 2015 is shown in Figure 2.3; the majority of use of dicloran is in the western United States (USGS).

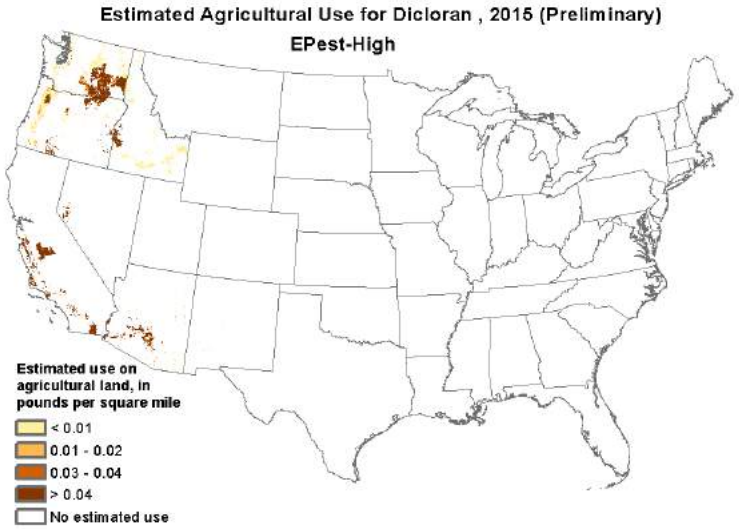



Figure 2.3. The reported (estimated) use of dicloran in the United States in 2015 (USGS).

The data USGS uses in Figure 2.3 is a result of the National Water-Quality Assessment (NAWQA) Project, which manages water resources including drinking water, irrigation, energy, ecosystem needs, and recreation; USGS applies EPA maximum use data to estimate and predict the use of a given pesticide throughout the United States, overlain with known cropping areas. (USGS).

It is estimated by the EPA that 40-60% of all celery and 10-15% of lettuce crops in the United States are treated with dicloran (USEPA, 2006). California is the largest producing state for head lettuce, romaine lettuce, and leaf lettuce in areas such as Monterey County, which coincides with the estimated use of dicloran shown in Figure 2.2 (CA PUR; Geisseler et al., 2016; USDA, 2018). Three of the top ten crops grown in Washington State are registered for dicloran application; in 2016, potatoes were listed as the 3rd largest commodity bringing in $813

million, cherries were listed as the 6th largest bringing in $502 million, and grapes were listed as the 9th largest bringing in $359 million to the state. Washington ranks first in the nation in Concord grape and cherry production and second for potatoes, all grapes, and apricots. Washington also produces strawberries, Christmas trees, and rhubarb, all of which are included on the list of currently registered crops approved for dicloran application (USEPA, 2006; WSDA).

With a $K_{oc}$ of 760-1120 (Table 2.1), dicloran is reported to have low mobility in soils according to EPA. Dicloran is reported to be persistent in sediments and the soil degradation rate is faster in anaerobic sediments compared to aerobic sediments (USEPA, 2006). The photolysis half-life of dicloran has been reported at a light intensity of 65 $W/m^2$.

Table 2.1. Physical properties of dicloran (USEPA, 2006; Vebrosky et al, 2018).

| Water solubility | $K_{oc}$ | Log $K_{ow}$ | pKa | Half-life (DI water) | Half-life (seawater) | Soil $DT_{50}$ |
|---|---|---|---|---|---|---|
| 6.3 mg/L | 760-1062 | 2.8 | Not ionized | 7.6 hours | 7.4 hours | 39-78 days |

Data reported by the Food and Agriculture Organization of the United Nations (FAO) states that the photolysis of dicloran on sandy loam soil. After 360 hours of light exposure, 88% of dicloran was lost from the soil by degradation, and possibly volatilization, using radiolabeled [$^{14}$C]dicloran. In a field dissipation study, dicloran was not detected 15 months post-application in a sandy loam soil (FAO, 2003).

Dicloran is listed as moderately to highly toxic to aquatic organisms (Table 2.2). Reported levels of dicloran in ground water were estimated to be <2.0 µg/L, while levels were >100 µg/L in surface waters; therefore, surface water dwelling organisms were considered for toxicity testing (USEPA, 2006). Rainbow trout (*Oncorhynchus mykiss*) have a reported $LC_{50}$ ranging 0.9-1.6 mg/L; bluegill (*Lepomis macrochirus*) have a much higher tolerance ranging

from 4.7-37 mg/L, which exceeds the solubility limit of dicloran in aqueous solutions (Roberts et al., 1998B, Tomlin, 2006). Limited data is available on the impacts of dicloran exposure to estuarine and marine organisms.

Table 2.2. Toxicology data of dicloran reported for aquatic organisms (Roberts et al., 1998B; Tomlin, 2006).

| | Rainbow trout[1] | Bluegill[1] | Goldfish[1] | Daphnia[2] | Midge[3] |
|---|---|---|---|---|---|
| $LC_{50}$ | 1.6 mg/L | 37 mg/L | 32 mg/L | 2.1 mg/L | n/a |
| $LC_{50}$ | 0.9 mg/L | 4.1 mg/L | n/a | 2.1 mg/L | 2.4 mg/L |
| [1]96-hour $LC_{50}$; [2]48-hour $LC_{50}$; [3]Chronic toxicity. | | | | | |

Previous literature has suggested dicloran has the potential to photodegrade. Boscá reported a photoreduction of the nitro group when dicloran was exposed to artificial sunlight in methanol and chloroform (Boscá et al., 1998). Vebrosky reported photonucleophilic substitution of the nitro group when dicloran is exposed to artificial sunlight in distilled water and artificial seawater (3.2%), undergoing degradation through a quinone-hydroquinone photodegradation pathway (Figure 2.4).

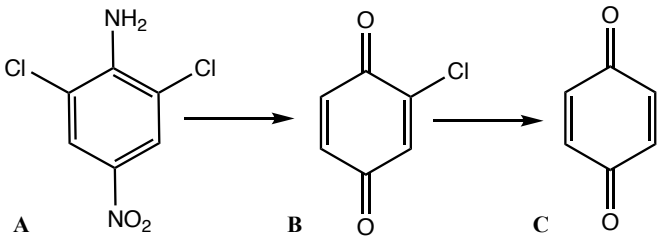



Figure 2.4. The partial photodegradation pathway of dicloran (A), 2-chloro-1,4-benzoquinone (B) and 1,4-benzoquinone (C).

Other pesticides such as 2,4-D (2,4-dichlorophenoxyacetic acid) and 4-chlorophenol have similar photodegradation pathways to dicloran, a quinone-semiquinone photodegradation pathway. Both compounds have been reported to photodegrade and form 2-chloro-1,4-benzoquinone at >10% formation, with 4-chlorophenol photodegrading to 1,4-benzoquinone. Vebrosky reported an equilibrium of 1,4-benzoquinone and hydroquinone, with detection of both

compounds by mass spectroscopy. Similar information is reported for the photodegradation of 4-chlorophenol; both 1,4-benzoquinone and hydroquinone are included in the photodegradation pathway and concentrations of the photoproducts vary depending upon the concentration of the parent compound prior to light exposure (Halmann, 1995; Vebrosky et al., 2018).

Limited published information is available on the influences of sunlight and sediment on the toxic response of aquatic organisms to dicloran exposure; previously published information suggests dicloran may be a model pesticide for photodegradation pathways and potential toxic responses of similarly structured compounds.

### 2.6.2. Chlorothalonil

Chlorothalonil, 2,4,5,6-tetrachloroisophthalonitrile, is an organochlorine, broad spectrum, non-systemic fungicide; its mode of action primarily interferes with glutathione-dependent enzymes and disturbs metabolism (Kelly, 2012). It is primarily used on peanuts in the United States, but it also applied heavily to tomatoes, potatoes, and turf, specifically turf for golf courses (USEPA, 1998). Chlorothalonil is manufactured under various trade names and companies, including Bravo® and Daconil Action® by Syngenta (Kelly, 2012). The mode of action of chlorothalonil is to impact enzymatic functions, such as energy production and respiratory metabolism; it is effective against four fungi classes, fungi imperfecti, basidiomycetes, ascomycetes, and oomycetes (Roberts et al., 1998B; Kelly, 2012).

Chlorothalonil (CHT) actively binds to the sediment where it then degrades; the primary soil degradation product is 4-hydroxychlorothalonil (4-OH-CHT), or 4-hydroxy-2,5,6-trichloroisophthalonitrile (Figure 2.5). The degradation of CHT in soils and sediment is predominantly a result of microbial activity. CHT has a $DT_{50}$ ranging between 5-30 days, dependent upon whether conditions are aquatic and if the system is aerobic or anaerobic (Table

2.3) (Roberts et al., 1998B). The EPA reports that photolysis is not a major pathway for the degradation for chlorothalonil and CHT is stable to hydrolysis, with the exception of pH 9 where it has a reported hydrolysis half-life of 40-60 days (USEPA, 1998). Armbrust reported that 4-OH-CHT is susceptible to photolysis and it has a very short half-life of 32 min in distilled water and 301 min in 2.0 M NaCl synthetic seawater (Armbrust, 2001; USEPA, 1998).

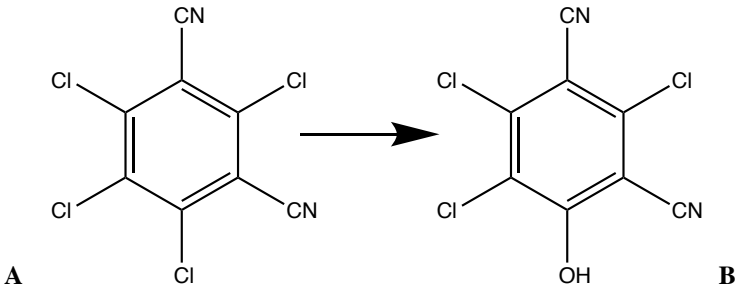



Figure 2.5. The chemical structures of chlorothalonil (A) and 4-hydroxychlorothalonil (B).

Table 2.3. The physicochemical properties of chlorothalonil (Roberts et al., 1998B).

| Water solubility | $K_{oc}$ | Log $K_{ow}$ | pKa | $DT_{50}$ |
|---|---|---|---|---|
| 0.6-1.2 mg/L | 1,600-14,000 | 2.88 | Not ionized | 5-36 days |

Chlorothalonil is applied throughout North America, Europe, Asia, and Africa; the distribution of its application throughout the United States is shown in Figure 2.6. It is applied to areas that runoff into freshwater ecosystems, such as inland ponds or lakes, and coastal regions, such as bays or estuaries, is likely to occur (Battaglin et al., 2008). Similar to dicloran, chlorothalonil is used on potatoes, cherries, Christmas trees, and apricots – all of which are grown in Washington, as well as celery, which is primarily grown in California. Peanuts account for a large percentage of crops with chlorothalonil applications; >90% of peanuts grown in the United States are grown in Georgia, Florida, North and South Carolina, Mississippi, and Texas. The application rate varies between crops; for the application season, a total of 9 lb ai/acre of CHT can be applied to peanuts and 9-18 lb ai/acres for vegetables (Kelly, 2012).

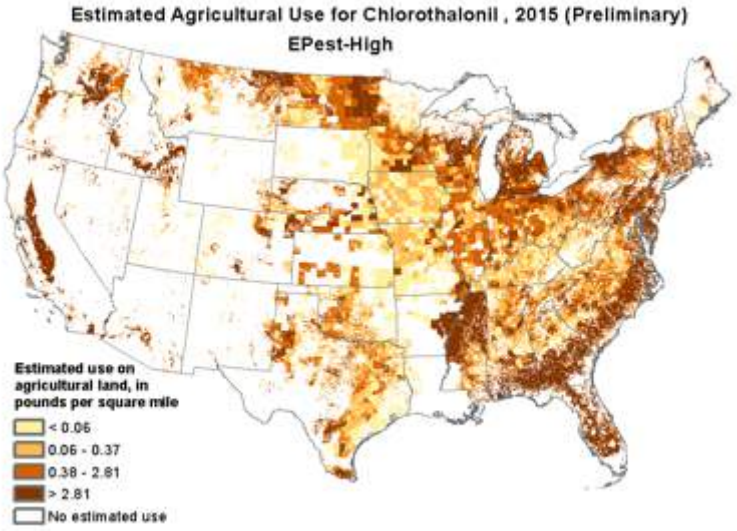



Figure 2.6. Estimated use of chlorothalonil throughout the United States in 2015 (USDA).

Chlorothalonil is lised by the US EPA "very highly toxic" to freshwater fishes and estuarine fishes and invertebrates (Table 2.3). On the contrary, 4-OH-CHT is listed as "slightly toxic" to freshwater fishes and invertebrates; no marine or estuarine organisms were reported for toxicity testing by EPA for 4-OH-CHT. Bluegill have a reported $LC_{50}$ of 15-45 mg/L after exposure to 4-OH-CHT, while bluegill exposed to CHT have an estimated $LC_{50}$ of 51-59.5 µg/L; *Daphnia magna* have an $LC_{50}$ of 26 mg/L after exposure to 4-OH-CHT (USEPA, 1998).

Table 2.4. Reported toxicological data for chlorothalonil on freshwater and estuarine organisms.

| | Rainbow trout | Bluegill | Channel catfish | Fathead minnow | Sheepshead minnow | Pink shrimp[1] | Eastern oyster[2] |
|---|---|---|---|---|---|---|---|
| $LC_{50}$ (µg/L) | 42.3 | 51-59.5 | 48 | 23 | 32 | 154 | 3.6 |
| [1]highly toxic; [2]$EC_{50}$ – shell deposition | | | | | | | |

Limited published information is available on 4-OH-CHT, or SDS-3701 as the EPA refers to it in the Reregistration Eligibility Decision (EPA RED) (Armbrust, 2001; USEPA, 1998; Kwon et al., 2006).

## 2.7. Herbicides

Herbicides are a class of pesticide used to rid unwanted or harmful plants or weeds. The last two decades have shown an increase in herbicide-resistant weed species, particularly in herbicides commonly used in rice fields (McKnight et al., 2018).

The many classes of herbicides include, but are not limited to, amides, anilides, benzoic acids, bis-carbamates, chloroacetanilides, dinitroanilines, dinitrophenols, hydroxybenzonitriles, imidazolinones, isoxazoles, organophosphorus herbicides, pyridazinones, quinolinecarboxylic acids, thiocarbamates, 1,3,5-triazines, 1,2,4-triazinones, triazoles, and uracils – each class containing unique characteristics including mode of action and chemical structures. Dinitroanilines, for example, are selective herbicides that are used for the pre-emergance control of broad-leaved weeds on turf and horticultural crops; the herbicides are absorbed by the roots of the seedlings, and phytotoxicity inhibits the binding of tubulin (Roberts et al., 1998A). Based upon their structures many have the potential to also exhibit phototoxicity to organisms that may be exposed to them.

### 2.7.1. Benzobicyclon

Benzobicyclon (BZB), 3-(2-chloro-4-(methylsulfonyl)benzoyl)-2-phenylthiobicyclo[3.2.1]oct-2-en-4-one), is a pro-herbicide registered under the trade name BUTTE® for use on California rice paddies. It was first registered for use in the United States on rice in California for the 2017 growing season with a maximum application rate of 0.27 lbs ai/acre. Benzobicyclon hydrolysate (BH) is the active ingredient in the herbicide (Figure 2.7). BZB rapidly hydrolyzes to form BH in water ($t_{1/2}$ <1 day), therefore BH is measurable in flooded rice paddies where the herbicide is applied (USEPA, 2017; Williams et al, 2016). BUTTE® is

used to control weed species such as barnyard grass that compete with rice crops in fields for resources including water and nutrients (McKnight et al., 2018; Smith, 1988).

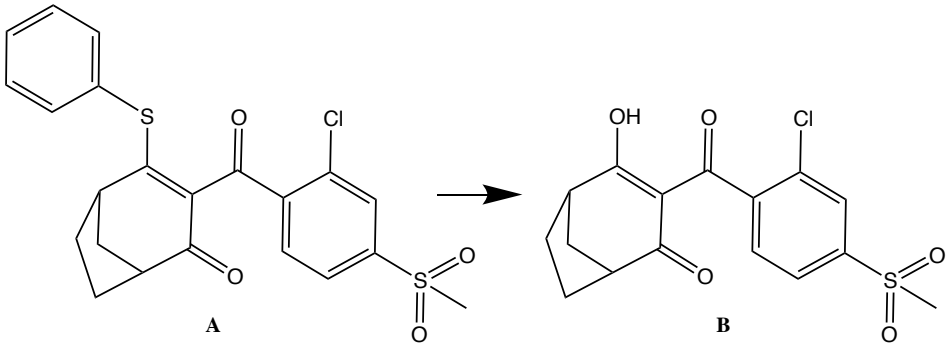



Figure 2.7. The chemical structures of benzobicyclon (A) and benzobicyclon hydrolysate (B).

BZB has a different mode of action compared to other currently registered rice herbicides; it bleaches the plants by inhibiting 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) and is up-taken by the roots of plants where it can cause death (USEPA, 2017). BUTTE® is applied in the granular form in California, therefore the risk for runoff or spray drift is minimal; transport of the herbicide may occur after the flooded field is drained due to remaining BH in the water (USEPA, 2017; Williams et al., 2017). Preliminary analysis and reports do not detect BZB in rice sediment by the end of the growing season.

Williams *et al* reported benzobicyclon hydrolysate to photodegrade in high-purity water and rice field water (California) in natural and artificial sunlight, accounting for varying light intensity at summer solstice and fall equinox. In rice water, BH had an estimated half-life of 46 hr after exposure to simulated sunlight (fall equinox) and 80 hr in natural sunlight; in high-purity water (pH 8), the half-life was 170 hr and 320 hr in simulated and natural sunlight respectively. Therefore, the behavior of BH appears to be dependent on water-type and light intensity for photodegradation rates (Williams et al., 2018). However, its structure and pattern of UV absorbance suggest that it could potentially be phototoxic to aquatic organisms.

The herbicide has previously been used in Asian countries such as Japan and Korea since 2001 where its behavior has been studied in local fields or simulated field conditions (Komatsubara et al., 2009). Its behavior in American rice fields, particularly in California and Louisiana, is currently being monitored and studied. LSU, for example, has been testing benzobicyclon at the H. Rouse Caffey Rice Research Station in Crowley, LA since 2015 (Gould, 2016A,B; McKnight et al., 2018).

The EPA reports that BH is persistent in water, while both BZB and BH can be persistent in sediments. Preliminary field analysis from the University of California at Davis and Louisiana State University does not show that BH is persistent in sediment; data suggests that BH is preferentially detected in water while BZB is persistent in sediments (up to 60 days).

Table 2.5. Physicochemical properties of benzobicyclon and benzobicyclon hydrolysate (USEPA, 2017; Williams et al., 2016; Williams et al., 2018).

| Chemical | Water Solubility (mg/L) | Log $K_{ow}$ | $K_{oc}$ | Hydrolysis half-life | Photolysis half-life |
|---|---|---|---|---|---|
| Benzobicyclon | 0.051 | 3.1 | 4,249-47,569 | 0.5-0.8 days | n/a |
| Benzobicyclon Hydrolysate | 133 (estimated) | 1.51 | 90-771 | 388 days | 7.8-12.9 days |

According to the Environmental Fate and Ecological Risk Assessment issued by the EPA, no acute toxicity effects for BZB or BH were reported on freshwater or estuarine/marine fishes (sheepshead minnow, *C. variegatus*; fathead minnow, *P. promelas*; and rainbow trout, *O. mykiss*) and invertebrates (*Daphnia magna*); though, chronic impacts like growth and reproduction are reported for freshwater fishes for BZB and BH and growth for estuarine/marine fishes for BH. The fishes used for the toxicity testing of BZB in concentrations that did not exceed the 50 µg/L water solubility limit as a maximum dose. While acute exposure results in minimal impacts (example: little to no mortality), sub-lethal effects such as fishes floating on the bottom of their tanks and resuming normal behaviors after transfer to clean water was observed

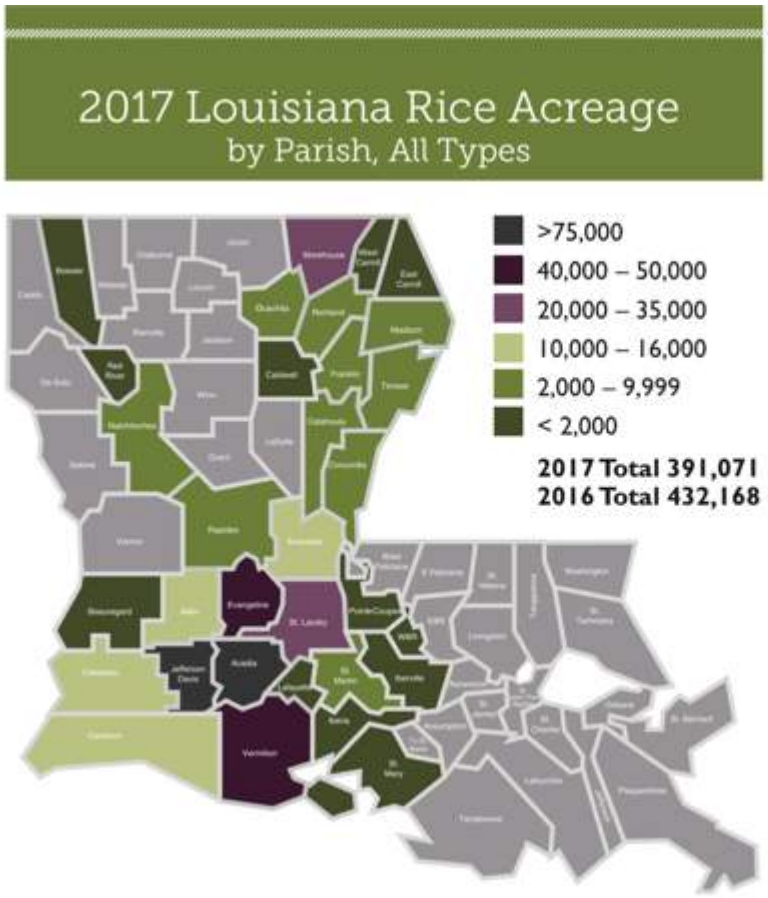
in exposures to both BZB and BH. Estuarine/marine invertebrates (Eastern oysters, *C. virginica*) were reported to have acute impacts such as lethargy after exposure to BH and chronic impacts such as growth; no acute effects were observed when exposed to BZB, reduced number of offspring was reported as a chronic effect. No acute or chronic effects were observed for sediment-dwelling invertebrates, however if BH does exhibit phototoxicity then it could potentially impact invertebrates, such as crayfish, residing in Louisiana rice fields (USEPA, 2017).

#### 2.7.1.1. Rice in Louisiana

Rice is an important crop for farmers in Louisiana. In 2014, an estimated 3.4 billion pounds of rice were harvested accounting for a production value of more than $670 million (Gould, 2016B; USDA, 2007). Crayfish, or "crawfish," harvest is typically rotated with rice in the same fields; the crawfish market in Louisiana exceeds $45 million annually (McClain et al., 2007). In recent years, herbicide-resistant weed species have increased in rice fields that compete for resources such as sunlight, nutrients, water, and surface area (McKnight et al., 2018; Smith, 1988).

The first account of herbicide-resistant weeds in rice production occurred in Arkansas in the 1990s, and observations continued throughout Louisiana and Mississippi through the 90s until today (Baltazar et al., 1994; Carey et al., 1995; Malik et al., 2010; McKnight et al., 2018). Weed species such as barnyard grass showed resistance to herbicides including propanil, quinclorac, clomazone, and imazethapyr (Malik et al., 2010; McKnight et al., 2018; Norsworthy et al., 2012). With competition between rice, weed, and grass species on the rise in rice paddies, the need for newly formulated herbicides with different modes of action than those currently registered and in use is pertinent for sustaining rice production and high crop yields.

In Louisiana, the Atchafalaya basin contains a large percentage of rice field acreage; this region is also where the majority of the commercial crawfish are harvested (Figure 2.8). Louisiana State University's AgCenter is conducting research on over eight new herbicides since 2017 to treat rice fields in hopes to combat herbicide-resistant weeds. Benzobicyclon (BUTTE®) is among the herbicides undergoing field testing, along with Provisia and Loyant (Schultz, 2018). For these new products it is imperative to understand how they degrade under Louisiana use conditions and how these products may impact organisms potentially exposed to them.




Top 10 Rice Parishes

| Parish | 2016 Total Acreage | 2017 Total Acreage |
|---|---|---|
| Acadia | 83,598 | 80,324 |
| Jefferson Davis | 81,013 | 76,377 |
| Vermilion | 48,857 | 45,380 |
| Evangeline | 43,970 | 41,829 |
| St. Landry | 24,051 | 21,329 |
| Morehouse | 27,504 | 20,624 |
| Allen | 15,646 | 14,452 |
| Avoyelles | 13,666 | 12,203 |
| Calcasieu | 12,291 | 11,136 |
| Cameron | 11,415 | 10,312 |

Figure 2.8. A map of Louisiana generated by the LSU AgCenter with the total acreage of rice shown by Parish (Schultz, 2018).

## 2.8. Toxicity tests: study organisms

All pesticides in the United States must be registered by the US EPA; this registration requires both toxicological and environmental testing. Testing may include, fish life cycle analysis, bioaccumulation studies, acute freshwater or estuarine fish, etc., but not all testing is required as pesticides are registered (and tested) on a case-by-case basis (Crosby, 1998).

Toxicity tests, or assays, have been established for regulatory agencies such as the United States Environmental Protection Agency (EPA) for both marine and freshwater aquatic organisms. Toxicity tests range from static to flow-through systems, static renewal and non-renewal, and chronic to acute, dependent upon the organism, study design, and overall goal of the research study. The durations of exposure for each test that characterizes the tests as acute are those consisting of a single dose or over a short period of time (24-96 hours), or chronic, constant and/or long-term exposure (USEPA 1994a, 1994b, 2006b, 2002a, 2002b, 2010, 2016a, 2016b). Toxicological analyses, by classical definition, narrowly focus on adverse impacts on small populations, or individuals, on replaceable species and often times exclude external influences on the chemical under evaluation (Crosby, 1998).

Toxic responses to chemical exposure are often plotted as dose-response relationships; dose-response curves may show the no observable effect limit (NOEL), toxic threshold, and increasing doses leading to the lethal dose or concentration. The relationship between the dose and acute response of the organisms is reported as the lethal dose to 50% of the population ($LD_{50}$) for oral exposure, concentration lethal to 50% of the population ($LC_{50}$) for aquatic exposure, or the effective concentration, where a chosen effect is observed for 50% of the population ($EC_{50}$) (Crosby, 1998).

EPA toxicity testing includes WET (Whole Effluent Toxicity) Testing where larval fishes are exposed to wastewater effluent at 24 hours or less post-hatch and growth and survival are the measures considered for testing. Fathead minnows (*Pimephales promelas*) are a commonly used freshwater fish species for WET testing. Larval fish are often used for toxicity testing, but specifically for WET testing. Wet testing is a seven-day, sub-chronic toxicity analysis used to monitor the effluent of dischargers under the NPDES program to evaluate the potential impact on organisms in the receiving waters (USEPA, 2002).

EPA standards also include assays under FIFRA (the Federal Insecticide, Fungicide, and Rodenticide Act) to meet requirements for acute toxicity testing on freshwater and estuarine/marine organisms. Juvenile fishes weighing <3.0 grams are chosen for analysis; among the chosen test species are fathead minnows (*Pimephales promelas*), zebrafish (*Danio rerio*), sheepshead minnow (*Cyprinodon variegatus*), and inland silversides (*Menidia beryllina*). The typical exposure time for chemical(s) is 96-hours, with $LC_{50}$ values calculated each 24-hours (USEPA, 2016a, b).

Ecosystems as a whole have the potential to be influenced by a "toxic" chemical; ecotoxicity takes multiple components within an ecosystem into account when analyzing the potential impacts on the system. Ecotoxicology encompasses the vastness of environmental toxicology to understand the fate of the toxic chemicals throughout the ecosystem (Crosby, 1998). Fishes and invertebrates would be most susceptible to photoinduced toxicity in shallow water environments. However, up to this point there are no standard protocols for assessing phototoxicity of pesticides to aquatic organisms as a part of the registration process.

### 2.8.1. Fathead minnows

Fathead minnows (*Pimephales promelas*) are potentially an ideal species to investigate phototoxicity in freshwater systems. They are a freshwater fish species in the family, Cyprinidae. Cyprinidae is an ecologically important freshwater fish family, which also encompasses species such as the common carp (*Cyprinus carpio*). Fathead minnows have gray/olive appearance but are slightly translucent and very lightly pigmented as juveniles; they have a dark/black line that can be observed from their mouth to their dorsal fin. Their native habitat ranges throughout the United States, Canada, and Mexico. These minnows are tolerant of a wide range of environmental factors including pH, alkalinity, temperature, and hardness throughout both lentic and lotic habitats (Ankley et al, 2006).

The use of fathead minnows in toxicity testing began prior to the formation of the EPA in the 1950s at facilities such as The Public Health Service in Cincinnati, OH (Ankley et al, 2006). Fathead minnows are used in toxicity analyses such as 48-96 hour acute assays, 30-day partial life-cycle assays, full life-cycle assays, and multigenerational assays with endpoints ranging from developmental inconsistencies, reproductive impacts, and lethality. As previously mentioned, WET testing uses fathead minnows 24 hours post-hatch, while FIFRA testing uses juvenile fathead minnows that are <3.0 grams in weight. For acute and some partial life-cycle assays, juveniles (typically 30-days post-hatch) are used. Regulatory requirements for toxicity assays vary from those used by other research institutions, such as academic research (Ankley et al., 2006; Broderius et al., 1985; USEPA, 2006b).

Similar fishes used in toxicological analyses include Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), bluegill (*Lepomis macrochirus*), and rainbow trout (*Oncorhynchus mykiss*). While Japanese medaka and zebrafish are both used extensively in regulatory and

research studies, the fathead minnow is the most widely used freshwater fish. In the 1980s, the EPA chose a large group of chemicals (>600 in total) to use for 96-hour exposure studies on fathead minnows. This extensive analysis generated data and baseline studies used in models and toxicological reports (Ankley et al, 2006; Russom et al., 1997; Veith et al., 1988).

Kagan *et al* used fathead minnows to monitor the potential for phototoxicity when exposed to the PAHs, pyrene, anthracene, and fluoranthene. $LC_{50}$ values at environmentally relevant concentrations of PAHs was observed; the $LC_{50}$ for pyrene was 0.22 mg/L, 0.20 mg/L for fluoranthene, and 0.36 mg/L for anthracene and sunlight. Dark controls did not result in any toxic response from the minnows. Therefore, the activation of PAHs by UV-light results in a phototoxic response when fathead minnows are exposed (Kagan et al., 1985; Kagan et al., 1987).

### 2.8.2. Inland silversides

Inland silversides (*Menidia beryllina*) are potentially a suitable fish species to investigate the potential for phototoxicity in estuarine and deltaic environments. They are a euryhaline fish species that can tolerate a wide range of salinities, typically 0.0-3.0%, but populations have been found in hypersaline regions of as high as 7.0%. Silversides are used by agencies such as EPA as an estuarine/marine fish species for toxicological analyses (USEPA, 2016a; Middaugh et al., 1992). Their native habitat ranges from the Northeast United States, just below Maine, throughout the East Coast and across the Gulf of Mexico to Texas; they are an introduced species in California. Coastal and inland populations exist as well. Similar to inland silversides, the Atlantic silverside (*Menidia Menidia*) and tidewater silverside (*Menidia penninsulae*) also co-exist in some of the same waters (Gosline, 1948; Little et al., 2000).

Pillard *et al* used mysid shrimp (*Mysidopsis bahia*), sheepshead minnow (*Cyprinodon vareigatus*), and inland silversides (*Menidia beryllina*), all euryhaline organisms, to determine

their response to varying salinities and establish a threshold with artificial seawater. Inland silversides appear to be the most variable of the salinity gradient. Low mortality was observed in freshwater, though nearly 100% survival was observed in low salinities and at 5.0%; 40% mortality was observed at 3.1% ASW. Mysid shrimp survival decreased at the high (4.3%) and low (0.375%) salinities, and they appear to thrive in the intermediate salinities. Sheepshead minnows appear to be highly tolerant of freshwater, and 100% mortality was not observed until exposed to concentrations of seawater exceeding 8.0%. Therefore, all species tested can tolerate a range of salinities but inland silversides are more variable, tolerating 0-30 ppt, among the chosen organisms (Middaugh et al., 1992; Pillard et al., 1999).

Osmoregulation is a key factor in euryhaline organisms maintaining homeostasis to survive in varying salinities. For example, when euryhaline fishes are in fresh waters they are considered hyper-osmotic and in saline water they are considered hypo-osmotic. When hyper-osmotic, fish ingest water and lose salts (ions) by excretion and diffusion; when hypo-osmotic, fish gain salts by diffusion and ingestion and lose water by excretion. Energy exertion to maintain homeostasis is dependent on the species but is a contributing factor to salinity tolerances of euryhaline organisms (Thompson et al., 1992).

*Menidia spp.* have been used in a variety of toxicological studies, including the effects of endocrine disrupting chemicals (EDCs) by Brander *et al*. Juvenile inland silversides were exposed to bifenthrin and sublethal effects were monitored. Gene expression analysis showed sublethal responses of silversides to low (ng/L) concentrations of bifenthrin; genes effected by estrogen (i.e. BAIAP212, GPR30, TR$\alpha$, and MAPK14) showed a downregulation for exposure levels (Brander et al., 2016).

Silversides belong to the family, Atherinopsidae, which also includes neotropical fishes in the genus, *Odontesthes*. Also referred to as silversides, the species in this genus are known to be euryhaline. The relative gene expression for *O. humensis* was studied for any variation salinity imposes. In all reference genes, Silveira *et al* reported no variation in gene expression between salinities. The acclimation of fishes between salinities resulted in both up- and down-regulation of the genes in this study. Fish that were acclimated to a single salinity did not show much change in gene expression, but fish that were acclimated to freshwater then back to brackish water (or vice versa) exhibited responses at the gene expression level (Silveira et al., 2018).

Previous studies using another euryhaline species, white shrimp (*Litopenaeus vannamei*) monitored the impact exposure to pesticides including DDT, chlordane, lindane, folidol, diazinon, gusathion, lorsban, and tamaron had on the osmoregulating processes at three salinities (1.0, 3.0, and 5.0%). All pesticides, with the exception of DDT, reduced the capability of osmoregulation of the shrimp at 1.0% salinity while at 5.0% salinity, the organochlorine pesticides (DDT, lorsban, and lindane) appear to reduce osmoregulation, the organophosphorus pesticides (diazinon, gusathion, and folidol) appear to increase osmoregulation; the authors observed no differences at 3.0% salinity (Galindo-Reyes et al., 2000). Influences on the osmoregulatory processes to euryhaline organisms are especially important to understand how chemicals and salinity fluctuations could impact their livelihood (Chong-Robles et al., 2014).

### 2.8.3. Red swamp crayfish

Red swamp crayfish (*Procambarus clarkii*) are an ideal freshwater invertebrate species for toxicity testing in monitoring the potential effects of herbicides and other agrochemicals that may enter their ecosystems. They are a benthic species and therefore direct phototoxicity may be

minimal, but the potential for the crayfish to come into contact with UV-activated chemicals exists resulting in indirect phototoxicity in the environment. These crayfish are an ecologically and economically important species native to North America and have been introduced in other global regions such as Asia; red swamp crayfish are considered an invasive species to areas in the northern United States, such as Michigan, and their native range spans from northern Mexico throughout the Southern United States. In Louisiana, crayfish will be referred to as "crawfish," and are a large commodity in the economy and culture of the state. Millions of dollars in revenue annually in Louisiana are a direct result of crayfish harvest and sale, often exceeding $45 million (McClain et al., 2007; Salassi et al., 2008).

Louisiana is particularly dependent on the health and growth of crayfish; it is estimated that 90-95% of crayfish in the United States are harvested in Louisiana. Prior to the 1960s, crayfish harvested for consumption was solely wild-caught crayfish; the first record of land set aside for commercial farming of crawfish in Louisiana dates to the 1960s. Crayfish are often grown and harvested in rice paddies throughout the state but are concentrated within the Atchafalaya Basin. Ponds and rice paddies are stocked with crayfish that are commercially purchased from aquaculture facilities, but farmers often rely on the offspring from the prior harvest to spawn and keep the reproduction and replenishing cycle in the systems (McClain et al., 2007).

Red swamp crayfish, along with other species such as Allegheny crayfish, are often used as bait for fishing. This may be the reasoning for the introduction of red swamp crayfish as an invasive species outside of its native range.

*P. clarkii* often co-inhabit systems with *P. zonangulus* or *acutus* (white river crayfish), though red swamp account for ~80% of the catch and harvest in Louisiana; they often may be

mistaken for one another in the juvenile life-stages. Red swamp crayfish are a burrowing species of crayfish; this is a result of their adaption to the flooded and dry cycles of the climates in the regions they inhabit. In the Southern United States, red swamp crayfish can spawn year-round with smaller but more plentiful eggs when compared to white river crayfish spawning. Spawning rates are high in autumn, and a large number of juvenile crayfish can be found in shallow waters in late winter/early spring in Louisiana. Red swamp crayfish are tolerant of high temperatures (exceeding 86°F) and low dissolved oxygen (DO) concentrations (Hobbs et al., 1993; Momet et al., 1978; Nyström et al., 2002).

Crayfish typically spawn in the autumn; once the eggs hatch and juveniles are detached from females, the emergence of juveniles can be seen in late winter/early spring. Within the first year, juvenile crayfish will molt 7-11 times; frequency of molting is dependent upon the growth rate for the individual. Growth rate can be impacted by factors including abundance of food, oxygen levels, temperature, and population density. During and shortly after molting, when the exoskeleton is soft, crayfish are more susceptible to disease and chemical exposure. As juveniles, red swamp crayfish do not exhibit the dark red coloration of their carapace as is widely observed in mature males and females; juvenile crayfish appear gray or translucent in their early life-stages (McClain et al., 2007).

Burrowing crayfish species, such as red swamp crayfish, may be more susceptible to exposure to pesticides that bind to the sediment. Crayfish are benthic organisms that are typically detritivores, therefore they inhabit and consume potentially contaminated sources. They may also exhibit opportunistic feeding behaviors; crayfish will consume vegetation, decomposing organic matter, small fish or other invertebrates, and they have also shown cannibalistic behavior (McClain et al., 2007).

Commercial crawfish ponds, or rice paddies, are shallow water systems with water depths typically ranging from 8-24 cm. Shallow water systems are highly susceptible to runoff and chemical alteration by processes previously discussed such as photolysis and hydrolysis, as well as dissipation of the chemicals into underlying sediments. Since crayfish are benthic organisms, they inhabit the sediment-water interface where unique chemical processes occur due to the abiotic and biotic influences in this environment. While typically anaerobic, this influences the chemical behavior and potential for uptake and exposure by the crayfish. The use of crayfish as an unconventional toxicity assay is beneficial to locality and the potentials risks herbicides and other pesticides pose to their overall health (Benli et al., 2007; Jolly et al., 1978 Kaila et al., 1977; Muncy et al., 1963).

## 2.9. Environmental fate of pesticides

The fate of pesticides in the environment is crucial from a management and registration standpoint to ensure the health and wellbeing of not only humans but to aquatic (and terrestrial) organisms that may come into contact with the pesticides or that may become a food source for humans. The US EPA has established a suite of tests to assess the processes most important to the ultimate fate of a chemical as well as its potential to impact organisms. However, there are situations where phototoxicity can be significant thus it is important to be able to have aquatic models where this may be assessed. It has been previously stated that larvae and juveniles are among the most sensitive populations to pesticide and other chemical exposure. Both larvae and juvenile aquatic organisms have limited mobility, minor pigmentation, are commonly found in shallow-water areas, and immature mechanisms of defense for potential toxicants to be flushed from their bodies (i.e. gills are still forming, therefore the ability of a chemical to enter an organism's bloodstream through the gills increases) (Barron et al., 2003; Pelletier et al., 1997).

In this dissertation, three juvenile aquatic organisms (fathead minnows, inland silversides, and red swamp crayfish) will be investigated for their response to exposure to irradiated and non-irradiated pesticides to determine the risk and fate of these pesticides and organismal response.

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# CHAPTER 3. DEGRADATION OF DICLORAN IN IRRIATED WATER-SEDIMENT SYSTEMS

## 3.1. Introduction

Dicloran (2,6-dichloro-4-nitroaniline) is a substituted aniline fungicide registered throughout the southern and western United States and Hawaii, but primarily used throughout the Pacific Northwest and California on a variety of specialty crops including grapes, sweet potatoes, and strawberries (USEPA, 2006). It is applied via aerial spray, sprinkler irrigation, or dip tank to prevent the fungal spore germination of fungal diseases including *Sclerotinia spp.* and *Botrytis spp.* (Gowan; Roberts et al., 1999; Tomlin, 2006). Many of the areas where these crops are grown are in close proximity to estuarine ecosystems, particularly shallow water bodies. The potential therefore exists for dicloran to run off into freshwater or brackish water when it is applied by methods allowed by the label. Dicloran has previously been shown to degrade by photolysis through a series of quinone-hydroquinone intermediates to small aliphatic acids. No variation in half-life was observed between synthetic freshwater and seawater although the formation of intermediate photoproducts differed between media (Vebrosky et al., 2018). The US EPA (United States Environmental Protection Agency) reports a low mobility in sediments for dicloran, with a reported $K_{oc}$ of 760-1062, and the capability for the fungicide to be persistent. Mobility of dicloran is dependent on soil type and conditions and the degradation of dicloran in sediment is reported to be faster under anaerobic conditions (USEPA, 2006).

Previously published analyses of dicloran have only taken aqueous conditions into account, including freshwater and saltwater (3.2%), with and without the presence of artificial sunlight (Vebrosky et al., 2018). The photodegradation of dicloran has been analyzed in organic solvents including methanol and chloroform via laser flash photolysis (Boscá et al., 1998). Under these conditions, dicloran was reported by Boscá *et al* to undergo photoreduction of the

nitro group as opposed to photonucleophilic substitution of the nitro group in water as reported by Vebrosky (Boscá et al., 1998; Vebrosky et al., 2018). It was reported that salinity did not impact the rate of photodegradation of dicloran, however the inclusion of sediment may alter the behavior and photodegradation of the chemical. While sunlight and salinity have both been reported to impact the behavior of dicloran, natural systems include other components such as sediment with organic material and metals that have previously been shown to significantly impact the behavior, persistence, and degradation of chemicals such as fungicides (Armbrust, 2001; Herbert et al., 1990; Hladick et al., 2008; Kwon et al., 2004; Kwon et al., 2006A; Lui et al., 2015; Muir et al., 1982; Shibata et al., 2011; Vebrosky et al., 2018).

Dicloran is listed as moderately to highly toxic to non-target aquatic organisms, including rainbow trout (*Oncorhynchus mykiss*), bluegill (*Lepomis macrochirus*), and water flea (*Daphnia magna*) (USEPA, 2006). An *in vitro* study using the cardiomyocyte culture from eastern oysters (*Crassostrea virginica*) reports an upregulation in the expression levels of heat shock protein 70, calmodulin, and defensin, along with a decrease in heartbeat counts after exposure to dicloran and irradiated dicloran (Xu et al., 2018).

Dissipation and accumulation of chemicals in sediments can pose increased threats for potential toxic-responses from sediment-dwelling organisms. With the potential for toxicity to increase (or vary) with the inclusion of sunlight in the experimental design, sediment may also impose variation in the response of aquatic organisms to dicloran exposure, especially epibenthic organisms. The purpose of the current investigation was to consider multiple environmental factors influencing the behavior of dicloran, taking sediment, water, salinity, and light into account for the degradation and persistence of dicloran in a laboratory designed shallow water system.

## 3.2. Materials and methods

### 3.2.1. Chemical reagents and materials

Analytical grade dicloran (2,6-dicloro-4-nitroaniline) (Figure 3.1) was obtained from Sigma Aldrich (St. Louis, MO). All solvents were high performance liquid chromatography (HPLC) grade; distilled water and acetonitrile (ACN) from VWR (Radnor, PA). Artificial seawater was prepared from Instant Ocean (Blacksburg, VA) and distilled water; salinity was monitored using a YSI Model 30 salinity, conductivity, and temperature meter (Yellow Springs, OH).

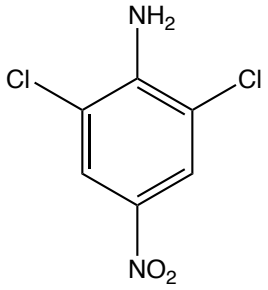


Figure 3.1. Molecular structure of dicloran.

### 3.2.2. Irradiated water-sediment experimental design

Savillex Teflon jars (240 mL Standard Jar) purchased from Savillex Corporation (Eden Prairie, MN) were 6 cm in diameter and 9 cm in height. Each jar contained 25 g (dry weight) of sediment obtained from a rice field in Crowley, LA, stored in a -80°C freezer until use, ball-milled to a fine consistency, and dried in an oven for 24 hours to ensure uniformity throughout trials. Distilled water or 25 ppt (2.5%) artificial seawater (ASW) was added to each jar (150 mL) and refrigerated for a minimum of 24 hours to allow the sediment to settle and prevent turbidity from impacting photodegradation. The sediment and water depth measured ~1 cm and ~5 cm respectively in each jar. The sediment was characterized by AgVise Laboratories (Northwood, ND) using the Series 1 Characterization testing (Table 3.1).

Table 3.1. Characterization of rice field sediment collected in Crowley, LA and analyzed by AgVise Laboratories according to the Series 1 Characterization.

| Sediment Characterization (Crowley, LA) | |
|---|---|
| % Sand | 16 |
| % Silt | 60 |
| % Clay | 24 |
| USDA Textural Class | Silt Loam |
| Bulk Density (gm/cc) | 1.10 |
| Cation Exchange Capacity (meq/100 g) | 15.6 |
| % Moisture at 1/3 Bar | 32.8 |
| % Organic Matter (Walkley-Black) | 2.1 |
| pH (water) | 6.5 |
| % Total Nitrogen | 0.119 |
| Olsen Phosphorus (ppm) | 13 |
| Cation %: Calcium | 59.4 |
| Cation %: Magnesium | 22.0 |
| Cation %: Sodium | 1.9 |

The water in each jar was spiked with 15 µL of a 10,000 mg/L stock solution (150 µg) of dicloran in ACN; jars were either placed in an ATLAS SUNTEST XXL+ environmental chamber (Mount Prospect, IL), fitted with a recirculating water system for temperature control (Fisher Scientific, Pittsburgh, PA) flowing through a water bath, or in a dark incubator (40°C) for experimental analysis. The environmental chamber was outfitted with a daylight filter that simulated the irradiance of natural sunlight at 40 $W/m^2$ (Figure 3.2) and 20% relative humidity, as measured across wavelengths ranging between 300-400 nm. The irradiance of sunlight from a July 2018 day at 12:00PM in Baton Rouge, LA and the irradiance of simulated light from the environmental chamber were measured using a Black Comet UV-VIS spectral radiometer (Stellar Net, Inc., Tampa, FL). The jars were removed from the chamber and incubator at 0, 2, 4, 8, or 24 hours for analysis. Water samples were analyzed first; 1 mL was pipetted from the middle of the water column and samples were filtered through 22 µm PTFE syringe filters (Fisher Scientific, Hampton, NH) into 2 mL borosilicate glass vials (Agilent Technologies, Santa Clara, CA) and analyzed using an Agilent 1260 Infinity HPLC with a ZORBAX C-8 Eclipse

Plus Analytical 4.6 x 150 mm 5 µm column, using a water and acetonitrile gradient mobile phase and monitoring the wavelengths using photodiode array detection at 254 and 380 nm (Vebrosky et al., 2018).

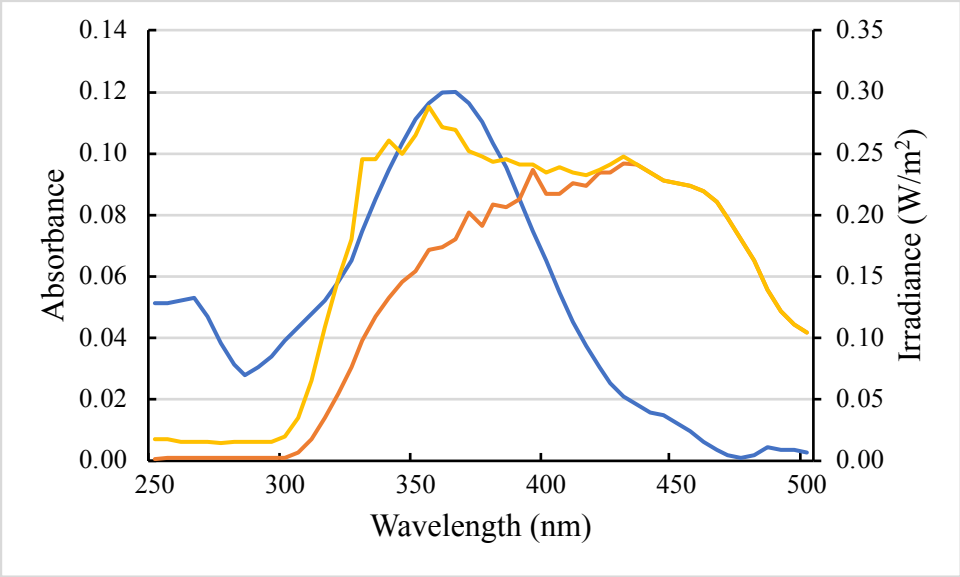


Figure 3.2. The absorbance of dicloran (blue) in water and the irradiance of natural sunlight in Baton Rouge, LA in July at 12:00PM (yellow) and artificial sunlight from the ATLAS XXL+ environmental chamber (orange).

The remaining water was decanted off of the sediment and 40 mL of 0.05% formic acid (Fluka, St. Louis, MO) in ACN was added to each jar. The tubes were shaken for 1 hour using a Pilot-Shake® rack shaker (Kühner AG; Birsfelden, Switzerland). After shaking, 1 mL of ACN solution from each jar was filtered, transferred to a sample vial, and analyzed using HPLC. Each sample was completed in triplicate and analyzed in duplicate.

### 3.2.3. Photolysis of dicloran without sediment

Dicloran in water samples were also irradiated and analyzed by HPLC. Savillex Teflon jars containing distilled water or artificial seawater, without sediment, were irradiated and analyzed for comparison by the same experimental design as discussed above. Samples were also irradiated in 2 mL borosilicate clear glass vials following the same experimental design as the water and water-sediment samples in distilled water and artificial seawater.

### 3.2.4. Data analysis

The percent remaining dicloran was averaged in water and sediment sampling for each time point (t = 0, 2, 4, 8, and 24 hours). The pseudo-first order rate constant was calculated by the following equation:

$$\ln C/C_0 = -kt$$

Where k is the rate constant, C is the concentration, $C_0$ is the initial concentration, and t is time. The half-life was calculated using the rate constant with the following equation:

$$t_{1/2} = \ln 2/k \textit{ or } t_{1/2} = 0.693/k$$

Where k is the previously calculated rate constant and $t_{1/2}$ is the half-life. A paired two-tail t test ($\alpha = 0.05$) and one-way ANOVA analysis of variance with Tukey's multiple comparisons test were used to determine statistical differences between trials and media (n=3) (Prism 7.0, GraphPad, Inc., La Jolla, CA).

## 3.3. Results and discussion

### 3.3.1. Fate of dicloran in water-sediment systems

Dicloran was recovered with >90% efficiency in water and 80% in sediment. The degradation and dissipation of dicloran was monitored over a 24-hour period in both dark (Figure 3.3.) and light (Figure 3.4) trials in distilled water and artificial seawater. After 24 hours in freshwater systems, 7.8 µg (5.2 %) of the applied dicloran was measured in sediment while 43.5 µg (29 %) remained in the water exposed to light; 91.6 µg (61.1 %) of the applied dicloran remained in the water column and 12.2 µg (8.2 %) in the sediment in dark trials. For seawater, after 24 hours of light exposure 4.8 µg (3.2%) of applied dicloran was measured in the sediment and 74.4 µg (49.6 %) remained in the water; in dark trials, 9.3 µg (6.2 %) of applied dicloran was measured in the sediment and 108.0 µg (72 %) in water (Table 3.2).

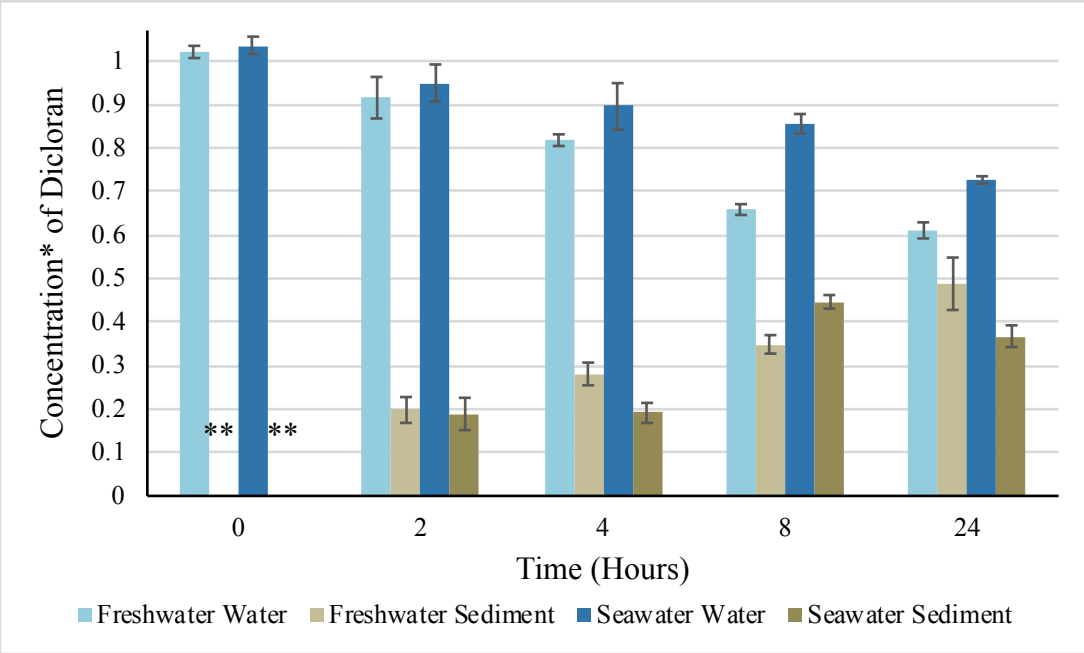


Figure 3.3. The dissipation of dicloran in the dark in water (blue) into sediment (brown) of dicloran in freshwater and seawater (in legend) over a 24-hour exposure period (*) water concentration measured in mg/L and sediment in mg/kg. Asterisks (**) indicate 0 mg/kg dicloran in sediment at time 0 hours; error bars indicate standard error. One-way ANOVA was used to determine statistical differences (n=3).

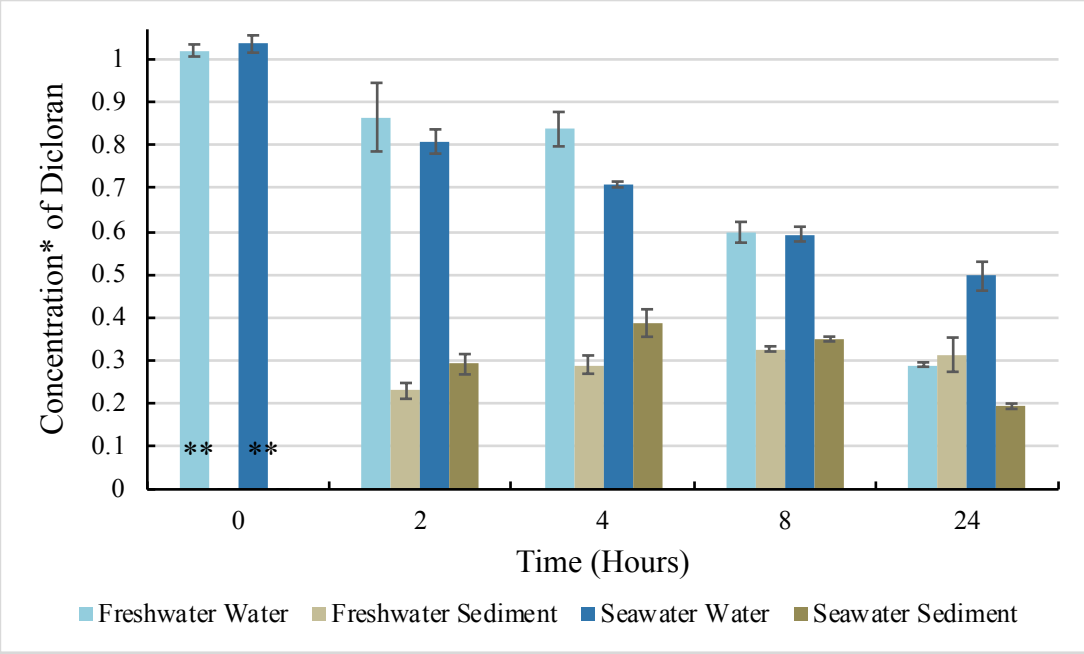


Figure 3.4. The photodegradation in water (blue) and dissipation into sediment (brown) of dicloran in freshwater and seawater (in legend) over a 24-hour light exposure period (*) water concentration measured in mg/L and sediment in mg/kg. Asterisks (**) indicate 0 mg/kg dicloran in sediment at time 0 hours; error bars indicate standard error. One-way ANOVA was used to determine statistical differences (n=3).

Table 3.2. The half-life, concentration, and mass of dicloran remaining after 24 hours of degradation or dissipation between distilled water and seawater in water-sediment systems, sediment-free systems (controls). One-way ANOVA (95%) and one-sided t-tests were used to determine statistical differences between treatments (n=3).

| | Distilled water and sediment | | Seawater and sediment | | Distilled water (without sediment) | Seawater (without sediment) |
|---|---|---|---|---|---|---|
| | Dark | Light | Dark | Light | Light | Light |
| Concentration water (µg/mL) | 0.61 ± 0.032 | 0.29 ± 0.010 | 0.72 ± 0.011 | 0.50 ± 0.058 | 0.41 ± 0.061 | 0.66 ± 0.055 |
| Half-life (hours) | n/a | 13.4 ± 0.987 | n/a | 27.0 ± 6.420 | 20.4 ± 2.800 | 45.3 ± 8.473 |
| Concentration sediment (µg/g) | 0.49 ± 0.069 | 0.31 ± 0.121 | 0.37 ± 0.062 | 0.19 ± 0.013 | No sediment | |
| Mass in water (µg) | 91.57 ± 4.800 | 43.54 ± 1.533 | 108.0 ± 1.698 | 74.37 ± 8.733 | n/a | |
| Mass in sediment (µg) | 12.19 ± 3.014 | 7.83 ± 1.726 | 9.25 ± 1.548 | 4.83 ± 0.313 | | |
| Total mass (µg) | 103.8 | 51.4 | 117.3 | 79.2 | | |
| Percent of applied | 69.2 | 34.2 | 78.2 | 52.8 | | |

A paired two-tail t-test ($\alpha = 0.05$) determined no statistical difference between treatments, with the exception of seawater water in dark and light, with a p-value of 0.0232 (n=3). One-way ANOVA analysis of variance with Tukey's multiple comparisons test ($\alpha = 0.05$) determined no statistical differences between water or sediment treatments, using average concentrations (n=3). Degradation and dissipation concentrations of dicloran appear similar within the first half-life for all measurements, values measured after the first half-life appears to be different between samples.

Dicloran is not ionized and therefore a difference in pH between distilled water and ASW is likely not a cause of the differences in distribution (USEPA 2006). In distilled water, dicloran has a shorter photodegradation half-life and partitions into sediment at a higher concentration within 24 hours compared to seawater. This may be due to a multitude of reasons, considering the mechanisms behind dicloran's behavior appear quite complicated. Although the competition

and reaction between ions in seawater may be a competing factor as to why dicloran partitions at a slower rate into sediment in seawater compared to distilled water.

### 3.3.2. Dicloran behavior without sediment

The photodegradation of dicloran was also monitored in Savillex jars without sediment in distilled water and artificial seawater for comparison of degradation rates when sediment was present and affecting the potential dissipation of the compound. Following methods previously described, the half-life of dicloran in distilled water without sediment was estimated to be 20.4 hours and after 24 hours of light exposure, 0.41-mg/L (41 % of the applied mass) of dicloran was measured in the water. For artificial seawater, the estimated half-life of dicloran was 45.3 hours, and 0.66-mg/L (66 % of the applied mass) of dicloran remained in solution after 24 hours of light exposure (Table 3.2). Sediment was excluded from the experiment as controls to determine how it impacts the photodegradation and partitioning in the irradiated-water sediment analyses.

### 3.3.3. Photodegradation of dicloran in vials at 40 W/m$^2$

The photodegradation of dicloran in distilled water and artificial seawater (32 ppt; 3.2%) in vials has previously been reported at a high light intensity (65 W/m$^2$) (Vebrosky et al., 2018). As this work suggested, multiple photodegradation processes and associated pathways were occurring simultaneously it seemed reasonable to repeat these experiments at lower light intensities so that the results could be compared to those in irradiated water-sediment investigations. To monitor the degradation of dicloran at a lower light intensity and lower salinity, kinetic experiments from this prior work were repeated using the same light intensity in the current investigation. At 40 W/m$^2$, the estimated half-life of dicloran in distilled water was 12.9 ± 0.898 hours and 14.9 ± 1.933 hours in artificial seawater (25 ppt; 2.5%) (Figure 3.5); in contrast Vebrosky reported a half-life of 7.6 hours in distilled water and 7.4 hours in artificial

seawater (3.2%) (Vebrosky et al., 2018). In previous work, 1 hour of exposure in the environmental chamber at 65 W/m$^2$ was estimated to be equivalent to 1.8 hours of sunlight during summer at 30°N latitude; 1 hour at 40 W/m$^2$ was estimated to be equivalent to 1.1 hours of sunlight (Pidwirny, 2006). The half-lives at this lower intensity are consistent with differences in daylight equivalency.

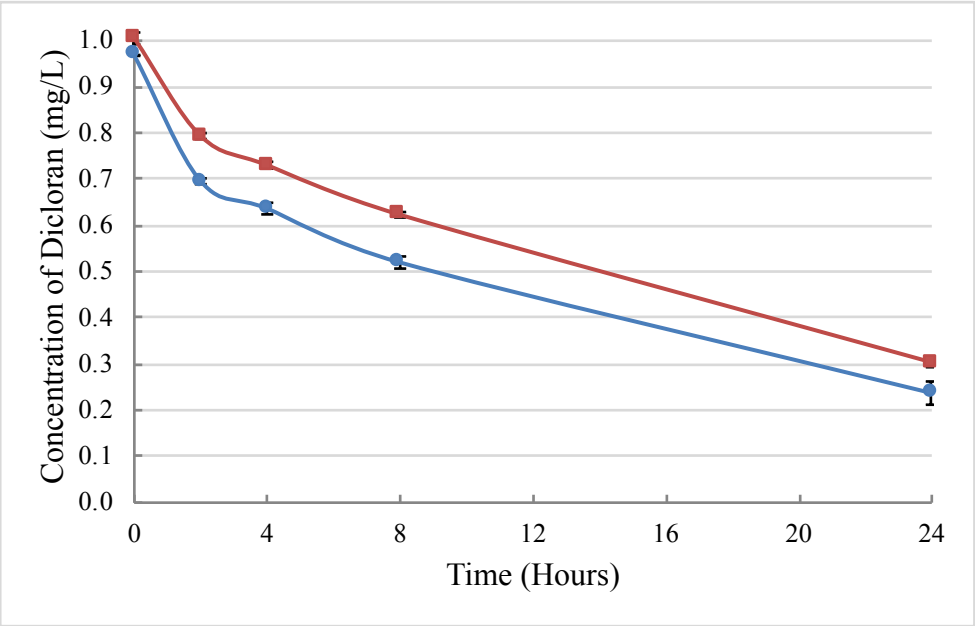


Figure 3.5. Photodegradation of dicloran in freshwater and seawater at 40 W/m$^2$ in borosilicate glass vials; distilled water (red) with a half-life of 12.9 hours and artificial seawater (blue) with a half-life of 14.8 hours, error bars indicate standard error. No degradation was observed in dark controls. One-sided t-test was used to determine statistical differences between media (n=3).

The intensity of light played a role in the half-life and rate of degradation, as well as differences in degradation between media for both parent and degradation products. The degradation of dicloran in distilled water and artificial seawater was not statistically different when irradiated at 65 W/m$^2$; at 40 W/m$^2$, there was a statistical difference between media. A one-sided paired t-test ($\alpha = 0.05$) produced a *p*-value of 0.0015, therefore salinity appeared to impact the photodegradation rate and half-life of dicloran the lower light intensity used in this study; salinity slows the rate of degradation of dicloran. Compared to a light intensity of 65

W/m$^2$, Vebrosky reported a *p*-value of 0.19 using a one-way ANOVA and no statistical difference between the photodegradation of dicloran in freshwater and seawater (Vebrosky et al., 2018). With a lower light intensity, it appears that there is a competition between degradation processes and mechanisms and the ions in seawater may be competing and reacting with dicloran, in turn slowing down its rate of degradation. At a higher light intensity, it appears that salinity is not a driving factor in the photodegradation rate.

Dicloran degrades to 2-chloro-1,4-benzoquinone (CBQ) and 1,4-benzoquinone (BQ) and ultimately to small acids. Salinity impacts the rate of formation and degradation of the benzoquinones. CBQ forms at higher concentrations than BQ; in distilled water, CBQ appears to be measurable at later time-points compared to seawater but it is fully degraded after 24 hours of light exposure. BQ appeared to form at higher concentrations in seawater compared to distilled water and both appear to follow a similar formation and degradation trend and are 0 ± 0.004 mg/L after 24 hours (Figure 3.6).

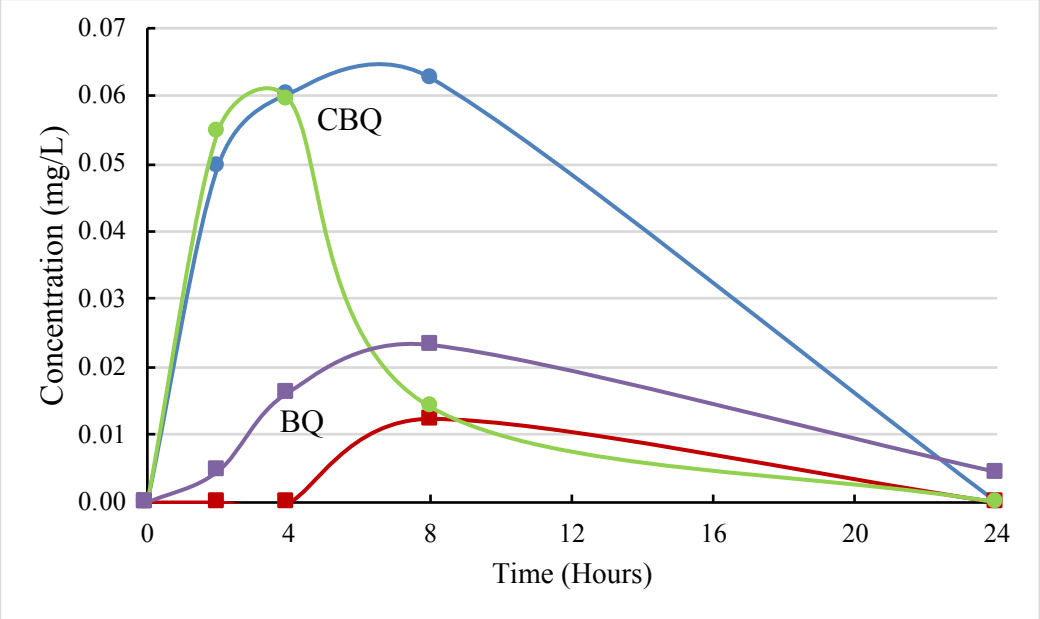


Figure 3.6. The formation and degradation of 1,4-benzoquinone (■) in distilled water (red) and seawater (purple) and 2-chloro-1,4-benzoquinone (●) in distilled water (blue) and seawater (green).

## 3.4. Discussion and conclusions

Sediment plays an important role in the behavior of chemicals that potentially enter aquatic ecosystems. The degradation of dicloran is impacted by salinity and sediment, and also impacts the distribution of photoproducts BQ and CBQ. CBQ in seawater appears to form highest in both systems and when sediment is present it forms at nearly twice the concentration by 8 hours of irradiation (Figure 3.7). CBQ in distilled water is below the LOQ in the sediment-free system (Figure 3.8), while it forms at a similar trend as in the vials when sediment is present. Neither CBQ or BQ were detected in any sediment sample at any time point. It is likely that in sediment both would quickly react to form covalent bonds with sediment organic matter similar to the process that organic intermediates naturally undergo during the humification process (Flores, 2014.; Harde 2008; Semenov et al., 2010).

Seawater was considered in the photodegradation and irradiated water-sediment analysis of dicloran as it has previously shown to impact the distribution of intermediate photodegradation products when irradiated at a higher light intensity than used in this dissertation (65 $W/m^2$) (Vebrosky et al., 2018). In south Louisiana, there is risk for saltwater intrusion due to coastal land loss and erosion, therefore the use of sediment obtained from rice fields in Louisiana was kept constant in this analysis. South Louisiana encompasses a large portion of the rice fields within the state and therefore the risk for saltwater intrusion due to climate change is rising. While the use of dicloran in rice fields is highly unlikely, the use of the fungicide on silt loam sediments is relevant. Therefore, understanding the mechanism of dicloran degradation and partitioning between freshwater and seawater with and without sediment is important and consistency within this study was considered for this model.

It is interesting to note that the product distributions were considerable different in sediment free systems when dicloran is irradiated in vials (Figure 3.6) vs Teflon jars (Figure 3.8), and that CBQ was not measured at detectable levels in distilled water in freshwater in irradiated samples in jars. In previous published works (Vebrosky et al., 2018) it was suggested that multiple mechanisms occurring simultaneously were likely responsible for dicloran's photodegradation. Changes in conditions such as modifications in salinity or light intensity could likely impact product distributions by favoring one mechanism kinetically or thermodynamically over the other, and these differences provide evidence for a multi-mechanistic degradation pathway. The primary mechanism for photodegradation of dicloran has previously been reported to be photonucleophilic substitution of the nitro group on the ring structure, although reductive processes also appear to be occurring simultaneously which may explain the formation of 1,4-benzoquinone (Vebrosky et al., 2018). This mechanism has previously been reported for other pesticides such as pentachlorophenol (Miille et al., 1983). A thorough investigation of these was beyond the scope of this thesis but may be the subject for future work.

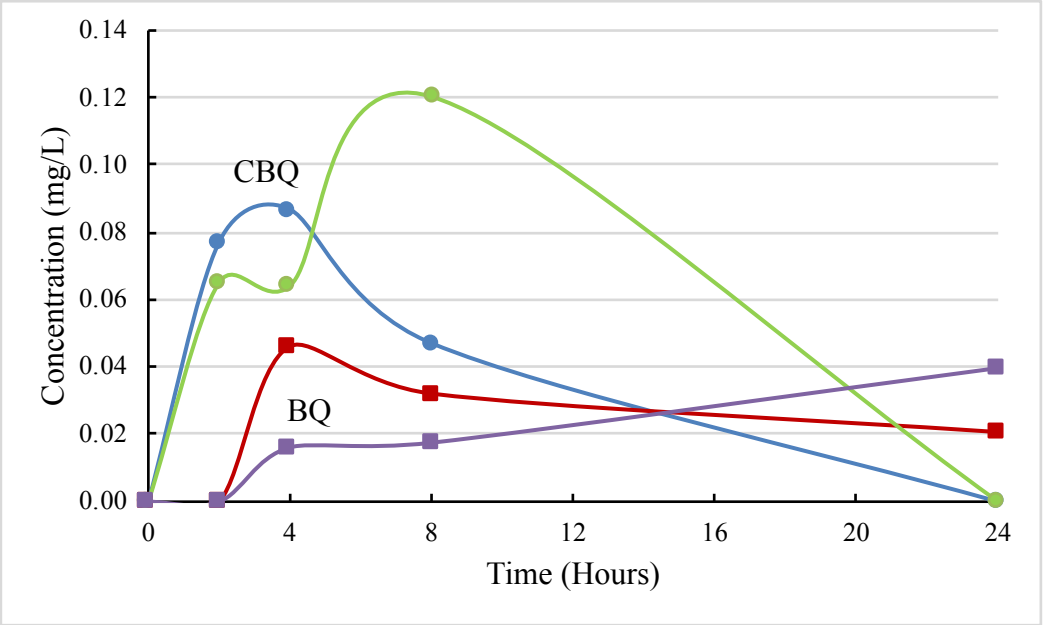


Figure 3.7. The formation and degradation of 1,4-benzoquinone (■) in distilled water (red) and seawater (purple) and 2-chloro-1,4-benzoquinone (●) in distilled water (blue) and seawater (green).

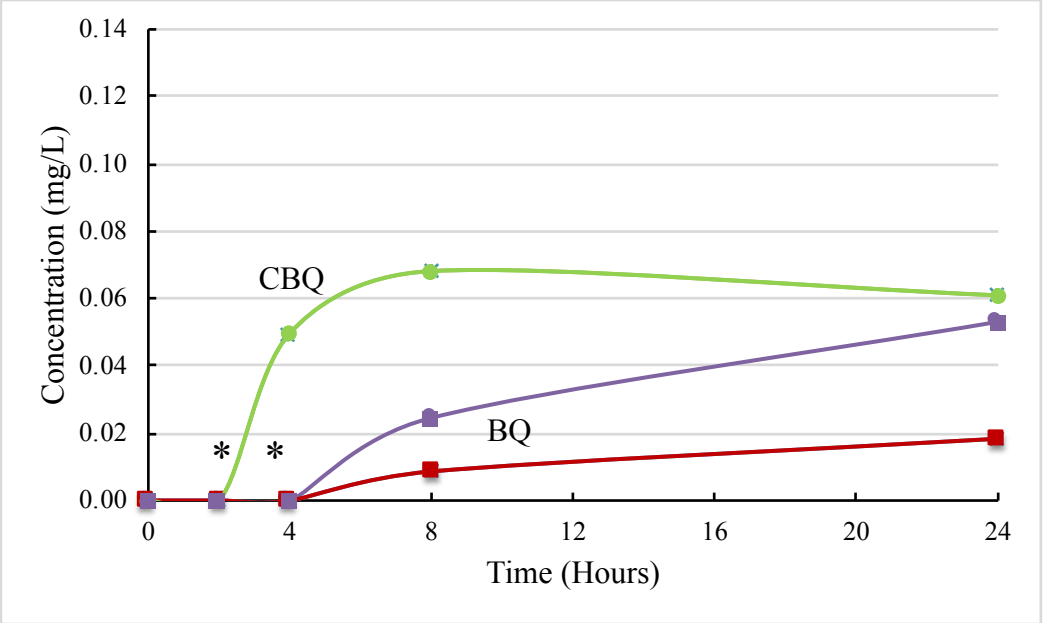


Figure 3.8. The formation and (photo)degradation of 1,4-benzoquinone (■) in freshwater (red) and seawater (purple) and 2-chloro-1,4-benzoquinone (●) in seawater (green) in Teflon jars without sediment. Asterisks indicate concentrations below the LOQ for BQ in freshwater; CBQ was not detected in freshwater in any sample.

The absence of 2-chloro-1,4-benzoquinone in freshwater in the sediment-free systems may be a result of mechanistic differences resulting from photodegradation in the Teflon jar

compared to the borosilicate glass vials. While lids are removed from the Teflon jars so the samples can be exposed to light, the vials are exposed to more light. As light penetrates the round vials, it refracts and more light enters the system for a faster degradation. The presence of ions in the ASW Teflon jars may be causing indirect photolysis and light refracting that degrades dicloran through the previously proposed photodegradation pathway that the absence of ions does not support.

In this study, water-type varied, and sediment remained constant. It appeared that dicloran dissipated into silt loam sediment at higher concentrations in freshwater than in seawater, in both dark and light trials. Direct photolysis of dicloran in water is apparent and previously reported, though indirect photolysis may also be playing a role in the photodegradation of dicloran in water and in the water-sediment systems. The behavior of a pesticide in low percentage sand sediment is of particular importance in coastal areas that are subject to both subject to both sediment runoff and saltwater intrusion. Areas that were typically or historically freshwater systems now are at risk of higher saline water encroachment and are relevant in areas such as south Louisiana with subsidence and land loss as well. External factors such as sediment type and salinity are not typically included into pesticide registration or re-registration decision processes but has local relevance and should be considered for the future.

Irradiation of chemicals in water-sediment systems has produced different half-lives and degradation products or product distributions in other published works. The fungicide chlorothalonil has been analyzed in a similar irradiated-water sediment experimental design as dicloran (Kwon et al., 2006A). Chlorothalonil (CHT) readily binds to sediments and its primary soil degradation product is reported to be 4-hydroxychlorothalonil (4-OH-CHT), which is re-suspended into the water column where it undergoes photolysis via a similar proposed

degradation pathway as dicloran (Armbrust, 2001). The behavior of CHT and 4-OH-CHT in both dark and light irradiated water-sediment systems showed differences in distribution of the compounds between sediment and water was dependent of the type of sediment used in the analysis and the presence of sunlight. With sunlight present, 4-OH-CHT rapidly photodegrades with a maximum reported half-life of 301 min. in artificial seawater without sediment present; therefore, the external factors such as sunlight, salinity, and sediment significantly impact the behavior of a chemical in laboratory exposure experiments (Armbrust, 2001; Hladik et al., 2008; Kwon et al., 2006A).

The fungicide benalaxyl was also studied within a water-sediment system, in the presence of sunlight. Benalaxyl (BX) has two enantiomers, with the *R*-(-)-BX reported as more active than the *S*-(+)-BX, which in turn may be more toxic to aquatic organisms (Lui et al., 2015). The herbicide flumioxazin has been reported to undergo photolysis and hydrolysis and been studied in water-sediment and sediment-free systems to determine differences in chemical distribution with and without light taking the presence of sediment into consideration (Kwon et al., 2006B; Shibata et al., 2011). Shibata reported a short half-life for flumioxazin in aqueous solutions, with and without light, and longer half-life when in the presence of sediment. In the absence of sediment, the half-life of flumioxazin in light is reported to be 0.1-0.3 days and with sediment, 0.2-1.5 days; similar trends were observed in the dark trials (Shibata et al., 2011). Kwon *et al.* also reported hydrolysis and photolysis of flumioxazin in aqueous solutions. In a sediment-free experimental design, the photolysis half-life of flumioxazin was estimated to be 4.9 and 42 hours in pH 7 and pH 5 buffers, respectively; at pH 9, flumioxazin rapidly hydrolyzed and resulted in a half-life of 0.25 hours (Kwon et al., 2006B). Differences in degradation product distribution were reported between Kwon and Shibata, likely a result of sediment influences on the parent

compound. In the sediment-free experimental analysis of the photolysis and hydrolysis of flumioxazin, two degradation products were reported; six degradation products were proposed for the water-sediment system. Both authors reported the presence of 6-amino-7- fluoro-4-(2-propynyl)-1,4,-benzoxazin-3(2H)-one (APF) in their proposed pathways, while the other reported degradation products differed between experimental design (Kwon et al., 2006B; Shibata et al., 2011).

Halogenated hydrocarbons have been used in anaerobic water-sediment systems to determine partitioning and degradation. Nitrogen-containing hydrocarbons appears to degrade predominately by nitro-reduction processes through a phenol pathway. Reductive dehalogenation was another primary degradation process reported for the halogenated hydrocarbons (Peijnenburg et al., 1992).

The Food and Agriculture Organization (FAO) reports 88% of dicloran applied to a water-sediment had been lost after 360 hours of exposure to a xenon lamp to simulate sunlight. It was also reported in this FAO report using a sandy loam sediment under both aerobic and anaerobic conditions in the dark to monitor the degradation of dicloran. They reported a 10-day half-life of dicloran in a flooded sandy loam soil. Data included in the FAO also reported a half-life of dicloran in phosphate buffer with 1% ACN using a mercury arc lamp, estimated to be 41 hours. All unpublished results reported by the FAO used [$^{14}$C]dicloran and followed the radioisotope throughout analyses (FAO, 1998).

Understanding the relationship between ecosystems and possible contaminants, such as pesticides, is pertinent for risk exposure studies and management. Some regions may require specialized or further monitoring or testing of combinations of factors due to sensitive ecosystems, at-risk or endangered species, previous [over] contaminated waters, historically

contaminated sediments, or other region-specific cases that may call for further or more in-depth analyses of pesticides.

The dissipation of dicloran from water appears to be faster in water-sediment systems, compared to sole water systems likely from the combined processes of sediment partitioning and photodegradation in the overlying water. The intensity of light also appears to kinetically play a role in the rate of degradation and half-life of dicloran as well as the distribution of its products and is media dependent.

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# CHAPTER 4. PHOTOTOXICITY OF DICLORAN TO JUVENILE FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

## 4.1. Introduction

The global population continues to increase, as does the demand for healthy, plentiful crop yields; therefore, the usage of pesticides, such as fungicides, continues to increase throughout the world (Phipps et al., 2002). Pesticides, including herbicides, fungicides, and insecticides, are relied upon by both developed and developing countries to enhance crop health to feed their populations and sustain demands (Ecochin, 2001; Tilman et al., 2009). With increased usage of pesticides, comes the risk of increased runoff into water bodies that may pose a threat to non-target organisms (Devine et al., 2007). As many of these pesticides enter waterways ranging from freshwater creeks to estuaries flowing into the ocean, they have the capability of persisting, accumulating in sediments, bioaccumulating in both aquatic and terrestrial organisms, or breaking down into other chemical components (Cuppen et al., 2000; Eichelberger et al., 1971).

Pesticides can break down by a variety of both natural and anthropogenic causes. When pesticides enter water bodies, they can potentially undergo hydrolysis; the pesticide disulfuton is known to undergo hydrolysis along with similar organophosphorus pesticides, breaking down in water (Zamy et al., 2004). Pesticides can also undergo photolysis in the presence of sunlight (Crosby, 1998). Pentachlorophenol (PCP) is an organochlorine pesticide that has been used as an herbicide, fungicide, bactericide, and insecticide, according to the EPA. PCP undergoes photolysis with a reported half-life as short as 0.9 hours (USEPA, 2008; Miille et al., 1983; Wong et al., 1981). Similar herbicides and fungicides such as 3,4-dichloroaniline and nitrofen have also shown to photodegrade when exposed to sunlight, breaking down into degradation products including quinones, hydroquinones, and aliphatic acids (Armbrust, 2001; Crosby, 1972;

Miille et al., 1983; Miller et al., 1979; Nakagawa et al., 1974; Wong et al., 1981). The degradation of the previously mentioned pesticides may be a result of both direct and indirect photolysis (Crosby, 1998).

Dicloran (2,6-dichloro-4-nitroaniline; Figure 4.1) is a substituted aniline fungicide and is the active ingredient in Botran®, which can be found in various formulations manufactured by Gowan Company, LLC.; it is used on crops throughout the southern and western United States including lettuce, celery, cherries, and grapes (Gowan). In Louisiana, dicloran is primarily used to prevent the spread of fungal diseases on sweet potatoes. Sweet potatoes are a multi-million-dollar industry in Louisiana, ranking second in sweet potato production in the country, behind North Carolina (Edmunds et al., 2003). Dicloran is applied by sprinkler irrigation, dip tanks, areal spray, and chemigation, therefore the risk for chemical drift into aquatic ecosystems is highly likely (USEPA, 2001).

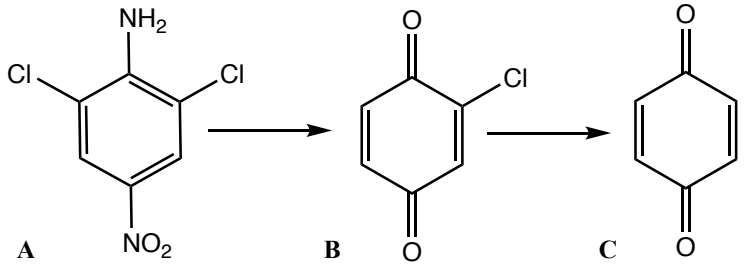


Figure 4.1. Chemical structures of (A) dicloran, (B) 2-chloro-1,4-benzoquinone, and (C) 1,4-benzoquinone.

In the presence of sunlight, dicloran has shown to undergo photonucleophilic substitution and ultimately degrade through a quinone-hydroquinone degradation pathway (Vebrosky et al., 2018). Two intermediate photodegradation products of dicloran include 2-chloro-1,4-benzoquinone and 1,4-benzoquinone (Figure 4.1, B and C respectively), similar to the proposed photodegradation pathways of pesticides including PCP, nitrofen, and chlorothalonil (Armbrust, 2001; Crosby, 1972; Miille et al., 1983; Miller et al., 1979; Nakagawa et al., 1974; Wong et al.,

1981). The similar photodegradation pathways of pesticides suggest a higher risk for non-target aquatic organisms to not only are exposed to the pesticide runoff but to also be exposed to the degradation products of these pesticides.

Fathead minnows (*Pimephales promelas*) are a commonly used freshwater fish species for toxicity assays. In their natural habitat, they inhabit slow-moving streams, ponds, and wetlands, but are widely tolerant of conditions and therefore can be found elsewhere; they range from Canada to Mexico. Fathead minnows belong to the Cyprinidae family and are a good probe for understanding the toxic impacts chemicals may have to others within the family including carp and true minnows (Ankley et al., 2006). The purpose of this investigation was to determine the potential lethal and sub-lethal toxic impacts dicloran and its photodegradation products pose to fathead minnows, taking sunlight into account as an amplifying factor.

## 4.2. Materials and methods

### 4.2.1. Fish exposures

Juvenile fathead minnows (Figure 4.2) aged 1.5 months (2 cm average length) were obtained from C. K. Associates (Baton Rouge, LA) and transported to Louisiana State University's Aquaculture Research Station (ARS; Baton Rouge, LA) where they were housed prior to experiments. Minnows were separated into 2.5-gallon glass fish tanks (Carolina Biological, Burlington, NC) containing 5 L of filtered well water (Baton Rouge, LA) and acclimated to experimental conditions for 24 hours prior to exposure experiments. Tanks were dosed with various concentrations of 10,000 mg/L stock solutions of 2,6-dichloro-4-nitroaniline (dicloran), 1,4-benzoquinone (BQ), or 2-chloro-1,4-benzoquinone (CBQ) (Sigma-Aldrich, St. Louis, MO) in acetonitrile (ACN; Fisher Scientific, Hampton, NH); the dosage of chemicals for each tank included 0.10, 0.17, 0.25, 0.50, and 0.75 mg/L of dicloran and 0.02, 0.04, and 0.06

mg/L for both BQ and CBQ. Dark and light trials were completed simultaneously with non-dosed "controls" for both treatments; trials were done in quadruplicate with 20-25 minnows per tank for a 48-hour exposure period.


Figure 4.2. Juvenile fathead minnow.

After 48 hours, total mortality per treatment was recorded to accurately generate a dose-response curve using Prism 7 (GraphPad Software, La Jolla, CA). Surviving minnows were either preserved in TRIzol® Reagent (Fisher Scientific, Hampton, NH) or 4% paraformaldehyde in phosphate buffer solution (4% PFA in PBS; affymetrix, Cleveland, OH) for further analysis.

To mimic sunlight, a combination of UVA 340 fluorescent UV bulbs (Q-Lab Corporation, Westlake, OH) and 40-Watt Linear Fluorescent Lamps (Grainger®, Lake Forest, IL) were housed in 48-inch shop light fixtures (Grainger®, Lake Forest, IL); the output of the simulated sunlight in comparison to natural sunlight in Baton Rouge, LA on a July day at 12:00 PM, measured with a BLACK-Comet Concave Grating Spectrometer (StellarNet, Inc.; Tampa, FL) is shown in Figure 4.3.

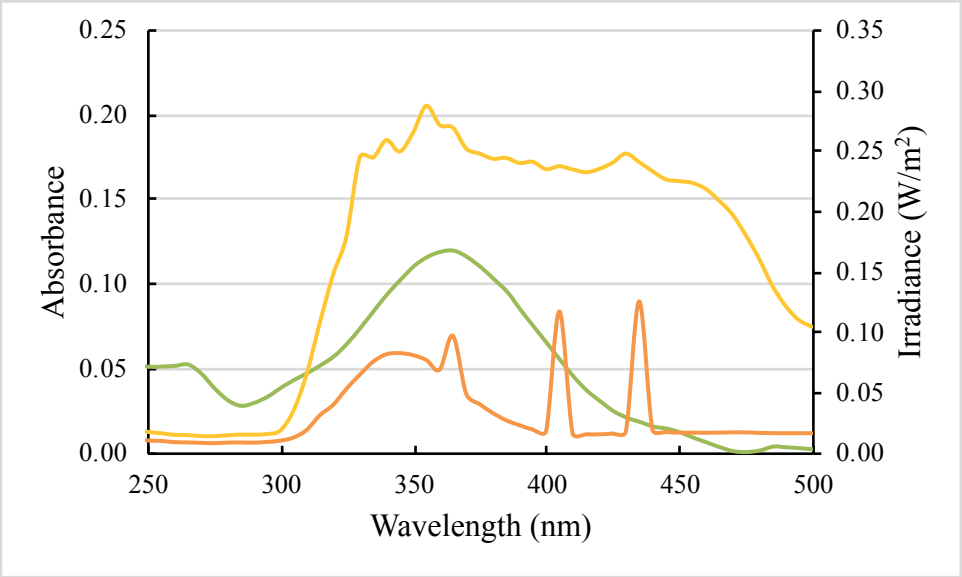


Figure 4.3. The absorbance of dicloran at 250-500 nm (green) and irradiance of natural sunlight in Baton Rouge, LA on a July 2018 day (yellow) and UVA and fluorescent bulb combination for phototoxicity experiments (orange). Wavelengths were monitored using a Black Comet Concave Grating UV-VIS spectrometer.

Lights were suspended from a shelving unit with light-exposed tanks surrounded by reflective paint and aluminum foil and dark tanks covered by tarps to enclose the system and keep unwanted light from entering (Figure 4.4).

Figure 4.4. The experimental design for the toxicity exposures of fathead minnows to dicloran; top shelves are dark and bottom shelves are light.

### 4.2.2. RNA extraction and measurement

Surviving minnows that were preserved in TRIzol® Reagent were kept in a -20°C freezer prior to RNA extraction. Whole fish were homogenized in 1 mL of TRIzol® using 800 µm glass beads (VWR, Radnor, PA) and shaken for 5 minutes on a Vortex-Genie 2 (Scientific Industries, Inc., Bohemia, NY) in 1.5 mL centrifuge tubes (Fisher Scientific, Hampton, NH). Immediately after homogenization, 200 µL of chloroform (VWR, Radnor, PA) was added to each tube and then centrifuged for 20 min; 400 µL of the supernatant was transferred to a 1.5 mL Eppendorf tube (Fisher Scientific, Hampton, NH) along with 400 µL isopropanol (VWR, Radnor, PA), shaken, and incubated in the -20°C freezer for a minimum of 30 min. The tubes were then centrifuged for 20 min, the supernatant was discarded, and 1 mL of 70% ethanol (VWR, Radnor, PA) prepared with RNase free water (Fisher Scientific, Hampton, NH) was added and centrifuged for another 10 min. The supernatant was removed and 25 µL of a "master mix" containing RNase free water, 10x DNase buffer, and TURBO DNase (Invitrogen, Carlsbad, CA) was added to each tube. The tubes were incubated in a 37°C water bath for 30 min, and 5 µL of DNase Inactivation Reagent (Invitrogen, Carlsbad, CA) was added and centrifuged; 25 µL of the supernatant was transferred to an Eppendorf tube and kept in the -80°C freezer until cDNA synthesis and qPCR analysis (Xu et al., 2018).

The concentration of RNA in each sample, along with the 260/230 and 280/260 values were measured using a GeneQuant Pro, values ranged 304-1006, 0.41-1.13, and 1.53-1.92 respectively (Amersham BioSciences, Little Chalfont, UK).

### 4.2.3. cDNA synthesis

Dependent upon the concentration of RNA for each sample, the correct amount of the RNA sample was aliquoted to PCR tube strips (VWR, Radnor, PA) and then diluted with 0.5 µL

deoxynucleotide mix [10 mM] (dNTP; G-Biosciences, St. Louis, MO) and 0.5 µL oligo DT (Integrated DNA Techologies, Coralville, IA). The strips were placed in the T100™ Thermal Cycler (Bio-Rad, Hercules, CA) and set to follow protocol. The protocol included a 5 min cycle at 65°C, 10 min at 4°C, and completed after 30 min. After 5 min at 4°C, 2 µL 5x SSIV buffer, 1 µL DTT, and 0.5 µL SSIV reverse transcriptase (Invitrogen, Carlsbad, CA) and 0.5 µL RNase free water was added into each tube, shaken, and put back into the thermal cycler to finish the sequence. The cDNA was then diluted with 90 µL of RNA-free water and stored in a -20°C freezer (Xu et al, 2018).

### 4.2.4. Quantitative PCR using cDNA

Primers for qPCR analysis for *P. promelas* were obtained from IDT (Coralville, IA); four primer pairs (Table 4.1) were chosen. GAPDH was chosen as the reference gene. The genes are representative of cell structure, endocrine disruption and immune function, all of which are critical systems of fishes.

Table 4.1. The genes, gene sequence, and source for genes chosen and analyzed for expression level analysis of fathead minnows.

| Function | Gene | Identity | Sequence |
|---|---|---|---|
| Reference | GAPDH | Glyceraldehyde 3-phosphate dehydrogenase | ACGAGGACACGACCAAATC<br>CCTTAACCTCACCTTGTACTT |
| Cell structure | BAIAP212 | Brain-specific angiogenesis inhibitor 1-associated protein | AGACGAAGGGAAGTGCATAAA<br>CGCTTGTTGAGTGCTGTTTAG |
| Endocrine | cyp1A | Cytochrome P4501A | AACAAGACGGAAGGAGAGAAAG<br>GCCCGTATTCTGGAGTCATATC |
| Endocrine | ESR1 | Estrogen receptor alpha | GTGACTATGCTTCCGGCTATC<br>CCTCCTTTCATCATGCCTACTT |
| Immune function | MMP9 | Matrix metalloproteinase 9 | GATGGACGAACTGATGGGTATC<br>CTGGTGCAGGAAGTGTATGT |

PCR samples were run on a 384-well plate; samples were prepared by combining 5 µL of the synthesized cDNA with 12 µL RNase free water, 5 µL 5x GoTaq polymerase colorless reaction buffer (Promega, Madison, WI), 0.5 µL dNTP, 0.25 µL of each primer (by pair, 50 µM)

(IDT, Coralville, IA), 1.25 μL Sybr Green 10x (Fisher Scientific, Hampton, NH), 0.5 μL ROX reference buffer (Fisher Scientific, Hampton, NH), and 0.25 μL GoTaq polymerase (Promega, Madison, WI) into two 96-well plates (VWR, Radnor, PA) then 10μL of each sample was aliquoted in duplicate to the 384-well plate. The samples were analyzed using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using sds2.4 software. Samples were prepared following qPCR protocol for each chosen primer pair; five individual samples per treatment were analyzed by PCR (Xu et al., 2018).

Normalized $C_t$ values were used to calculate the relative gene expression (mRNA) values for the selected genes in each sample (treatment) against controls (untreated fishes) using $2^{-\Delta\Delta Ct}$ method; data was analyzed using one-way ANOVA with Tukey's post-hoc multiple comparisons and Dunnett's multiple comparisons tests to generate p-values ($\alpha = 0.05$) and determine statistical differences between the treatments compared to controls (Prism 7, GraphPad, Inc., La Jolla, CA) (Brander et al., 2015; Livak et al., 2001; Xu et al., 2018).

### 4.2.5. Histology slide preparation

Surviving fish from each treatment were stored in 4 mL borosilicate glass vials (Fisher Scientific, Hampton, NH) containing 4% paraformaldehyde in a 4°C refrigerator. Individual, whole fish were placed in a premium processing/embedding tissue cassette between 2 biopsy pads (VWR, West Chester, PA) and preserved in 4% PFA. The minnows were cut into slides at the Louisiana State University School of Veterinary Medicine's Histology Laboratory (Baton Rouge, LA) and stained with hematoxylin and eosin (H&E) staining; the slides were analyzed using an AmScope Microscope with a Digital Camera attachment and AmScope (x86) software (United Scope, LLC., Irvine, CA) to observe anomalies in the gill filaments in comparison control fish with treated fish.

## 4.3. Results and conclusions

### 4.3.1. Fathead minnow response to chemical exposure

Fathead minnows showed a significant difference in response to dicloran exposure between dark and light trials. No mortality was observed in dark exposures ranging from control (0 mg/L) to 0.75 mg/L over 48-hours. Minnows exposed to 0.50-mg/L dicloran and light resulted in100% mortality, while no mortality was observed in light controls with no added dicloran. The dose-response of minnows exposed to dicloran and light is shown in Figure 4.5; the estimated $LC_{50}$ using best-fit values on a non-linear regression (curve fit) analysis of [agonist] vs. normalized response – variable slope is 0.19 mg/L ($\alpha = 0.05$).

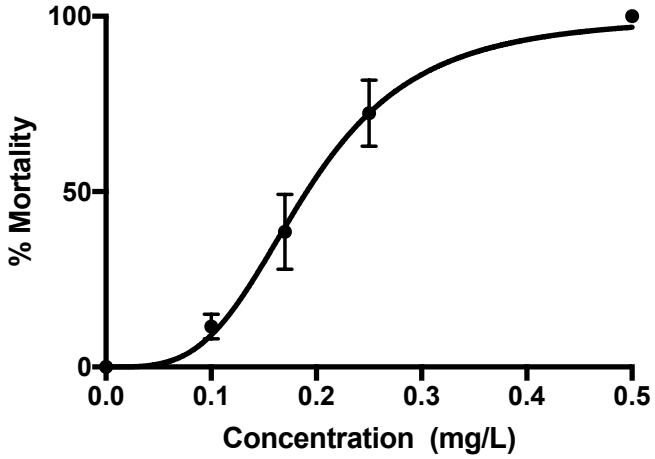


Figure 4.5. Dose-response results of concentration vs. % mortality for fathead minnows exposed to various concentrations of dicloran in the presence of simulated sunlight. Error bars indicate standard error.

Fathead minnows were also exposed to 2-chloro-1,4-benozquinone and 1,4-benzoquinone in dark and light trials. Both benzoquinones have shown to degrade in the presence of sunlight after <2 hours of light exposure; therefore, no effects for initial range finding trials were observed and no replicates were completed for the exposures using the benzoquinones and light. No mortality was observed for fathead minnows exposed to CBQ or BQ in the dark for 48 hours in four trials. Concentrations chosen for analysis were based upon concentrations of CBQ and

BQ quantified during the photodegradation of 1 mg/L dicloran in distilled water (Vebrosky et al., 2018).

Throughout experimental analysis, parameters such as dissolved oxygen (DO), water pH, and water temperature were monitored to ensure no negative impacts to fish were a result of water composition. Water temperature remained constant throughout the 48 hours of treatment in all tanks, both dark and light, and averaged 26ºC in the wet lab. Dissolved oxygen levels slightly fluctuated but were consistent between tanks, 4.5-5.0 mg/L and water pH remained between 7.8-7.9.

### 4.3.2. Gene expression

Surviving fathead minnows were analyzed using qPCR for potential sub-lethal effects that may impact gene expression. Whole body fish (n = 5) were used for analysis. GAPDH was used as the reference gene; the following genes were used to measure the relative gene expression (mRNA) of fathead minnows exposed to dicloran: cyp1a (Figure 4.6), ESR 1 (Figure 4.7), and MMP9 (Figure 4.8). Ten genes were chosen for analysis in total; cyp1a, ESR1, and MMP9 results depict the best observations of gene expression trends. One-way ANOVA analysis of variance with Dunnett's multiple comparisons test was used to determine statistical differences between the dark controls with the other treatments ($\alpha = 0.05$) (Brander et al., 2015, Xu et al., 2018).

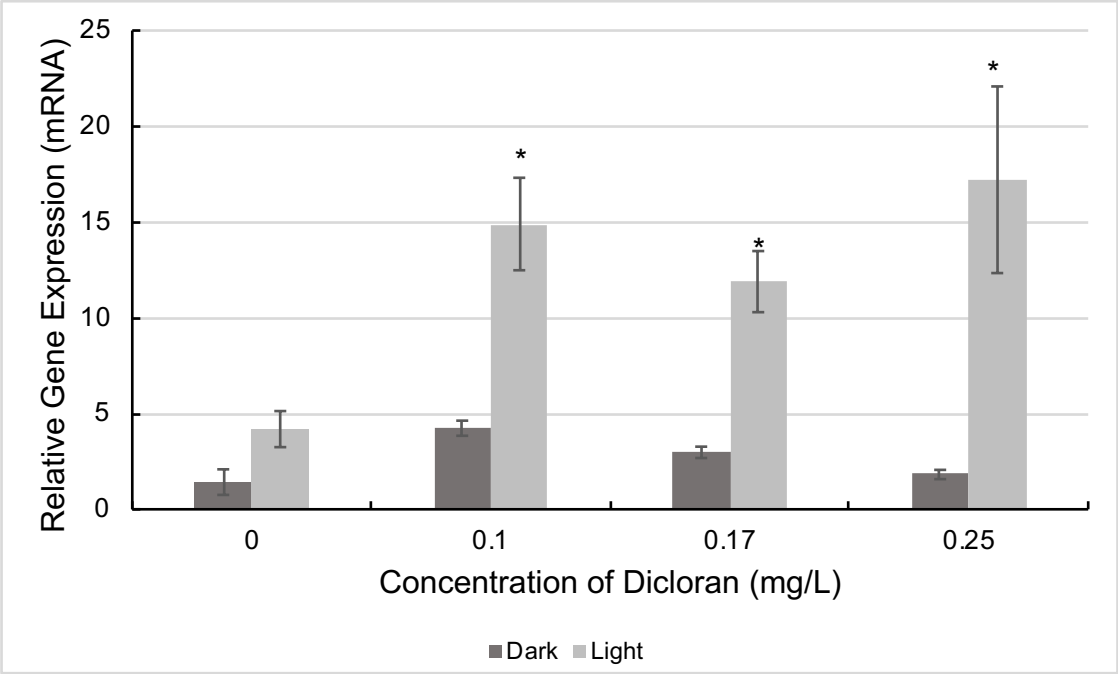


Figure 4.6. Relative gene expression of Cytochrome P4501A (cyp1a). Expression levels were compared to the untreated control samples, and asterisks (*) indicate significant differences. Error bars indicate standard error.

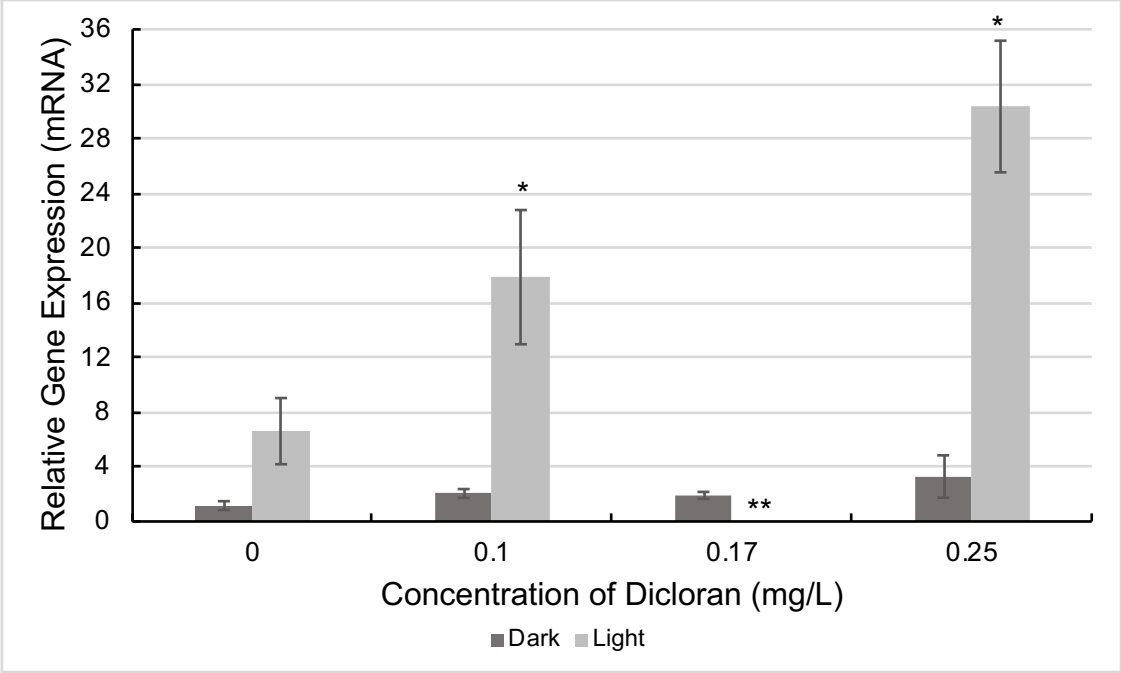


Figure 4.7. Relative gene expression of estrogen receptor alpha (ESR1). Expression levels were compared to the untreated control samples. Error bars indicate standard error; asterisks (*) indicate significant differences observed for gene expression ($\alpha$ = 0.05, 0.01) and (**) indicates not sampled.

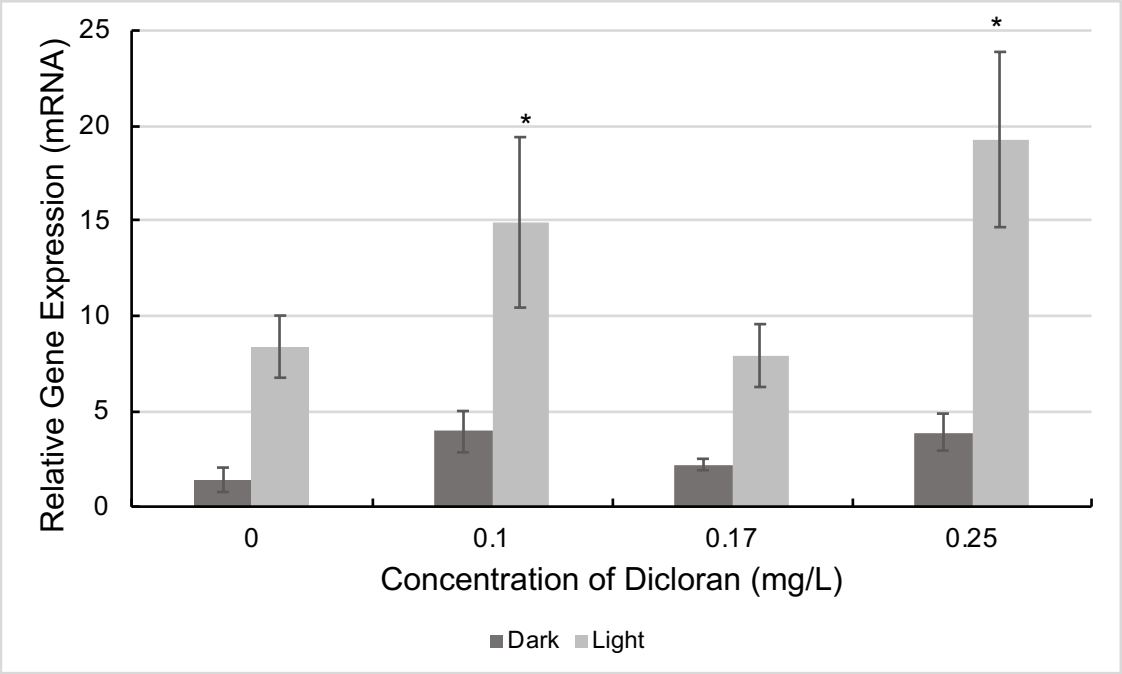

Figure 4.8. Relative gene expression of matrix metalloproteinase 9 (MMP9). Expression levels were compared to the untreated control samples. Error bars indicate standard error.

An upregulation in relative gene expression can be observed for MMP9, ESR1, and cyp1a in treatments that included both dicloran and light. An increased relative gene expression can be observed for light controls (0 mg/L), therefore UV exposure alone may impact gene expression; though, light and chemical in combination result in larger expression levels. While fish were dosed at 0.50 and 0.75 mg/L dicloran in the dark, no light samples at these concentrations were available for PCR analysis due to the $LC_{50}$ for irradiated dicloran being 0.19 mg/L and 100% mortality at these higher concentrations; therefore, these concentrations were not taken into consideration for qPCR analysis as there was no light samples for statistical comparison.

Significant differences between 0.10, 0.17, and 0.25 mg/L irradiated dicloran exposures were observed compared to dark controls, with p-values of 0.0194, 0.0455, and 0.0023 respectively for cyp1a as shown with (*) in Figure 4.6. No significant difference was observed

between dark controls and light controls. One-way ANOVA with Tukey's multiple comparison test generated significant differences between treatments for cyp1a gene expression Table 4.2.

Table 4.2. Statistical analysis of cyp1a across all treatments; (ns) indicate not statistically different and (*) indicates statistically different.

| | Dark control | 0.10 dark | 0.10 light | 0.17 dark | 0.17 light | 0.25 dark | 0.25 light |
|---|---|---|---|---|---|---|---|
| Light Control | ns | ns | ns | ns | ns | ns | * |
| 0.10 dark | ns | - | ns | ns | ns | ns | * |
| 0.10 light | ns | ns | - | ns | ns | ns | ns |
| 0.17 dark | ns | ns | ns | - | ns | ns | * |
| 0.17 light | ns | ns | ns | ns | - | ns | ns |
| 0.25 dark | ns | ns | ns | ns | ns | - | ns |
| 0.25 light | * | * | ns | * | ns | ns | - |

No statistical difference was observed for gene expression with the dark control compared to 0.10, 0.17, and 0.25 mg/L dark samples; statistical differences were observed for 0.10, 0.17, and 0.25 mg/L in light with one-way ANOVA ($\alpha = 0.05$) generating *p*-values of 0.0022 and <0.0001, respectively for ESR1. One-way ANOVA and Tukey's multiple comparisons test generated significant differences between treatments for relative ESR1 expression level (Table 4.3).

Table 4.3. Statistical analysis of ESR1 across all treatments; (ns) indicate not statistically different and (*) indicates statistically different.

| | Dark control | 0.10 dark | 0.10 light | 0.17 dark | 0.25 dark | 0.25 light |
|---|---|---|---|---|---|---|
| Light Control | ns | ns | ns | ns | ns | * |
| 0.10 dark | ns | - | * | ns | ns | * |
| 0.10 light | * | * | - | * | * | ns |
| 0.17 dark | ns | ns | * | - | ns | |
| 0.25 dark | ns | ns | * | ns | - | * |
| 0.25 light | * | * | ns | * | * | - |

Significant differences were determined by one-way ANOVA analysis of variance comparing treatment to the dark control; 0.10 mg/L and 0.25 mg/L irradiated dicloran generated *p*-values of 0.0251 and 0.0058 respectively. One-way ANOVA with Tukey's multiple

comparisons generated significant differences between each treatment (Table 4.4). Dicloran and light at the highest analyzed dose for gene expression (0.25 mg/L) was not significantly different compared to light control, 0.10 mg/L light, 0.17 mg/L light, and 0.25 mg/L dark.

Table 4.4. Statistical analysis of MMP9 across all treatments; (ns) indicate not statistically different and (*) indicates statistically different.

| | Dark control | 0.10 dark | 0.10 light | 0.17 dark | 0.17 light | 0.25 dark | 0.25 light |
|---|---|---|---|---|---|---|---|
| Light Control | ns | ns | ns | ns | ns | ns | ns |
| 0.10 dark | ns | - | ns | ns | ns | ns | * |
| 0.10 light | ns | ns | - | * | ns | ns | ns |
| 0.17 dark | ns | ns | * | - | ns | ns | * |
| 0.17 light | ns | ns | ns | ns | - | ns | ns |
| 0.25 dark | ns | ns | ns | ns | ns | - | * |
| 0.25 light | * | * | ns | * | ns | * | - |

Fish used in the gene expression analysis were chosen at random from the four trials, in which fish were obtained from the same source but from different batches and therefore the margin of dissimilarity increases for genealogical variations in samples. Light trials resulted in large standard errors, which could be in part due to the individual's response to the exposure of irradiated dicloran.

Variation in gene expression levels indicate a response to each treatment, in some cases an upregulation in the expression levels can be observed (i.e. ESR1 dark and light treatments). Expression levels were unique for each gene analyzed.

Genes were chosen based upon results from previously published literature and relevant to the health of fathead minnows; the chosen genes encompass major biological functions of fish. Cytochrome P4501A (cyp1a) is a gene related to steroid metabolism within the endocrine system. It has previously been reported to be representative of oxidative stress, oxidative DNA damage, tissue damage, and programmed death of cells in fathead minnows exposed to toxicants such as bifenthrin (He et al., 2012). It has also been considered a gene related to general stress to

fish; and downregulation in the expression of similarly related genes (cyp3a) have been reported in fathead minnows exposed to bifenthrin for 24 hours. Cyp3a is related to the detoxification of toxicants in fish, such as fathead minnows (Beggel et al., 2011).

Estrogen receptor alpha (ESR1) is representative of the disruption of estrogen receptors in a fish as a result of a toxicant (Filby et al., 2005). Therefore, a difference in expression for ESR1 is indicative of impacts to the endocrine systems such as steroid metabolism and hormone receptors. Matrix metalloproteinase 9 (MMP9) is a gene related to immune function of fishes. Also observed by immunohistochemistry analysis, MMP9 function is indicative of morphological features to cells and can potentially indicate cellular damage such as apoptosis (Thóme et al., 2010).

### 4.3.3. Histological analysis of gill filaments

Fathead minnows were preserved in 4% PFA in PBS for histological analysis. Whole body fish were cut into slides and stained with hematoxylin and eosin (H&E) at the Louisiana State University School of Veterinary Medicine's Histology Laboratory (Baton Rouge, LA) and analyzed at the Aquaculture Research Station. Control and the highest dose of dicloran (0.75-mg/L) gill filaments are shown in Figure 4.9 for dark trials; (A) shows the control fishes' gill filaments while (B) shows indications of oxidative stress. Although no mortality was observed at any concentration exposure of dicloran to fathead minnows, sub-lethal impacts to the gills is apparent.

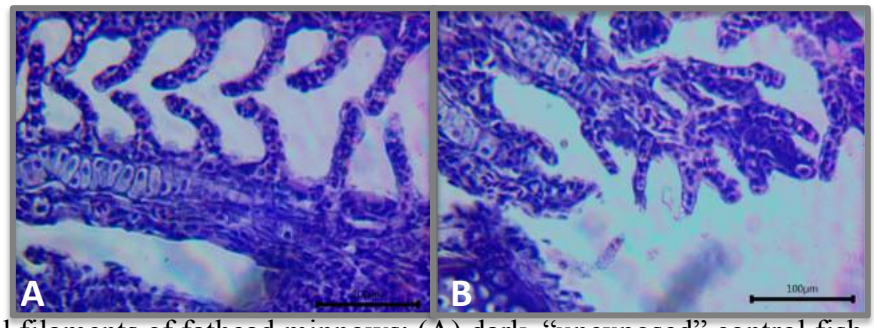

Figure 4.9. Gill filaments of fathead minnows; (A) dark, "unexposed" control fish, (B) dark, 0.75 mg/L exposure to dicloran fish.

Fish exposed to dicloran and sunlight showed negative impacts, ranging from death to lethargy. Slide images (40x) of gill filaments exposed to light and dicloran are shown in Figure 4.10. The slide image in Figure 4.10. (A) shows the healthy gill filaments of an experimental control and Figure 4.10 (A) shows the gill filaments of the experimental light control, without dicloran, indicating slight cell proliferation and minimal oxidative stress. The combination of light and increasing concentrations of dicloran negatively impacts the gills of fathead minnows, increasing oxidative stress; (B) at 0.10 mg/L, apoptosis can be observed, (C) at 0.17 mg/L, increasing cell proliferation can be observed, and (D) at 0.25 mg/L, increased stress resulted in further cell proliferation and apoptosis, possibly necrosis, of the gill filaments.

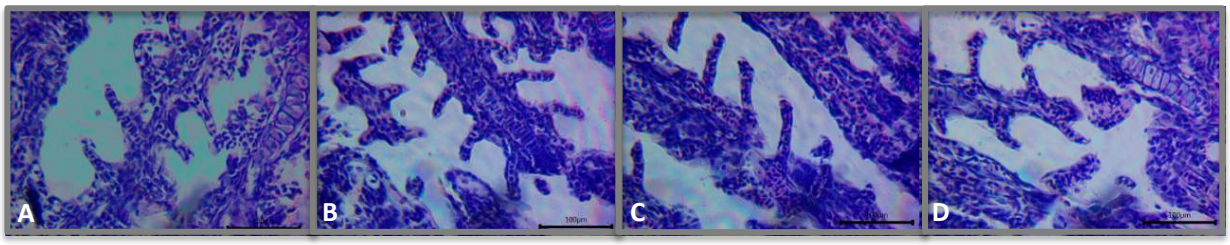

Figure 4.10. Gill filaments of fathead minnows exposed to light (A), light and 0.10 mg/L dicloran (B), light and 0.17 mg/L dicloran (C), and light and 0.25 mg/L dicloran (D).

### 4.3.4. Implications of results

While exposure to non-activated (by UV-light) dicloran did not result in lethal effects for fathead minnows, sub-lethal effects were observed. At the highest treatment in dark samples, cell proliferation is apparent in the gill filaments of the minnows. Analysis of the relative gene

expression levels for cyp1a, ESR1, and MMP9 did not result in statistical differences between control and dark treatments. Cell proliferation is observed in a higher treatment of dicloran in the dark in Figure 4.9 compared to the concentrations used in gene expression analysis, therefore there is a potential upregulation in gene expression that has not been analyzed. At levels 0.25 mg/L and below in the dark, the minnows appear to be under minimal stress.

Trends vary drastically when light is added to the experimental design. Exposure to UV-activated pesticides are possible for aquatic organisms as a result of runoff from treated fields during a sunny day. Dicloran appears to be phototoxic to fathead minnows at concentrations that are nontoxic in the dark. Both lethal and sublethal effects can be observed for fish exposed to irradiated dicloran, ranging from upregulation in gene expression to apoptosis in their gill filaments.

## 4.4. Discussion

Exposure to dicloran and sunlight resulted in both lethal and sublethal impacts to fathead minnows. The estimated $LC_{50}$ for fathead minnows exposed to irradiated dicloran is 0.19 mg/L, which is comparable to concentrations of dicloran detected in environmental samples.

Cytochrome P4501A (cyp1a) functions by metabolizing with the goal to detoxify toxicants that enter the fish (Garcia-Reyero et al., 2009; Wiseman et al., 2013). Colavecchia *et al* exposed fathead minnows (7 days post-hatch) to tar sands to monitor the expression of cyp1a of specific organs and tissues. PAHs are a common toxicant that fishes have the potential to come into contact to and are found in tar sands; PAHs are lipophilic and bioaccumulate in fishes. Non-chlorinated PAHs can be metabolized as a result of Phase I Metabolism due to P-4501A monooxygenase genes (Colavecchia et al., 2007; Crosby, 1998). In the kidneys of fathead minnows, high expression levels for cyp1a were observed for the fish exposed to tar sands. A

positive correlation between an increase in mortality and increase in cyp1a expression was observed for fathead minnows (Colavecchia et al., 2007).

Analysis of relative gene expression indicated sublethal impacts to fathead minnows. The upregulation in the expression level of cyp1a may indicate an increase in cellular (DNA) damage as a result of activated dicloran. Sullivan *et al* exposed fathead minnows to biosolids (including PAHs) and monitored the expression levels of the cyp1a gene as well as DNA damage in hepatocytes using a COMET assay. They observed a correlation between the upregulation of cyp1a and DNA damage using the Pearson correlation index; therefore, the expression level of cyp1a may be an indicator of cellular damage due to chemical exposure (Sullivan et al., 2007).

ESR1 expression levels may indicate a response to potential endocrine disrupting chemicals (EDCs), as ESR1 is the estrogen receptor 1 hormone gene. Filby and Tyler observed increases in ESR1 and ESR2 after exposing male fathead minnows to estradiol (Filby et al., 2005). When fathead minnows are exposed to endocrine disrupting compounds (EDCs), other estrogen-sensitive genes have shown responses. Johns *et al* exposed fathead minnows (24-hr post-hatch) to EDCs to monitor the expression of the genes, growth hormone (gh), vitellogenin (vtg1), and insulin-like growth-factor 1 (igf1), and an upregulation in response to EDC exposure was observed. Impacts observable at young life-stages as limited as gender cannot be determined at that young age. Decreased growth, including weight and body length, are commonly observed for fishes in response to upregulation in estrogen-sensitive genes (Johns et al., 2009).

The MMP9 gene indicates damage to connective tissues. Similar trends can be observed for fathead minnows' gene expression for MMP9 compared to ESR1 and cyp1a, an upregulation in response as concentration of irradiated dicloran increases. Relative gene expression may be

an indicator for the potential for lethal responses of fishes to chemical exposure. An increase in mortality was directly correlated to an upregulation in the relative gene expression for the three genes analyzed for fathead minnows.

Oxidative stress of gill filaments is another obvious sublethal impact that increases in severity with an increasing concentration of irradiated dicloran. While a high dose (0.50 mg/L) of dicloran in the dark indicated cell proliferation and increased stress to fathead minnows, the degree of proliferation is not as extensive as observed in light trials. Apoptosis can be observed at 0.10 mg/L of irradiated dicloran. The gill filaments of the fish exposed to 0.25 mg/L appears to show signs of an aneurism and necrosis in the lamellae. Gills are an important barrier for fishes to keep potential toxic substances from entering the bloodstream. Responses such as cell proliferation as a defense mechanism where there is an attempt to increase the surface area to prevent the toxicant from entering through the gills. Increases in stress as a result of high concentration or more toxic chemicals influence further responses such as apoptosis, which is the programed death of a cell by an organism which differs from necrosis which is the death of a cell due to injury (Cengiz, 2006). Sunlight is potentially a contributing factor to pesticide toxicity to non-target aquatic organisms, as shown in the data presented.

Toxicological analyses in regulatory studies do not account for sunlight to enhance the potential toxic responses to organisms when registering pesticides, though variation in the response of fathead minnows with and without sunlight is apparent for dicloran toxicity.

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# CHAPTER 5. SALINITY IMPACTS ON THE PHOTOTOXICITY OF DICLORAN TO *MENIDIA BERYLLINA*

## 5.1. Introduction

Euryhaline organisms are a unique group of aquatic species that can tolerate a wide range of salinities through osmoregulating processes (Kültz, 2015; Wedderbum et al., 2016). Physiological mechanisms within the fishes and invertebrates regulate the intake of salts keep an osmotic balance by diluting salt concentration from within the fish so that they can survive across salinity gradients. Environmental salinities and salinity tolerances are critical environmental factors impacting both stenohaline and euryhaline species (Cataldi et al., 2005; Evans, 2010; Kidder et al., 2006; Kültz, 2015).

Inland silversides (*Menidia beryllina*) are a small, euryhaline fish species native to the East Coast of the United States and Gulf of Mexico consisting of both coastal and inland populations. Silversides are tolerant of salinities ranging from 0.0-3.0% with populations tolerant of hypersaline conditions reaching 7.0% salinity. *Menidia spp.* including tidewater (*M. pennisulae*) and Atlantic (*M. menidia*) silversides are found in estuarine/marine areas throughout North America (Brander et al., 2013, 2016). *M. beryllina* are most commonly found inhabiting estuarine regions where the salinity is 1.9% and below, while *M. menidia* are commonly found in regions of 2.5% (Fluker et al., 2011).

The United States Environmental Protection Agency (US EPA) has adopted methods for the use of inland silversides as a standard marine/estuarine fish for toxicity testing. US EPA also uses sheepshead minnows (*Cyprinodon variegatus*) for marine and estuarine testing on fishes. Both regulatory and research agencies have used inland silversides for a variety of toxicity testing, including endocrine disrupting compounds (EDCs), pesticides, and PAHs (Brander et al.,

2016; DeCourten et al., 2017; Genthner et al., 1992; Middaugh et al., 1988; Middaugh et al., 1995; USEPA, 2002A, B; USEPA, 2010; Weis et al., 1976).

The fungicide dicloran (2,6-dichloro-4-nitroaniline, DCNA) is a substituted aniline fungicide manufactured by Gowan Company under the tradename, Botran®. Dicloran is registered for use on crops throughout the southern and western United States, including many coastal regions in California, Washington, and Oregon. The potential for dicloran to enter shallow water systems such as bayous, brackish wetlands, and estuarine water bodies increases due to the risk of runoff and spray drift as a result of its application processes. Dicloran is applied at a maximum application rate of 4 lb ai/acre for all registered crops with the exception of fennel and celery, which are 5 lb ai/acre, and potatoes, which is 7.5 lb ai/acre (USEPA, 2006).

Dicloran has previously been reported to undergo photolysis through a quinone-semiquinone photodegradation pathway, and the difference in behavior has shown to be a result of factors including salinity, sediment presence, and light intensity (Figure 5.1) (Vebrosky et al., 2018). Other pesticides, for example pentachlorophenol, 4-chlorophenol, 2,4-D, and nitrofen, have been reported to photodegrade via similar pathways resulting in similar intermediate degradation products as dicloran, including 1,4-benzoquinone and/or 2-chloro-1,4-benzoquinone.

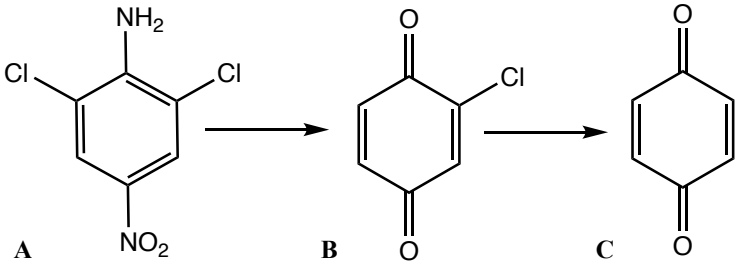


Figure 5.1. The molecular structures of dicloran (A), 2-chloro-1,4-benzoquinone (B), and 1,4-benzoquinone (C).

Dicloran has previously shown phototoxicity to the freshwater fish species, fathead minnows (*Pimephales promelas*), and the estuarine invertebrate, eastern oysters (*Crassostrea*

*virginica*) (Xu et al., 2018). Euryhaline fishes, such as inland silversides, inhabit wide ranges of salinities and the rate of formation and degradation of the benzoquinones have previously been reported to vary dependent upon salinity; therefore, the potential for variation in response to the phototoxicity of dicloran exists (Vebrosky et al., 2018).

The purpose of this investigation was to use the fungicide dicloran as a probe for the potential variation in phototoxic responses of a euryhaline fish species exposed to a toxicant in the presence of sunlight across a salinity gradient.

## 5.2. Materials and methods

Similar methods were described in Chapter 4 for the exposure of fathead minnows to irradiated dicloran.

### 5.2.1. Chemical reagents

A standard of 10,000-mg/L analytical grade dicloran (Sigma Aldrich, St. Louis, MO) in HPLC grade acetonitrile (ACN; VWR, Radnor, PA) was used to dose fish and monitor photodegradation rates. Artificial seawater (ASW) was made using Instant Ocean® (Blacksburg, VA) in HPLC grade distilled water (VWR, Radnor, PA) for chemical analyses and Crystal Sea (Marine Enterprises International, LLC, Baltimore, MD) in filtered well water (Baton Rouge, LA) for toxicity experiments; the salinity was monitored using a YSI Model 30 salinity, conductivity, and temperature meter (Yellow Springs Instruments, Yellow Springs, OH).

### 5.2.2. Photodegradation of dicloran in various salinities

The photodegradation of dicloran was completed following previously published methods (Vebrosky et al., 2018). Borosilicate glass vials (Agilent Technologies, Santa Clara, CA) containing 1 mL of 1 mg/L dicloran in 0.5%, 1.2%, and 2.5% seawater were placed in an ATLAS SUNTEST XXL+ environmental chamber (Mount Prospect, IL) outfitted with a

daylight filter for 24 hours at 40 W/m$^2$. Vials were removed at 0, 2, 4, 7 or 8, and 24 hours and analyzed using an Agilent 1260 Infinity HPLC (Santa Clara, CA) with a ZORBAX C-8 Eclipse Plus Analytical 4.6 x 150 mm 5 μm column, using a water and acetonitrile gradient mobile phase and monitoring the wavelengths using photodiode array detection at 380 nm. A one- way ANOVA with Tukey's multiple comparisons test ($\alpha = 0.05$) was used to determine statistical differences between the half-life of dicloran in each media. Each sample was completed in triplicate.

### 5.2.3. Phototoxicity experimental design

Juvenile inland silversides (1.5 months post-hatch) were obtained from C. K. Associates (Baton Rouge, LA) and Aquatic BioSystems (Fort Collins, CO). The fish were acclimated to experimental conditions for a minimum of 24 hours prior to the beginning of each trial. Silversides were acclimated to 3 salinities; 2.5% (the salinity at which they were inhabiting upon arrival to the Aquaculture Research Station) and were slowly acclimated to 1.2% and 0.5% salinities over a multi-week timeframe with daily 70% water changes and were fed dried *Artemia* twice daily (Cadmus et al., 2018; USEPA, 1975). Fish at 12 ppt salinity were acclimated over a 2-week period, while fish at 5 ppt were acclimated over a 3.5-week period. All fish exposed to dicloran were aged 1.5 months. Silversides needing acclimation periods were obtained from suppliers at a younger age to account for the acclimation period prior to experimental proceedings. The average length of juvenile silversides used in this experiment was about 2.5 cm (Figure 5.2).

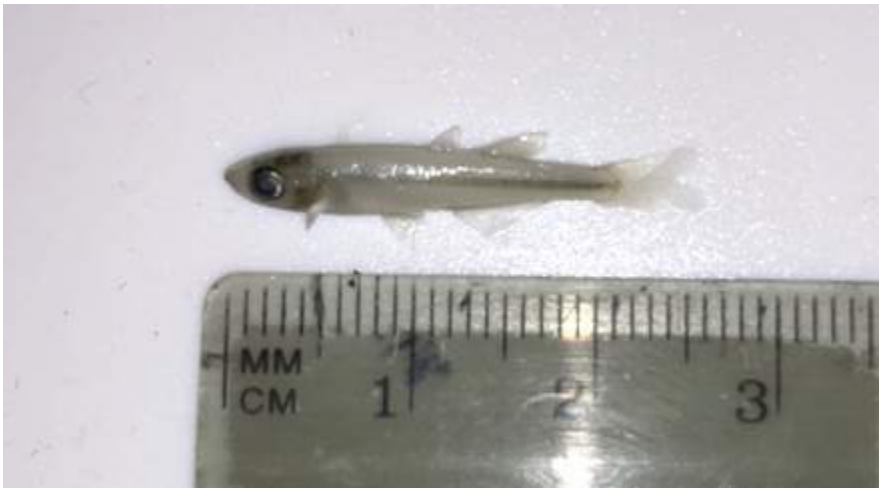

Figure 5.2. Juvenile inland silverside.

The silversides (20-25 fish per tank) were transferred to 2.5-gallon glass fish tanks (Carolina Biological, Burlington, NC) containing 5 L of water. The tanks were dosed with 0.05, 0.10, 0.17, 0.25, 0.50, or 0.75-mg/L dicloran from a 10,000-mg/L stock solution dependent upon the trial. Both light and dark trials were completed in triplicate for each treatment, including dark and light non-dosed control tanks. Silversides were also exposed to 0.04, 0.06 and 0.08 or 0.12-mg/L 1,4-benzoquinone (BQ) and 2-chloro-1,4-benzoquinone (CBQ) in dark trials. Previous analysis has shown BQ and CBQ photodegrade in <2 hr, therefore the inclusion of light would be counterproductive to the investigation. Fish were treated under experimental conditions for 48 hours.

### 5.2.4. Analysis: mortality curves and chemical fate calculations

Mortality curves were generated for each treatment using Prism 7 software (GraphPad Software, Inc., La Jolla, CA); $LC_{50}$ values were estimated using best-fit values on a non-linear regression (curve fit) analysis of [agonist] vs. normalized response – variable slope. The pseudo first-order half-life of dicloran was calculated using the average of the percent remaining dicloran in three salinities sampled and analyzed at various time points over a 24-hour period (t = 0, 2, 4, 7 or 8, and 24 hours), using the following equations:

$$\ln C/C_0 = -kt$$

$$t_{1/2} = 0.693/k$$

Where k is the rate constant, C is the concentration, $C_0$ is the initial concentration, and t is time; and k is the previously calculated rate constant and $t_{1/2}$ is the half-life. A small-sample one-sided t test ($\alpha = 0.05$) was used to determine statistical differences between trials and media for chemical analysis.

### 5.2.5. Analysis: sub-lethal impacts

Surviving fish from each treatment were preserved in 4% paraformaldehyde in potassium buffer solution (PFA in PBS) or TRIzol® reagent (Fisher Scientific, Hampton, NH). Silversides preserved in PFA were plated for histology; they were cut into slides stained with hematoxylin and eosin (H&E) stain by the Louisiana State University School of Veterinary Medicine's Histology Laboratory (Baton Rouge, LA).

The surviving fishes (n = 5) from each treatment that were preserved in TRIzol® reagent were later prepared for mRNA analysis of gene expression using qPCR. Whole fish were homogenized for 5 minutes on a Vortex-Genie 2 (Scientific Industries, Inc., Bohemia, NY) in 1.5 mL centrifuge tubes (Fisher Scientific, Hampton, NH) containing 1 mL TRIzol® and 1 mL 800 µm glass beads (VWR, Radnor, PA), then 200 µL of chloroform (VWR, Radnor, PA) was added to each tube and the tubes were centrifuged for 20 min; 400 µL of the supernatant was transferred to a 1.5 mL Eppendorf tube (Fisher Scientific, Hampton, NH) and 400 µL isopropanol (VWR, Radnor, PA) was added to each tube, the tubes were briefly shaken, and incubated in the -20°C freezer for a minimum of 30 min. The tubes were then centrifuged for 20 min, the supernatant was discarded, and 1 mL of 70% ethanol (VWR, Radnor, PA) prepared with RNase free water (Fisher Scientific, Hampton, NH) was added to each tube and they were

centrifuged for another 10 min. The supernatant was again removed and 25 µL of a mixture of RNase free water, 10x DNase buffer, and TURBO DNase (Invitrogen, Carlsbad, CA) was added to each tube. The tubes were incubated in a 37°C water bath for 30 min, then 5 µL of DNase Inactivation Reagent (Invitrogen, Carlsbad, CA) was added and centrifuged for 5 min; 25 µL of the supernatant was transferred to an Eppendorf tube and kept in the -80°C freezer until cDNA synthesis and qPCR analysis.

The concentration of RNA in each sample was measured using a GeneQuant Pro (Amersham BioSciences, Little Chalfont, UK). The RNA concentration was then measured using 4 µL of the extracted RNA diluted with 196 µL RNA-free water in an Eppendorf tube; the concentration, 260/230, and 280/260 values were recorded, values ranged 394-1186, 0.49-0.94, and 1.52-2.09 respectively.

cDNAs were synthesized and used for qPCR analysis. RNA was aliquoted to PCR-strips (VWR, Radnor, PA) with deoxynucleotide mix [10 mM] (dNTP; G-Biosciences, St. Louis, MO), oligo DT (Integrated DNA Techologies, Coralville, IA), 5x SSIV buffer, SSIV reverse transcriptase, 0.1M DTT (Fisher Scientific, Hampton, NH), and RNase Free water in the T100™ Thermal Cycler (Bio-Rad, Hercules, CA) and set to follow protocol. The protocol included a 5 min cycle at 65°C, 10 min at 4°C, and completed after 30 min. The cDNAs were diluted with RNase Free water and stored in a -20ºC freezer until PCR analysis (Xu et al., 2018).

Genes were chosen based off of previously published literature by Brander *et al.* and Jeffries *et al.* (Table 5.1). In a 384-well plate, cDNA (n = 4 for each treatment) was combined with dNTP, primers pairs (IDT, Coralville, IA), ROX reference dye, 10x Sybr Green (Fisher Scientific, Hampton, NH), 5x GoTaq polymerase colorless reaction buffer, GoTaq polymerase (Promega, Madison, WI) and RNase free water, centrifuged, and analyzed by the $\Delta\Delta C_t$ method

using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and sds2.4 software (Brander et al., 2016; Jeffries et al., 2015; Livak et al., 2001; Xu et al., 2018).

Table 5.1. Genes used for qPCR analysis.

| Function | Gene | Identity | Sequence |
|---|---|---|---|
| Reference | GAPDH | Glyceraldehyde 3-phosphate dehydrogenase | CAGTGGTGGACCTGACATGC<br>TTCTTGACGGCGTCCTTGAT |
| Reference | EF-1a | Elongation factor 1-alpha like | CATCGCCTGCAAGTTCAGC<br>CCCAGACTTCAGCGCCTT |
| Cell structure | BAIAP212 | Brain-specific angiogenesis inhibitor 1-associated protein | GAGGGGCACCAATCCATTC<br>GCCGATCTGTCATTGGTGGT |
| Hormone receptor | GPR30 | G-protein coupled estrogen receptor | CGTCCTCTCCGGCCTCTAC<br>TGAGGATGTTCCCAATGAAGC |
| Hormone receptor | ESR1 | Estrogen receptor alpha | CTCCATTGTGCCAGTGCAGA<br>ACGCTTCCGCATGCTCA |
| Immune function | MMP9 | matrix metalloproteinase | CAACCAGCAGCTTTGACAGTG<br>TCCGATTACAGCAGTGTCACG |
| Immune function | CCL28 | c-c motif chemokine 28 precursor | GCTCTGGCCATCACCTTCAC<br>AAATTAACGGGCCTTATGCTGA |
| Leukotriene synthesis | ALOX5AP | arachidonate 5-lipoxygenase-activating protein | GATGTCCTCTGTGGGCATCTTT<br>GTACTCTTTGTAGTCGCTGCCGTA |
| Osmoregulation | ATP1A1a | sodium potassium-transporting ATPase alpha 1a subunit | TGATCTGGTGGAGGTGAAAGG<br>TGGGCGGAGATGATTCTCA |
| Osmoregulation | ATP1A1b | sodium potassium-transporting ATPase alpha 1b subunit | GGTTATCATGGTGACGGGTGA<br>AGATGATACCCACGCCTTTAGC |
| Oxidative stress | CYBA | cytochrome b-245 light chain-like | CACGACCTCCTGCGGATTAT<br>CATAGGGTTGTCGTAGGCTGCT |
| Prostaglandin synthesis | PTGS2 | prostaglandin g/h synthase 2-like | GGTGACTGAGCACGGCATAAG<br>CCCGTCCTGCAATCTGCTT |
| Stress defense | GSR | Glutathione reductase | CTCCAATGTATCACGCCATCAC<br>TTCTCCTCTTTGCCAACACACA |
| Structural | C1QTNF5 | complement c1q tumor necrosis factor-related protein 5-like | AGTACTGAAGTTTCGCAATGTGCT<br>GCCATCCAAGGGACAAGTGA |

One-way ANOVA analysis of variance with Tukey's multiple comparisons test was used to determine statistical differences between gene responses between all treatments (light/dark and dicloran exposure) and salinities (Prism 7, GraphPad, Inc.; La Jolla, CA).

Whole fish were preserved in 4% PFA and cut into slides by the Histology Lab at the Louisiana State University Veterinary School of Medicine. The slides were stained with H&E and impacts to the gill filaments due to exposure to dicloran and/or light were observed. The images were taken at a 40x zoom on the AmScope Microscope with a Digital Camera attachment and AmScope (x86) software (United Scope, LLC., Irvine, CA) to monitor the cell response to exposure more closely.

## 5.3. Results: chemical and toxicological

### 5.3.1. Chemical analysis of dicloran

The photodegradation of dicloran was analyzed and half-lives were calculated (n=5). Dicloran in each aqueous phase (0.5, 1.2, and 2.5% ASW) was approximately 70% degraded within 24 hours. While the half-life of dicloran differed between salinities, a paired t-test ($\alpha$ = 0.05) indicated no statistical difference between the half-lives; the estimated half-life of dicloran is 12.6, 13.4, and 14.8 hours in 5 ppt, 12 ppt, and 25 ppt (0.5, 1.2, and 2.5%) salinities respectively. The photodegradation of dicloran in each salinity is shown in Figure 5.3.

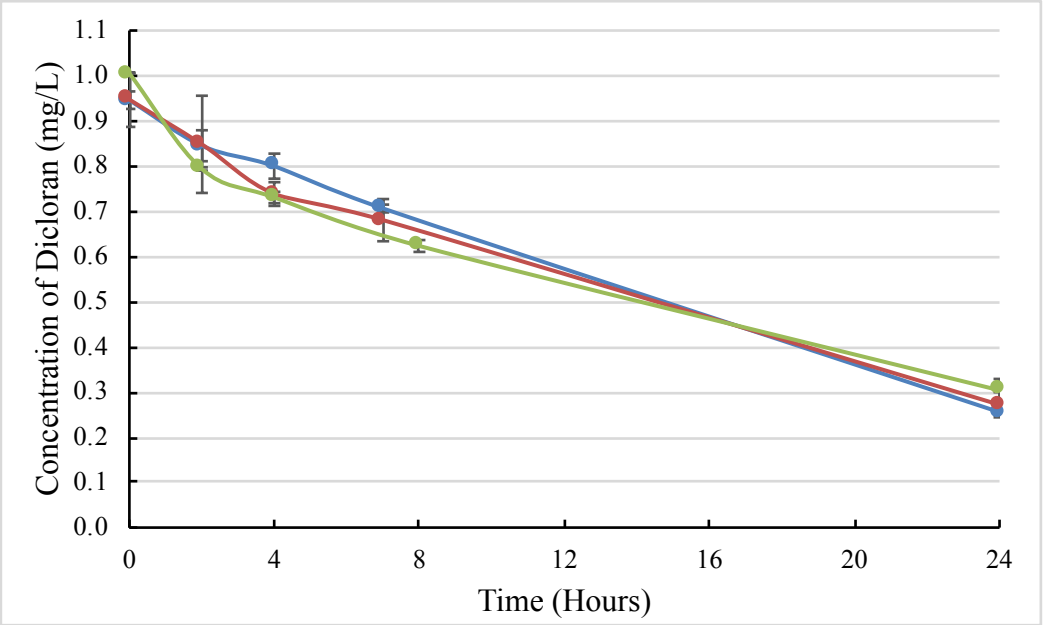

Figure 5.3. The photodegradation of dicloran in 5 ppt (0.5%) seawater (blue), 12 ppt (1.2%) seawater (red), and 25 ppt (2.5%) seawater (green); error bars indicate standard error (n=5).

### 5.3.2. Lethal effects of dicloran exposure to inland silversides at multiple salinities

While not statistically significant, the photolysis half-life of dicloran did appear to show a slight dependency on the salinity of the aqueous phase; in distilled water, dicloran has an estimated half-life of 12.9 hours and in 2.5% artificial seawater it has an estimated half-life of 14.8 hours, suggesting a possible difference in toxicological behavior. Figure 5.4 shows the toxicological differences in response of inland silversides to dicloran exposure at three salinities.

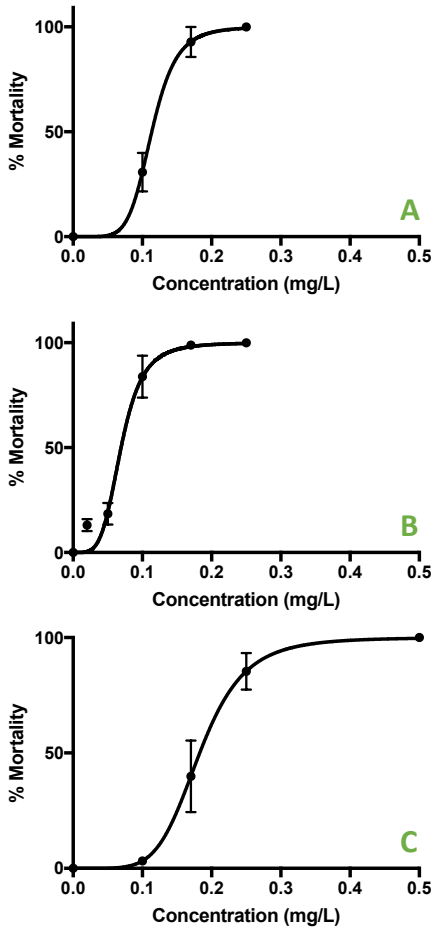


Figure 5.4. Dose-response curve depicting the % mortality vs the log [concentration] of inland silversides exposed to dicloran at (A) 0.5% salinity, (B) 1.2% salinity, and (C) 2.5% salinity; error bars indicate standard error.

At the lowest salinity used in this experiment (0.5%), the estimated $LC_{50}$ for fish exposed to dicloran and light is 0.11 mg/L. The intermediate salinity (1.2%) resulted in the highest mortality of silversides exposed to dicloran and light with an estimated $LC_{50}$ of 0.07 mg/L. The highest $LC_{50}$ was estimated from silversides exposed to dicloran and light at the highest salinity (2.5%), 0.18 mg/L. Mortality was observed for silversides exposed to dicloran without sunlight at 1.2% salinity, no morality was observed at 0.5% or 2.5% salinity. Approximately 50% of the fishes responded lethally to 0.75 mg/L of dicloran in the dark at 1.2% salinity.

### 5.3.3. Sub-lethal effects of dicloran exposure: relative gene expression

Quantitative PCR (qPCR) analysis was completed to monitor the relative gene expression levels for ten genes for silversides. The expression of ATP1A1a for 0.5% (Figure 5. 5), 1.2% (Figure 5.6), and 2.5% (Figure 5.7) shows variation between the salinities. ATP1A1a is expressed as an indicator of osmoregulation. The elevated expression level for fishes acclimated to 0.5% salinity may be an indication of stress on the organs responsible for osmoregulatory functions. Silversides at other salinities did not result in as high of expression levels as those in the 0.5% regime, this may be due to the average habitat of silversides to be 1.2-2.5% salinity and therefore the fishes are less stressed at the higher salinities prior to chemical exposure (Armesto et al., 2014).

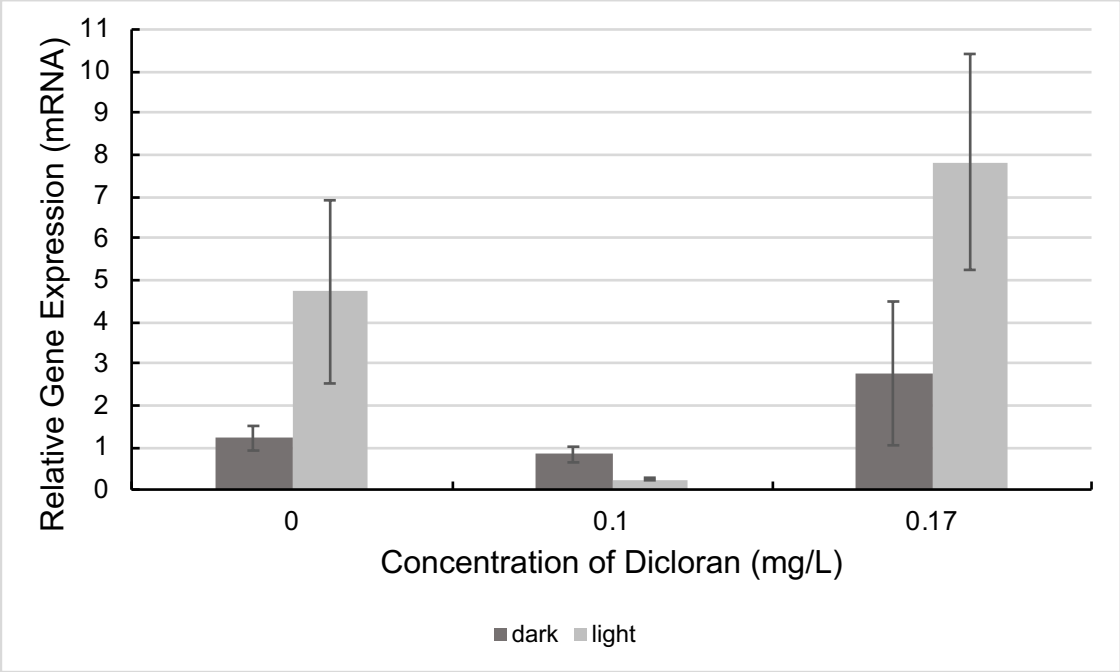


Figure 5.5. The relative gene expression for ATP1A1a for inland silversides exposed to dicloran in 0.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

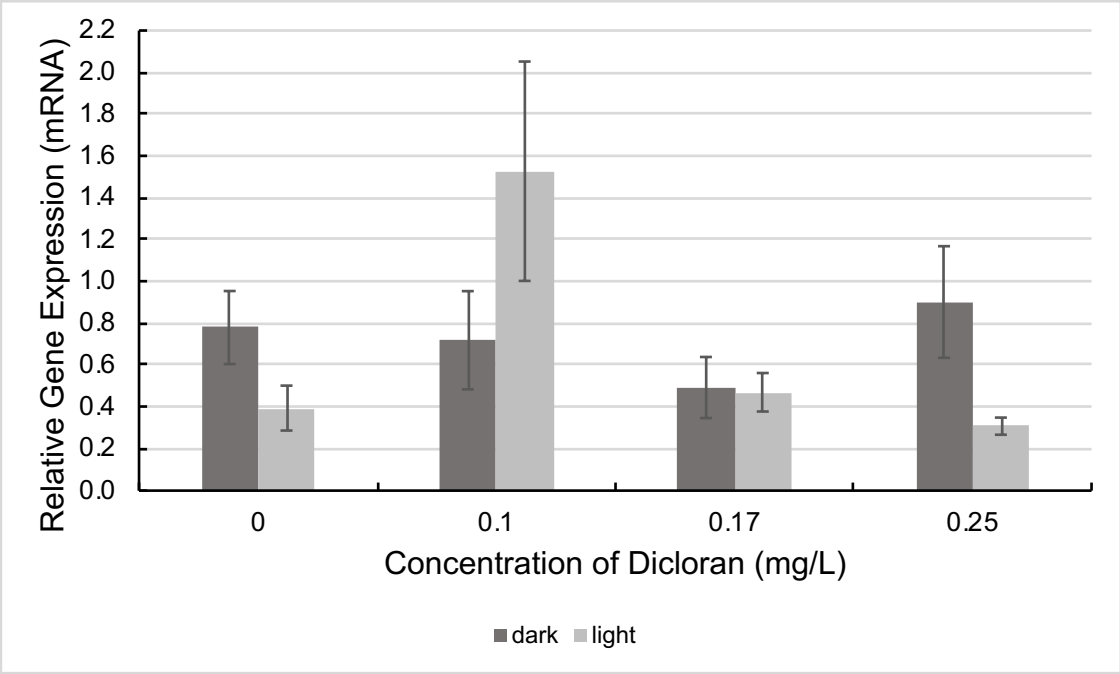


Figure 5.6. The relative gene expression for ATP1A1a for inland silversides exposed to dicloran in 1.2% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

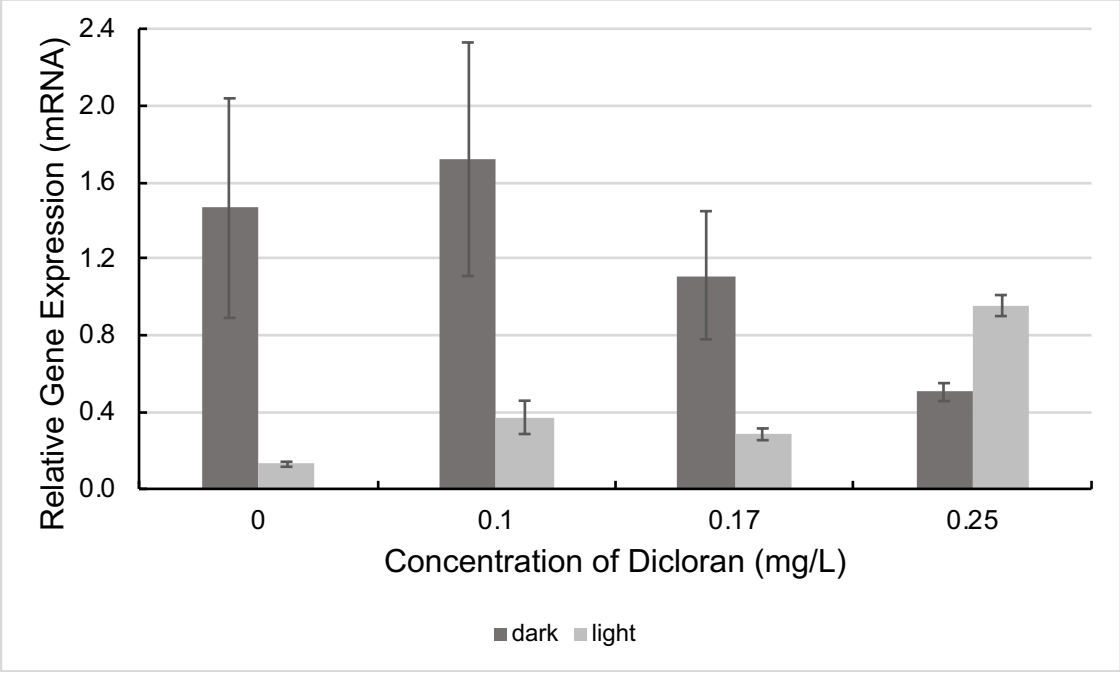


Figure 5.7. The relative gene expression for ATP1A1a for inland silversides exposed to dicloran in 2.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

Silversides exposed to dicloran in the dark (2.5%) showed a higher gene expression compared to those at 0.5 and 1.2%, and they show a downregulation with increased concentration in the dark and an upregulation with increased concentration in the light. No significant differences were observed between treatments for ATP1A1a for 0.5%, with *p*-values ranging from 0.4229 to >0.9999, for 1.2%, with *p*-values ranging from 0.5553 to >0.999, or for 2.5%, with *p*- values ranging 0.3213 to >0.9999 ($\alpha = 0.05$). A comparison of treatments between salinities resulted in few significant differences; 0.5% and 2.5% at 0.25 mg/L in the dark resulted in a *p*-values of 0.252, the remaining treatments were not statistically different. No significant differences were observed between lights treatments at different salinities. EF-1$\alpha$ was used as the reference gene for ATP1A1a. The trends in gene expression for BAIAP212 for 0.5% (Figure 5.8), 1.2% (Figure 5.9), and 2.5% (Figure 5.10) show different responses dependent upon salinity.

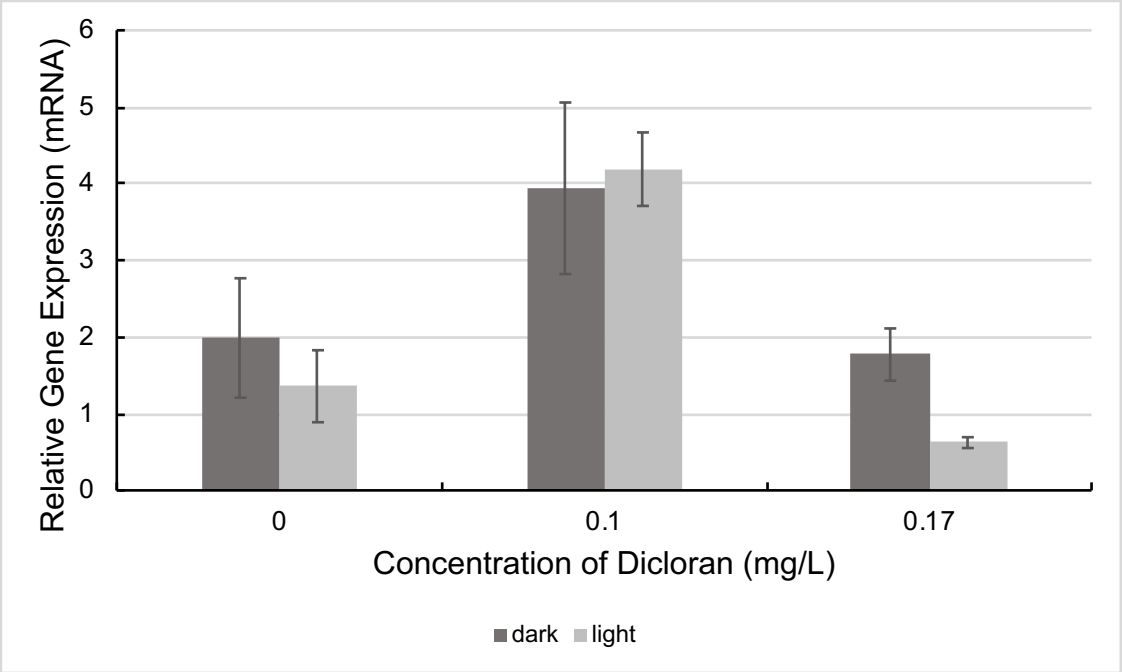


Figure 5.8. The relative gene expression for BAIAP212 for inland silversides exposed to dicloran in 0.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

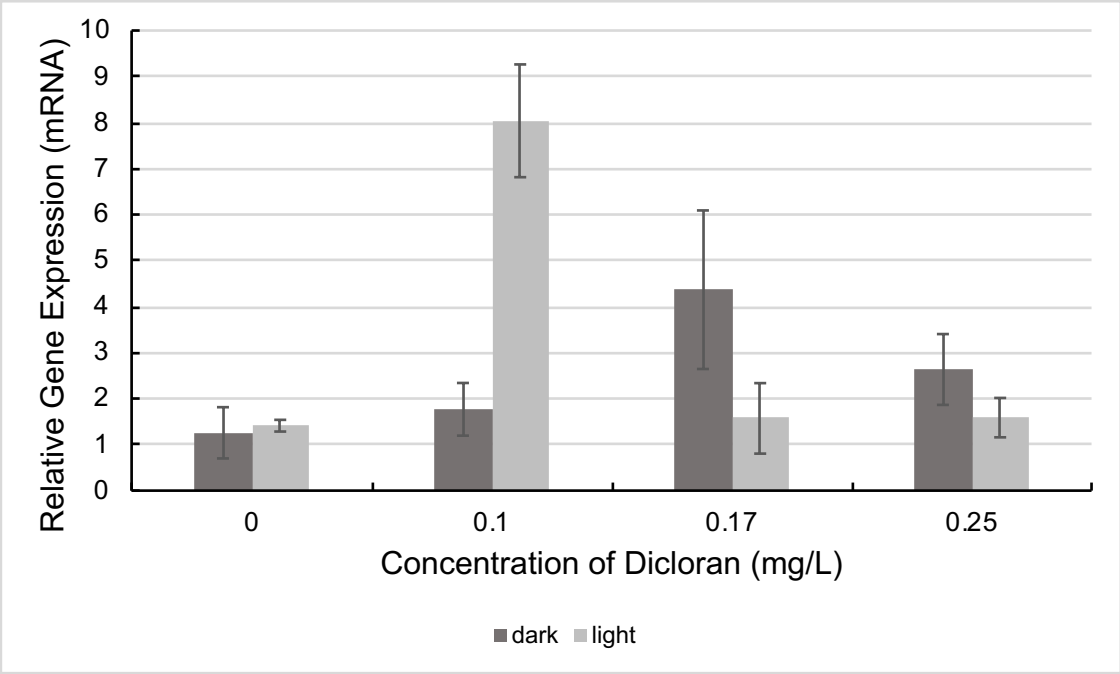


Figure 5.9. The relative gene expression for BAIAP212 for inland silversides exposed to dicloran in 1.2% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

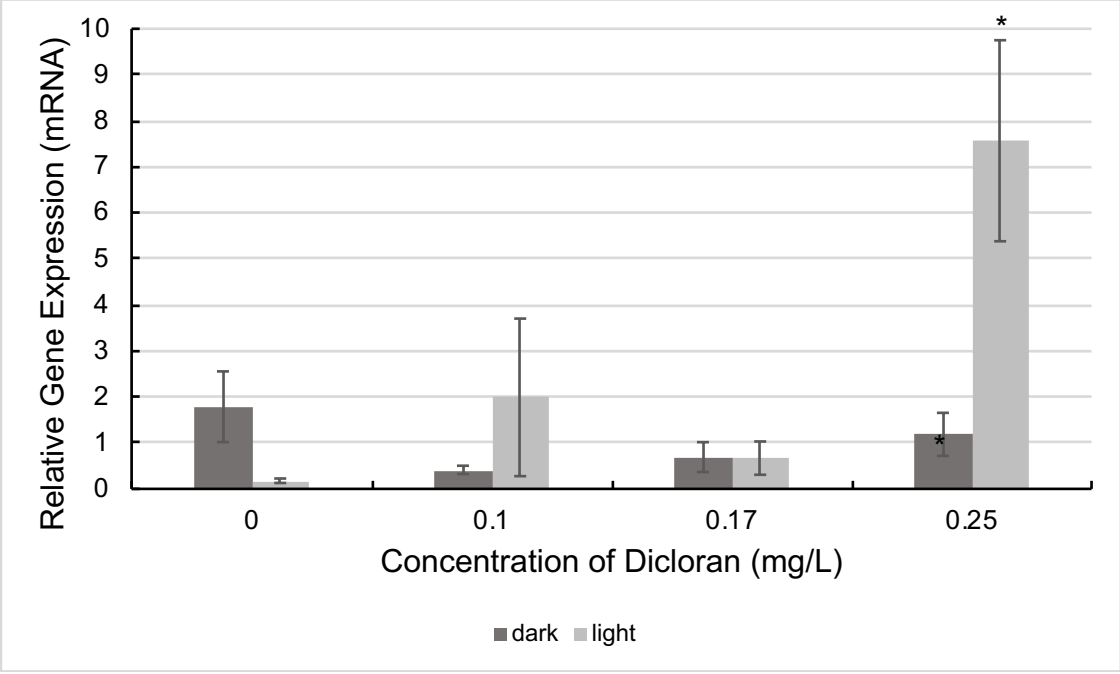


Figure 5.10. The relative gene expression for BAIAP212 for inland silversides exposed to dicloran in 2.5% salinity. Asterisks (*) indicate significant differences. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

No significant differences were observed between exposure concentrations and treatment at 0.5% salinity for BAIAP212, with *p*-values ranging 0.5395 to >0.9999 or 1.2% salinity with *p*-values ranging 0.2341 to >0.9999 ($\alpha = 0.05$). Significant differences were observed at 2.5% salinity; light control and 0.25 mg/L light with a *p*-value of 0.0132 and 0.25 mg/L dark and light with a *p*-value of 0.0239. One-way ANOVA with Dunnett's multiple comparisons test also produced a *p*-value of 0.0125 between dark control and 0.25 mg/L light. EF-1α was used as the reference gene for BAIAP212. The gene expression of C1QTNF5 silversides exposed to dicloran in 0.5% (Figure 5.11), 1.2% (Figure 5.12), and 2.5% (Figure 5.13) salinity shows an upregulation between treatments, using GAPDH as the reference gene.

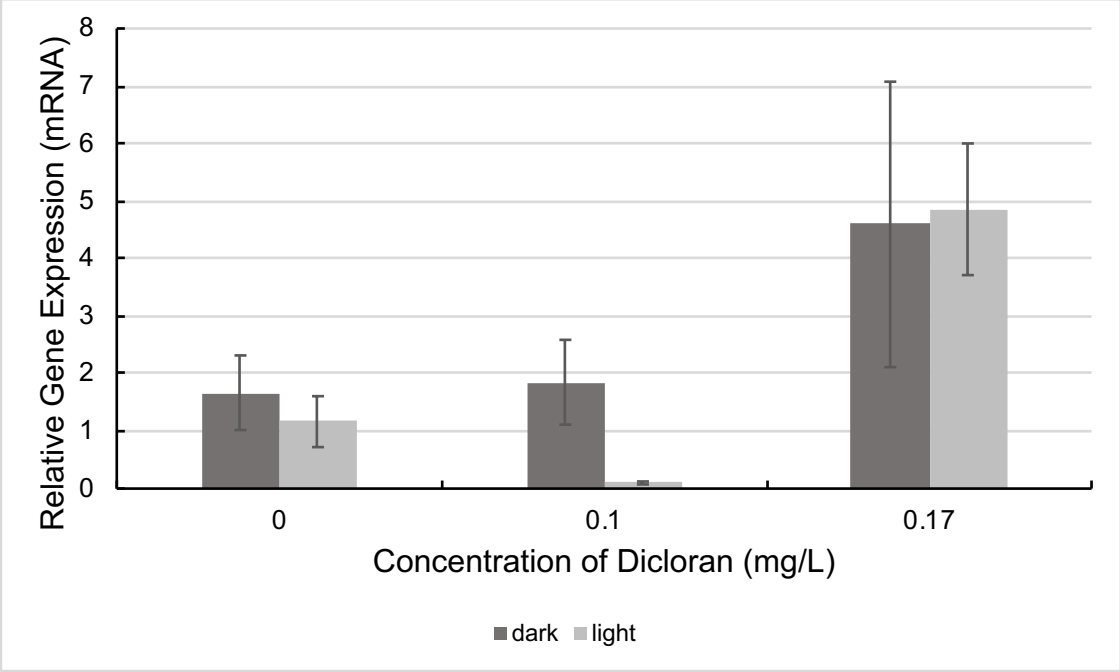


Figure 5.11. The relative gene expression of C1QTNF5 for inland silversides exposed to dicloran in dark and light treatments at 0.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

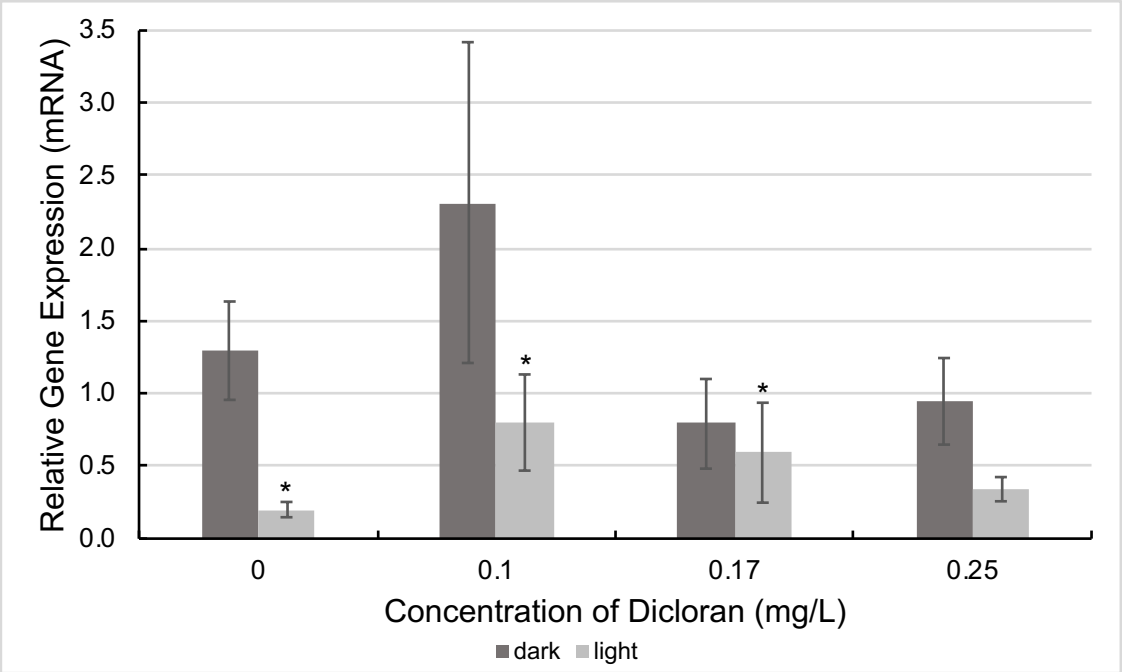


Figure 5.12. The relative gene expression of C1QTNF5 for inland silversides exposed to dicloran in dark and light treatments at 1.2% salinity. Asterisks (*) indicate significant differences. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

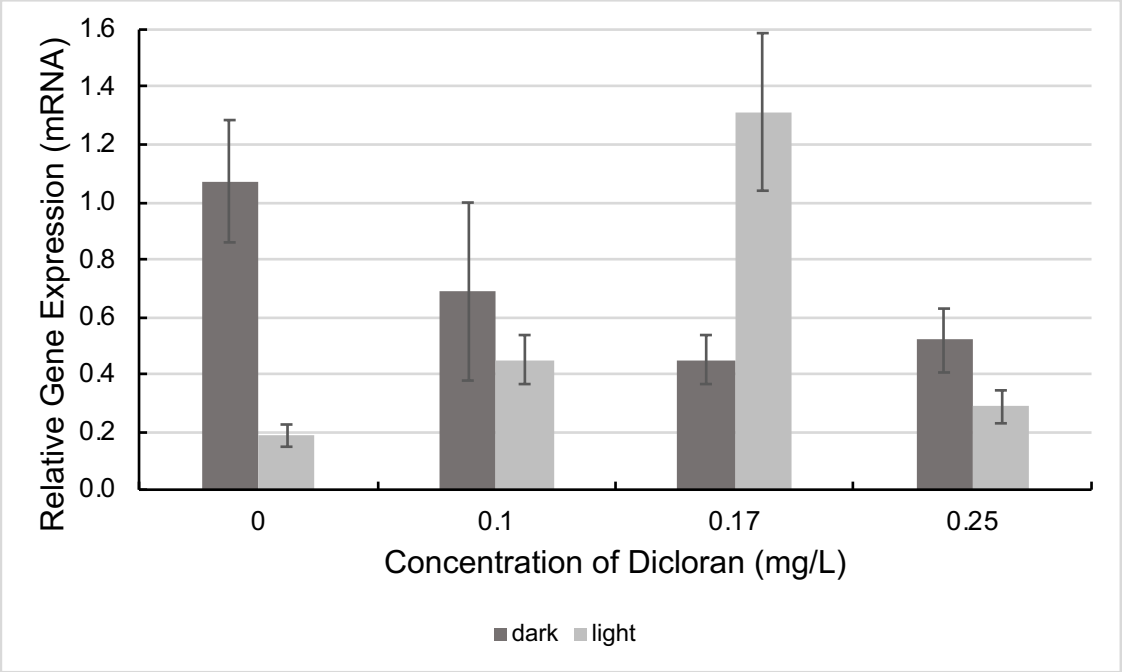


Figure 5.13. The relative gene expression of C1QTNF5 for inland silversides exposed to dicloran in dark and light treatments at 2.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

The level of gene expression decreases as salinity increases, with higher relative expressions observed at 0.5% salinity. An upregulation in expression levels was observed for both dark and light treatments at 0.5% salinity, with the exception of 0.10 mg/L light which was low. At 1.2% salinity, there was an initial increase in expression from controls to 0.10 mg/L followed by a decrease in expression levels. Downregulation in expressions was observed for 2.5% salinity. Different trends were observed for expression levels when comparing all three salinities. Significant differences were observed between light control and 0.17 mg/L light at 0.5% salinity with a *p*-values of 0.0085, 0.10 and 0.17 mg/L light at 0.5% with a *p*-value of 0.0030, 0.16 mg/L at 0.5% and 0.17 at 1.2% with a *p*-value of 0.0096, and 0.17 mg/L at 1.2% and 0.17 at 2.5% with a *p*-value of 0.0014. The relative gene expression of CCL28 for 0.5% (Figure 5.14), 1.2% (Figure 5.15), and 2.5% (Figure 5.16) was quantified using EF-1α as the reference gene.

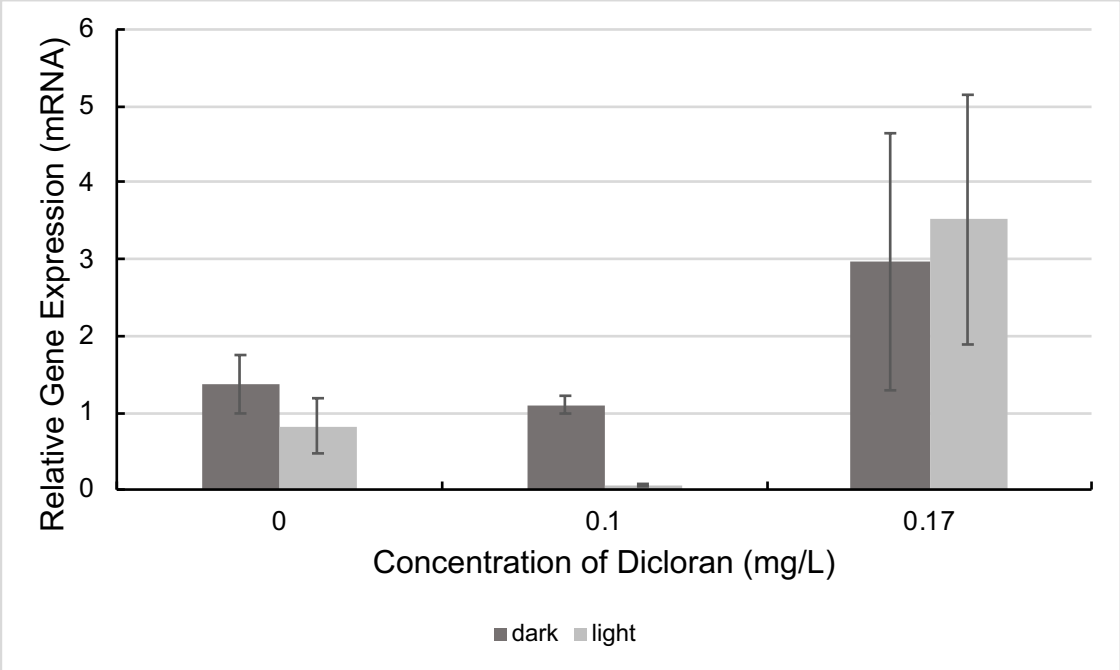


Figure 5.14. The relative gene expression of CCL28 for inland silversides exposed to dicloran in dark and light treatments at 0.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

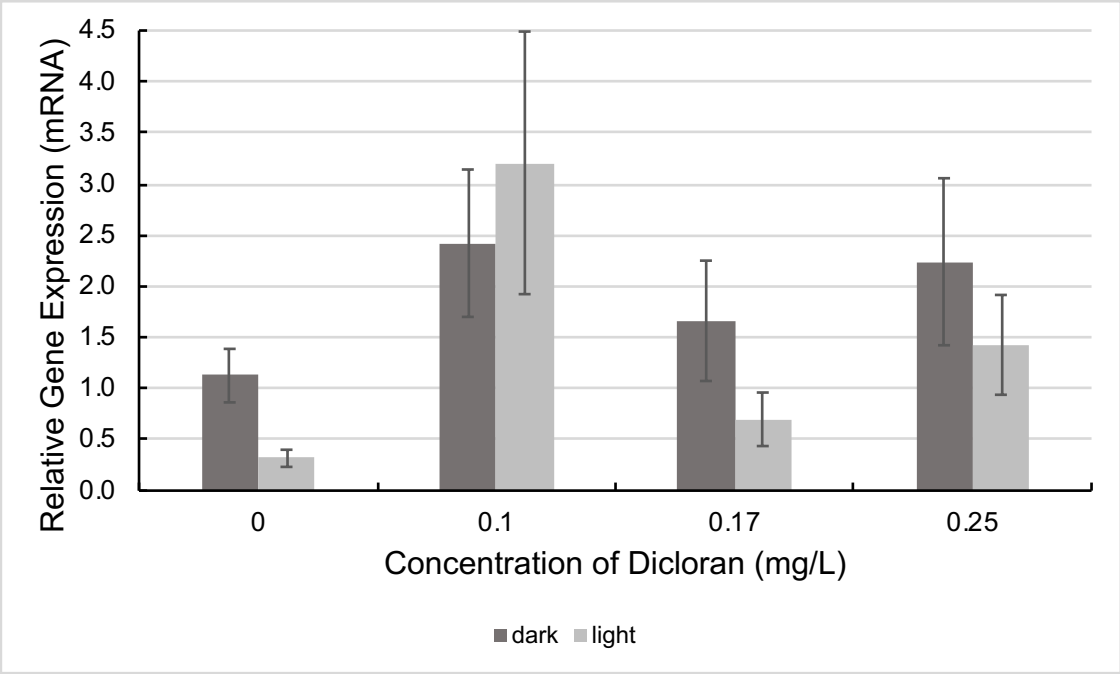


Figure 5.15. The relative gene expression of CCL28 for inland silversides exposed to dicloran in dark and light treatments at 1.2% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

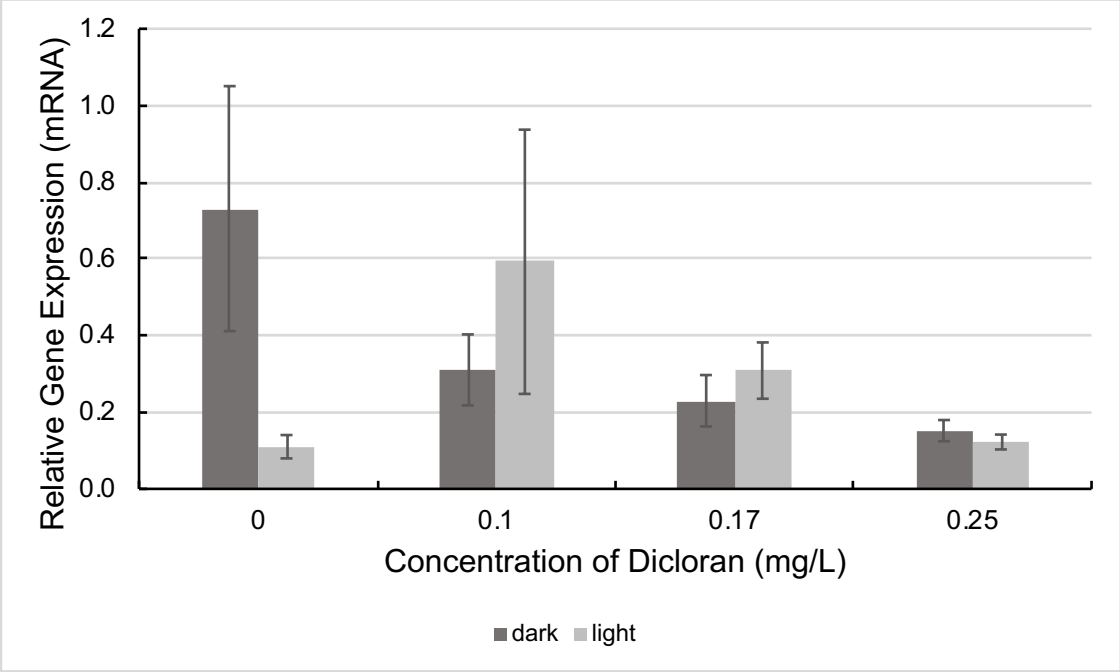


Figure 5.16. The relative gene expression of CCL28 for inland silversides exposed to dicloran in dark and light treatments at 2.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

The expression levels for 0.5% salinity treatments were higher than those at 1.2 and 2.5% salinities. An upregulation in gene expression was observed for 0.5% treatments, while the expression was not consistent for 1.2% salinity. Downregulation for dark treatments was observed for 2.5% salinity and while there was an initial increase in expression level for light treatments between light control and 0.10 mg/L, it is followed by a downregulation for 0.17 and 0.25 mg/L. No significant differences between any treatment or salinity was observed for CCL28. The relative gene expression of CYBA for inland silversides at 0.5% salinity (Figure 5.17) used EF-1α as the reference gene and 1.2% (Figure 5.18) and 2.5% salinity (Figure 5.19) used GAPDH as the reference gene.

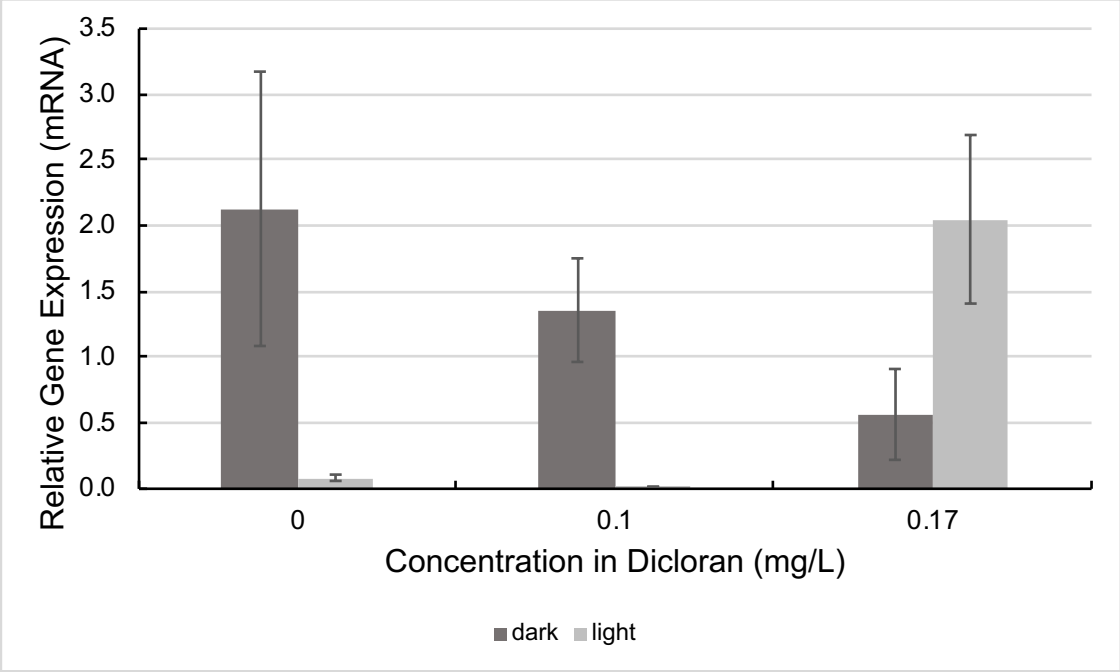


Figure 5.17. The relative gene expression of CYBA for inland silversides exposed to dicloran in both dark and light treatments at 0.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

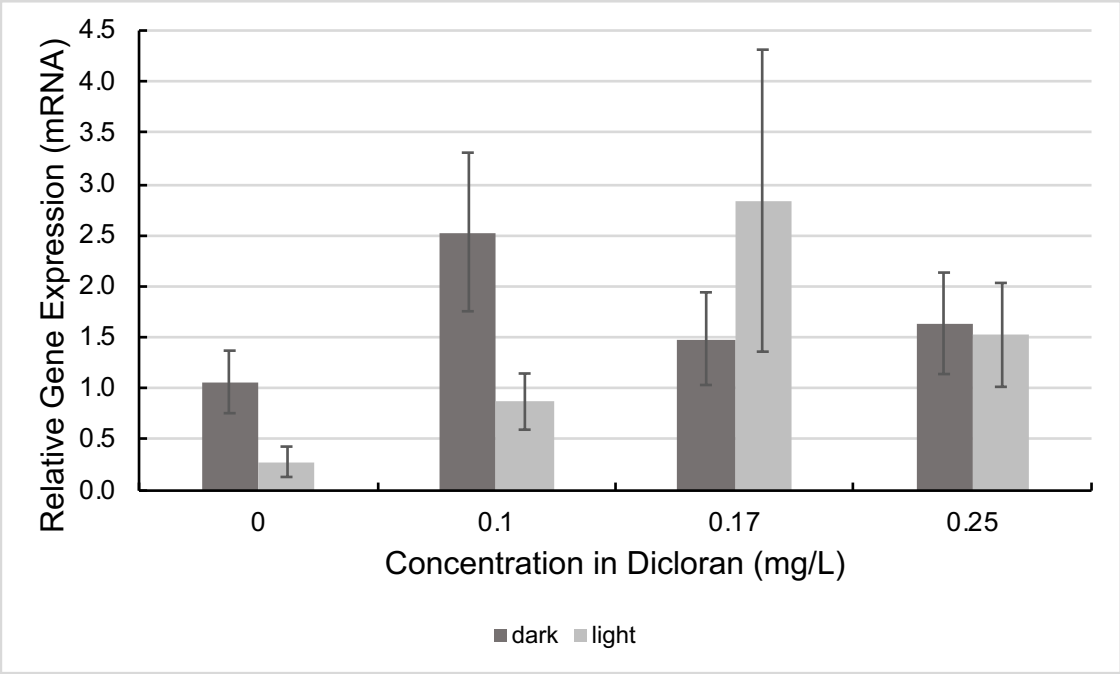


Figure 5.18. The relative gene expression of CYBA for inland silversides exposed to dicloran in both dark and light treatments at 1.2% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

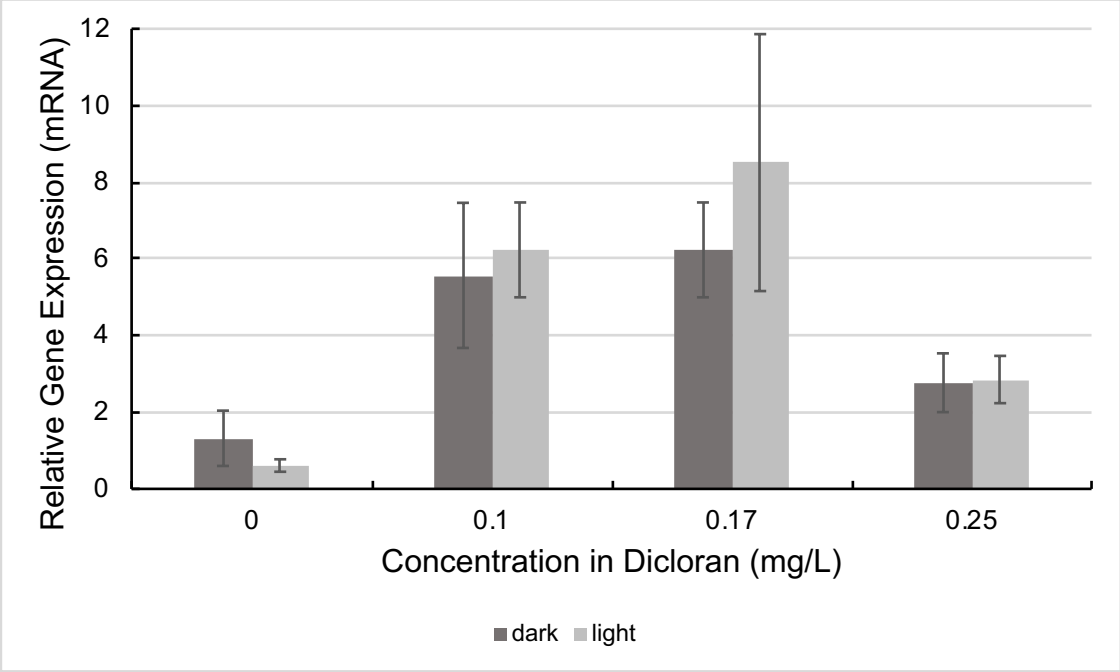


Figure 5.19. The relative gene expression of CYBA for inland silversides exposed to dicloran in both dark and light treatments at 2.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

No significant differences were calculated for CYBA expression levels. There is a downregulation in 0.5% salinity dark treatments at low relative levels. An upregulation for gene expression in 1.2% light treatments was observed, with a decrease in relative expression at 0.25 mg/L. The same trend can be observed for 2.5% salinity, although the gene expression for 2.5% is nearly triple compared to 1.2% salinity. The relative gene expression for ESR1 for 0.5% salinity (Figure 5.20) and 1.2% salinity (Figure 5.21) was quantified using EF-1α as the reference gene and 2.5% salinity (Figure 5.22) with GAPDH.

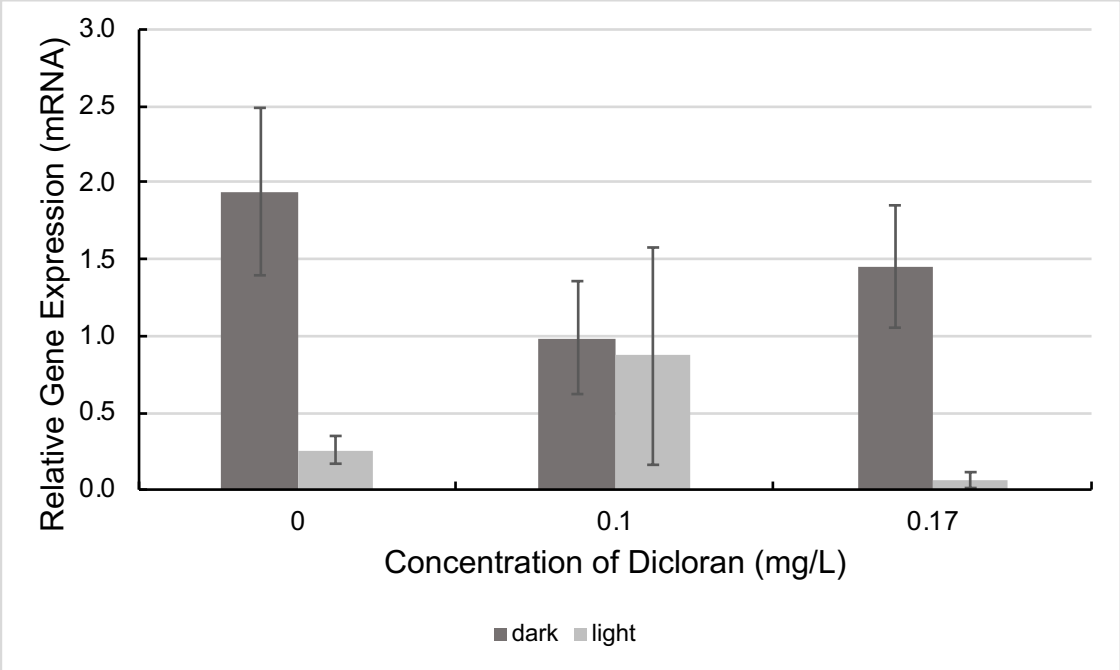


Figure 5.20. The relative gene expression of ESR1 for inland silversides exposed to dark and light treatments of dicloran at 0.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

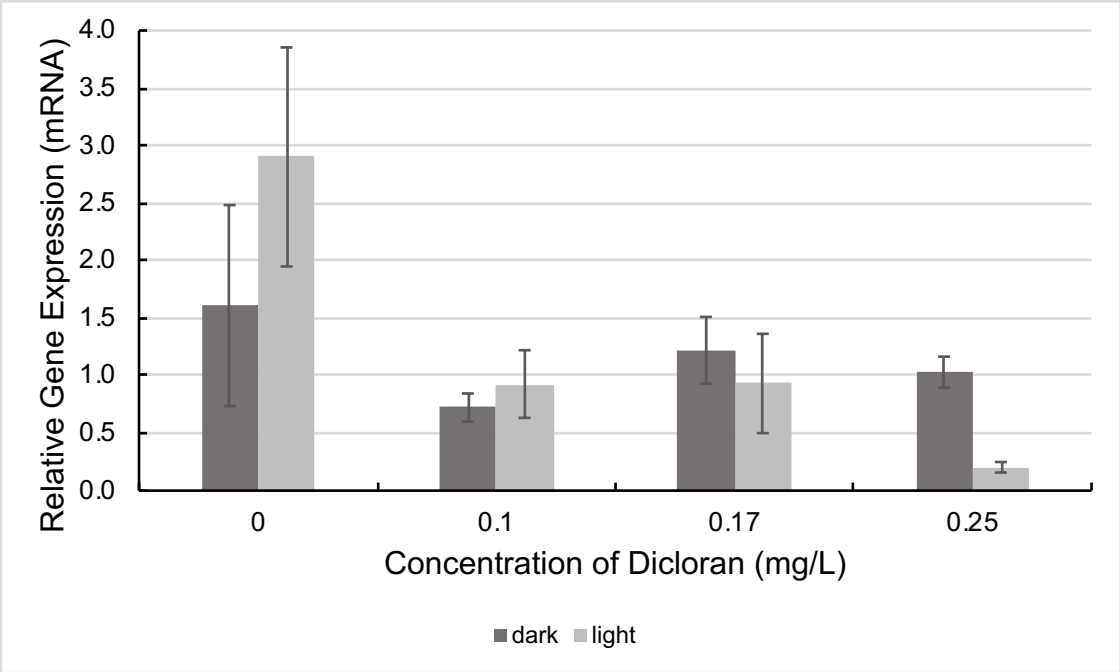


Figure 5.21. The relative gene expression of ESR1 for inland silversides exposed to dark and light treatments of dicloran at 1.2% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

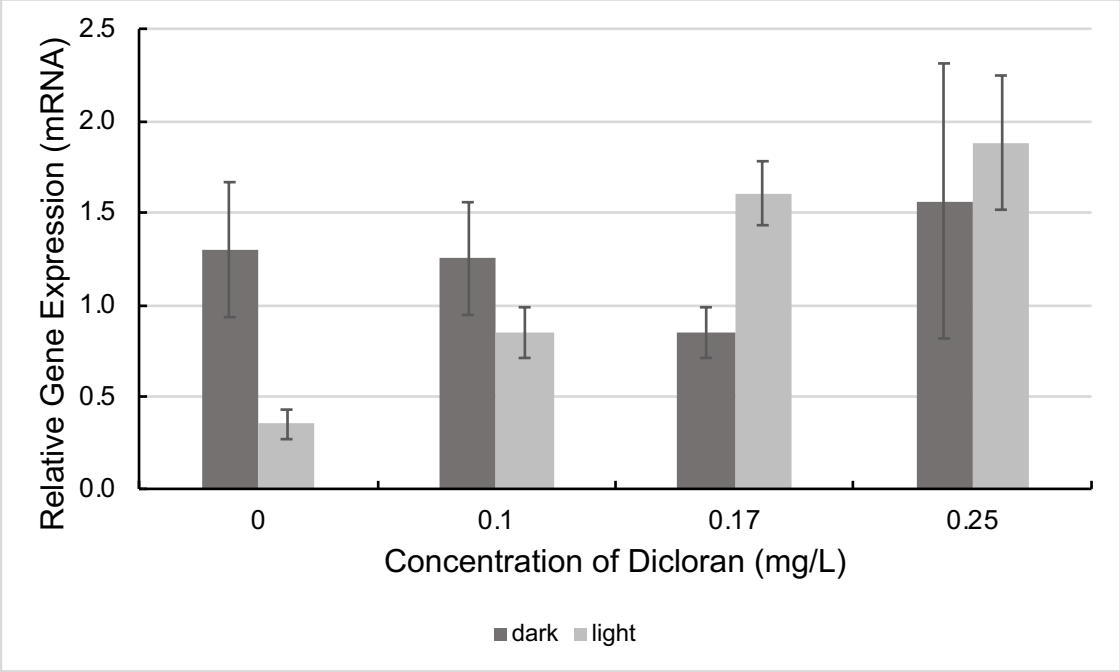


Figure 5.22. The relative gene expression of ESR1 for inland silversides exposed to dark and light treatments of dicloran at 2.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

A significant difference between light and dark controls at 0.5% salinity was observed with a *p*-value of 0.0165, and no other significant differences were observed between treatment within a single salinity. Differences between salinities and treatments included, 0.5% and 1.2% light control (*p*-value of 0.0006) and 1.2 and 2.5% salinity light control (*p*-value of 0.0025). The relative gene expression of GPR30 for inland silversides at 0.5% salinity (Figure 5.23) was calculated using EF-1α, and 1.2% salinity (Figure 5.24) and 2.5% salinity (Figure 2.25) used GAPDH

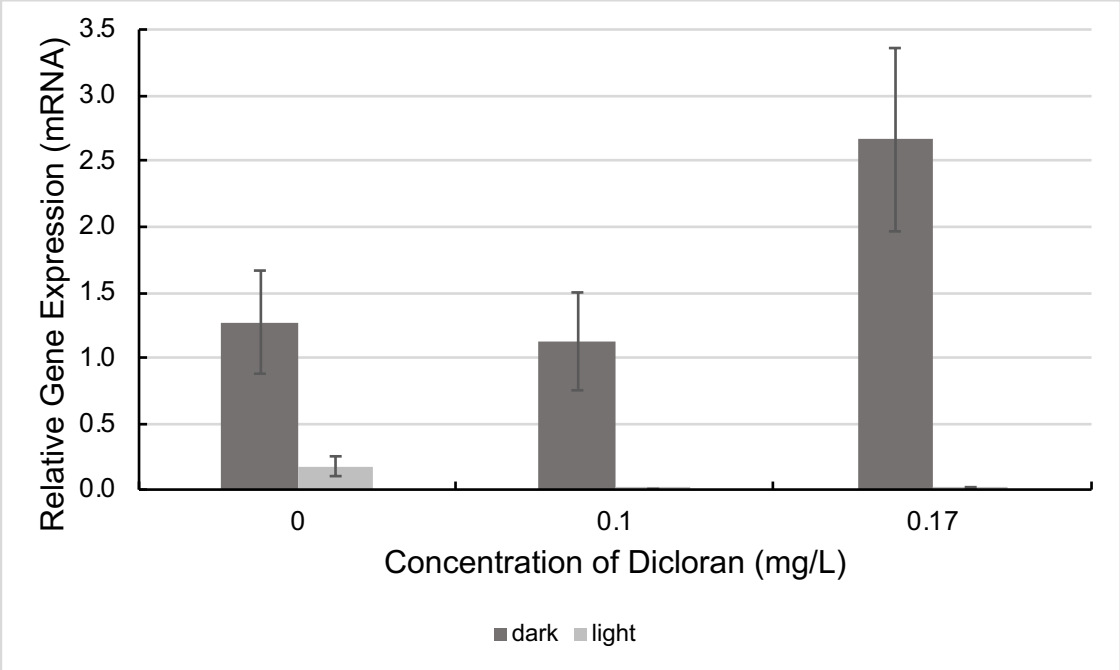


Figure 5.23. The relative gene expression of GPR30 for inland silversides exposed to dicloran in dark and light treatments at 0.5% salinity; limited expression is observed for light treatments. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

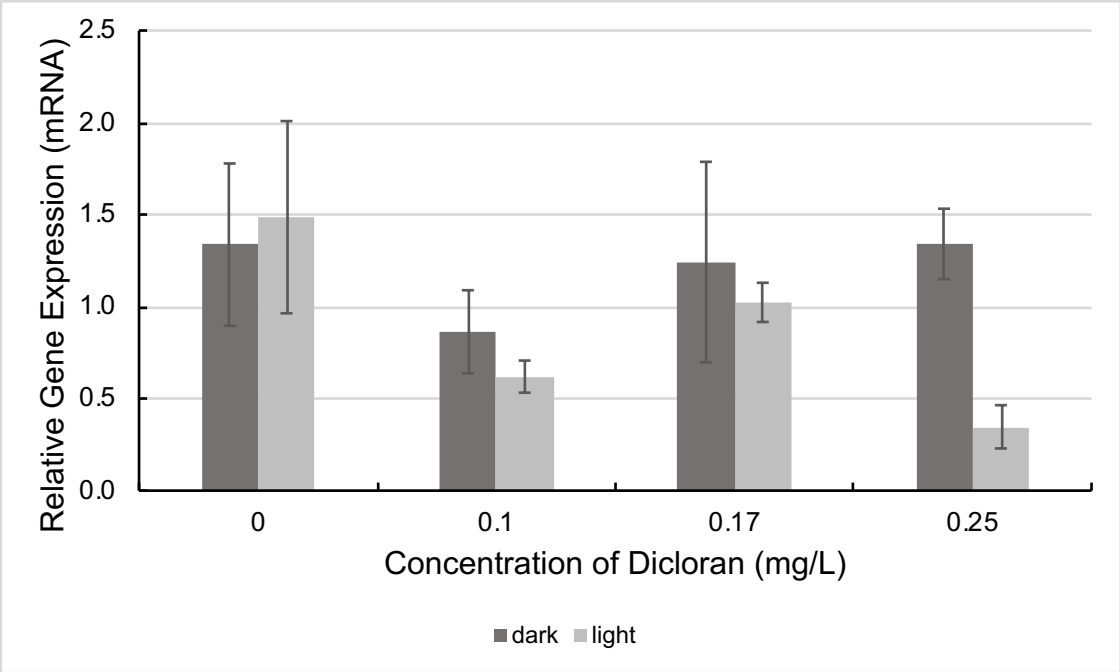


Figure 5.24. The relative gene expression of GPR30 for inland silversides exposed to dicloran in dark and light treatments at 1.2% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

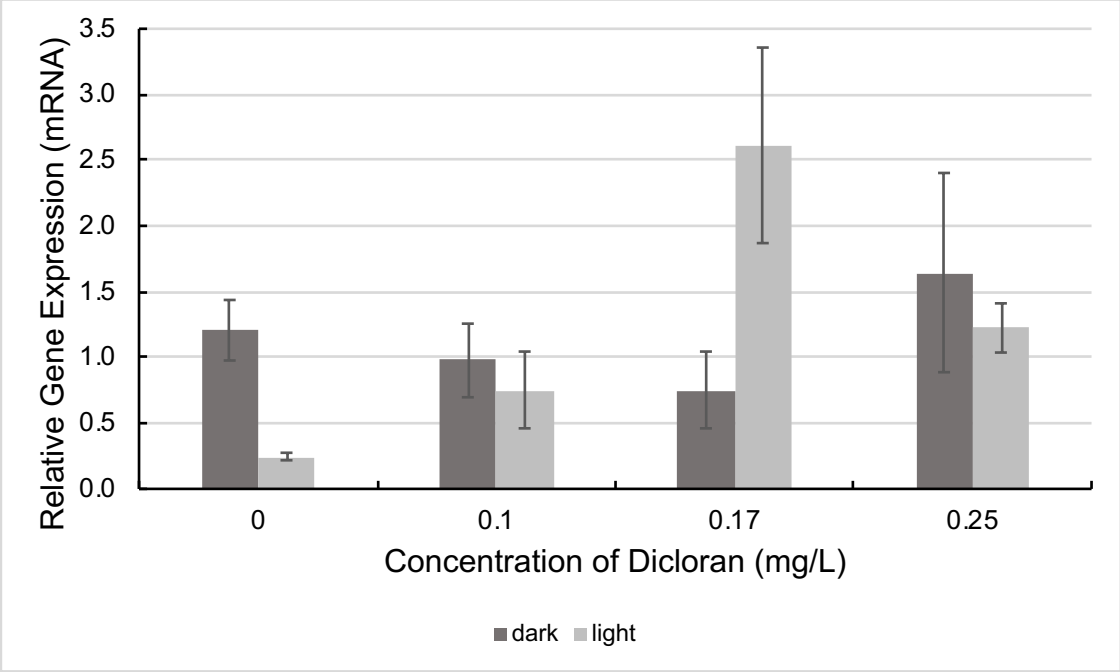


Figure 5.25. The relative gene expression of GPR30 for inland silversides exposed to dicloran in dark and light treatments at 2.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

Significant differences were not observed between any treatments in individual salinities; differences between salinities and treatments resulted in a significant difference between 0.75 mg/L dark in 0.5% and 1.2% salinity, with a *p*-value of 0.0460. Limited expression was observed for 0.5% salinity light samples, this may be due to a response from the fish or the small samples size analyzed. A higher expression of GPR30 can be observed for 1.2% salinity in the dark treatments in comparison to the light treatments. The gene expression of GSR for inland silversides at 0.5% salinity (Figure 5.26) and 1.2% salinity (Figure 5.27) was calculated using EF-1α and 2.5% salinity (Figure 5.28) with GADPH.

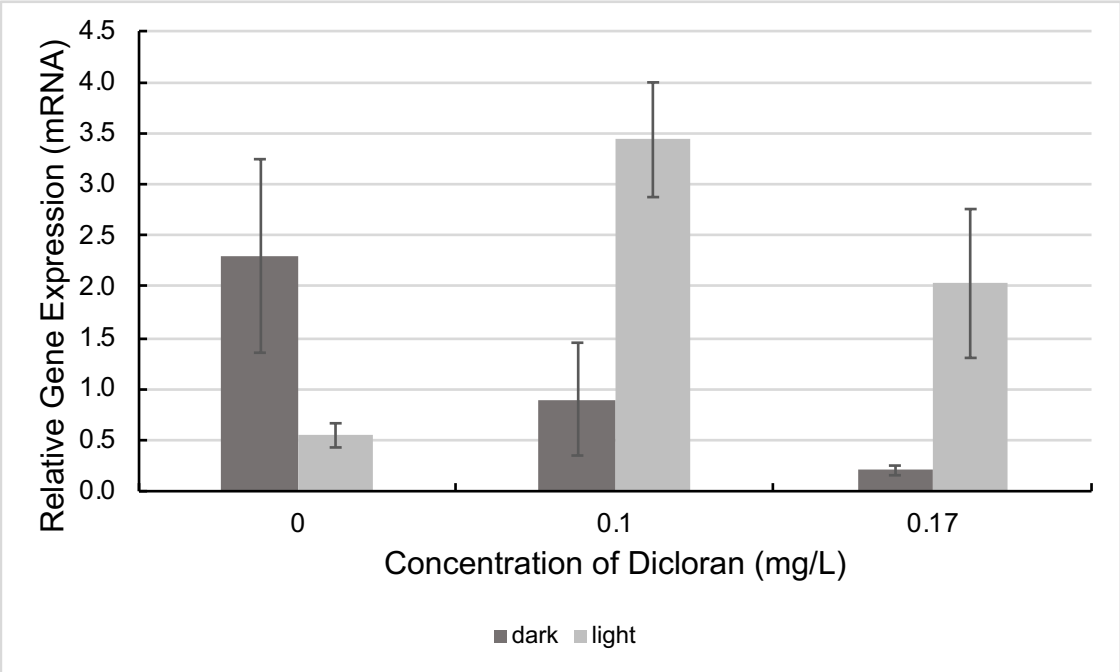


Figure 5.26. The relative gene expression of GSR for inland silversides exposed to dicloran in both dark and light treatments at 0.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

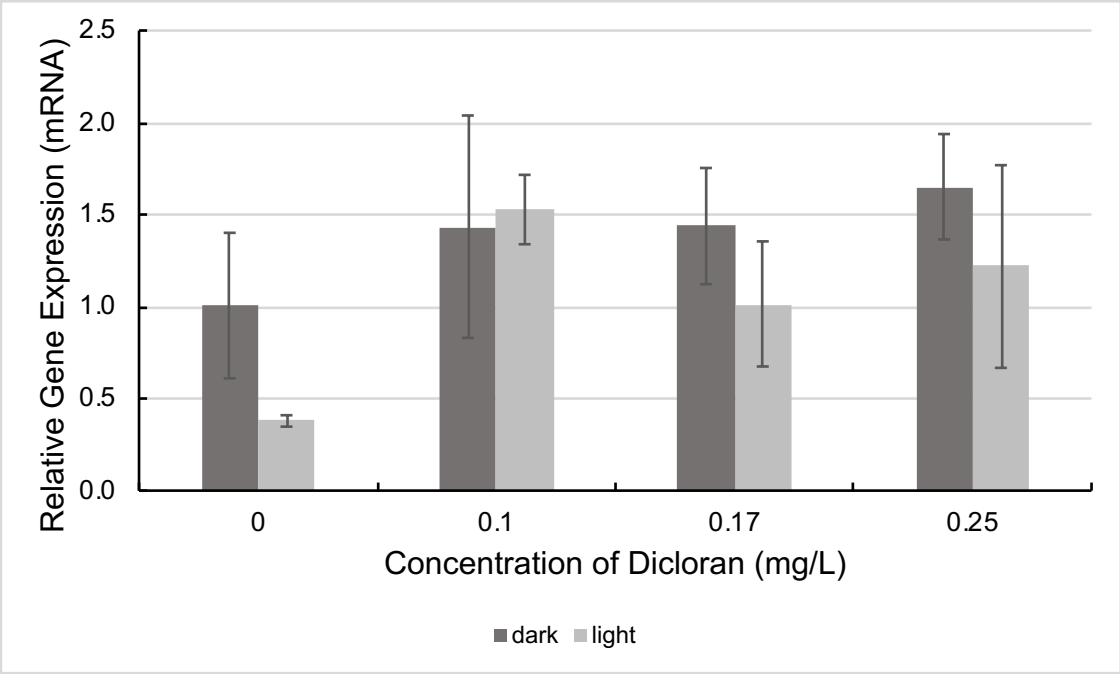


Figure 5.27. The relative gene expression of GSR for inland silversides exposed to dicloran in both dark and light treatments at 1.2% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

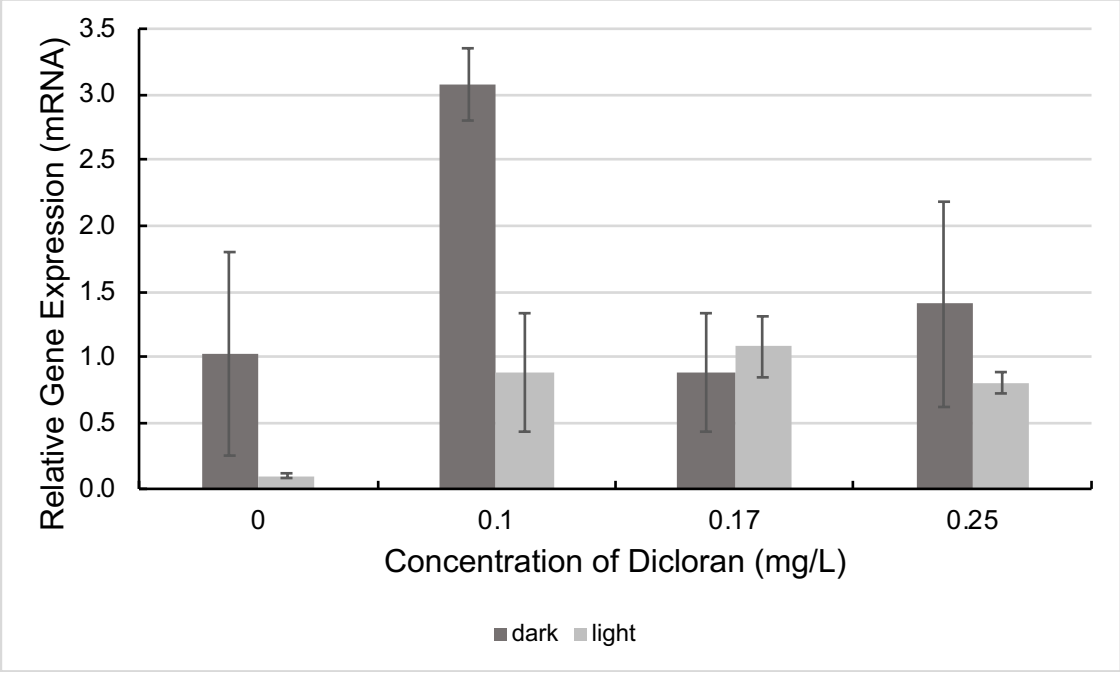


Figure 5.28. The relative gene expression of GSR for inland silversides exposed to dicloran in both dark and light treatments at 2.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

A significant different between 0.5% light control and 0.1 mg/L resulted in a *p*-value of 0.0072, while no other difference between treatments within a single salinity were observed. A significant difference between 0.1 mg/L dicloran at 0.5% and 2.5% salinities in light treatments was also observed with a *p*-value of 0.0052. The relative gene expression of PTGS2 for inland silversides at 0.5% salinity (Figure 5.29), 1.2% salinity (Figure 5.30), and 2.5% salinity (Figure 5.31) were calculated using GAPDH as the reference gene.

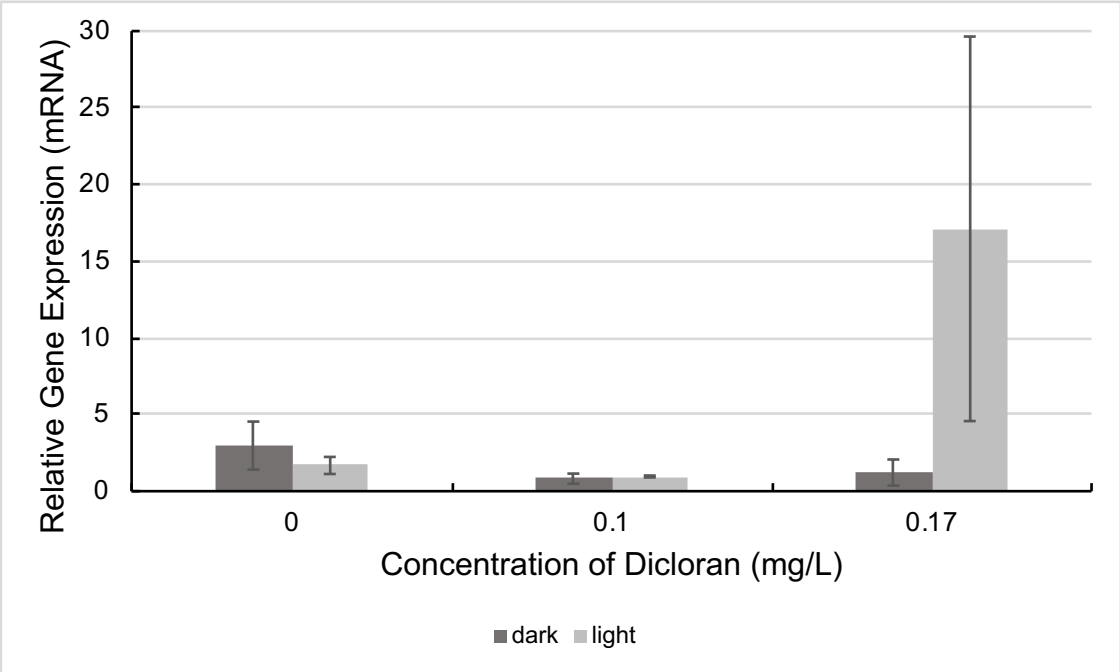


Figure 5.29. The relative gene expression of PTGS2 for inland silversides exposed to dicloran in dark and light treatments at 0.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

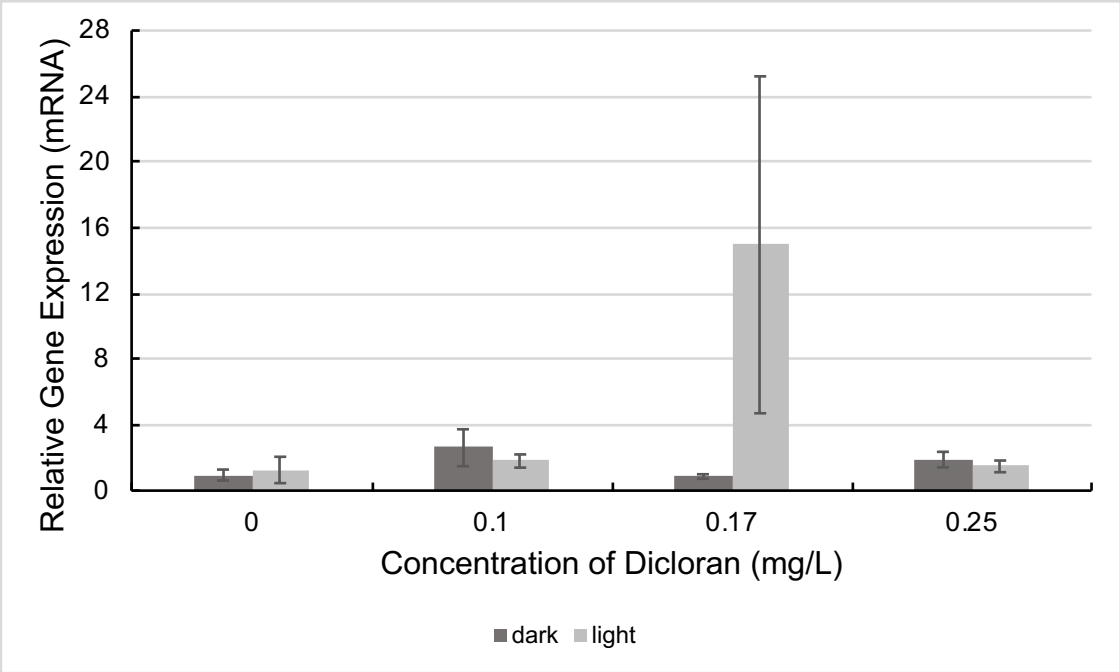


Figure 5.30. The relative gene expression of PTGS2 for inland silversides exposed to dicloran in dark and light treatments at 1.2% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

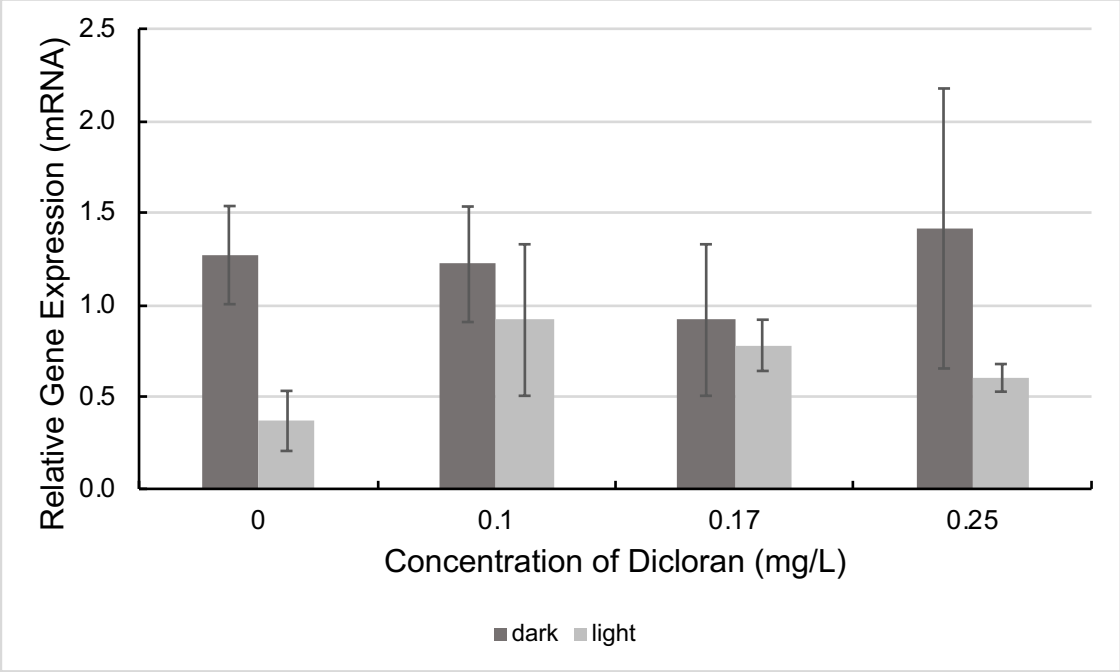


Figure 5.31. The relative gene expression of PTGS2 for inland silversides exposed to dicloran in dark and light treatments at 2.5% salinity. Significance was determined using a one-way ANOVA analysis of variance with Dunnett's multiple comparisons test, $\alpha = 0.05$ (n=4).

Gene expressions varied dependent upon salinity and treatment, with significant differences observed. Large standard errors within samples may be a result of the low sample size or due to genealogical variation within the fishes themselves. For 0.5% salinity, fishes were obtained from two sources and therefore the likelihood for variation in genes is much larger compared to 1.2% and 2.5% salinities, as they were obtained from a single source.

### 5.3.4. Sub-lethal effects of dicloran exposure: histology

Fish gills are a line of defense to prevent toxicants from entering a fishes' bloodstream and organs. Initial indications of stress include cell proliferation, where the gill filaments enlarge as a result of oxidative stress. Apoptosis occurs after increased or continual stress, for example exposure to a chemical or toxicant, within the gills; degeneration (and death) of the cells is controlled by the fishes' response to the toxicant to prevent further introduction or harm (Bonga et al., 1989). While apoptosis is controlled cellular death, necrosis is the death of the cells due to the exposure to the toxicants (AnvariFar et al., 2017; Hong et al., 1998). Figures 5.32 and 5.33 show impacts of exposure to dicloran in dark and light treatments and the response of inland silversides within the gills.

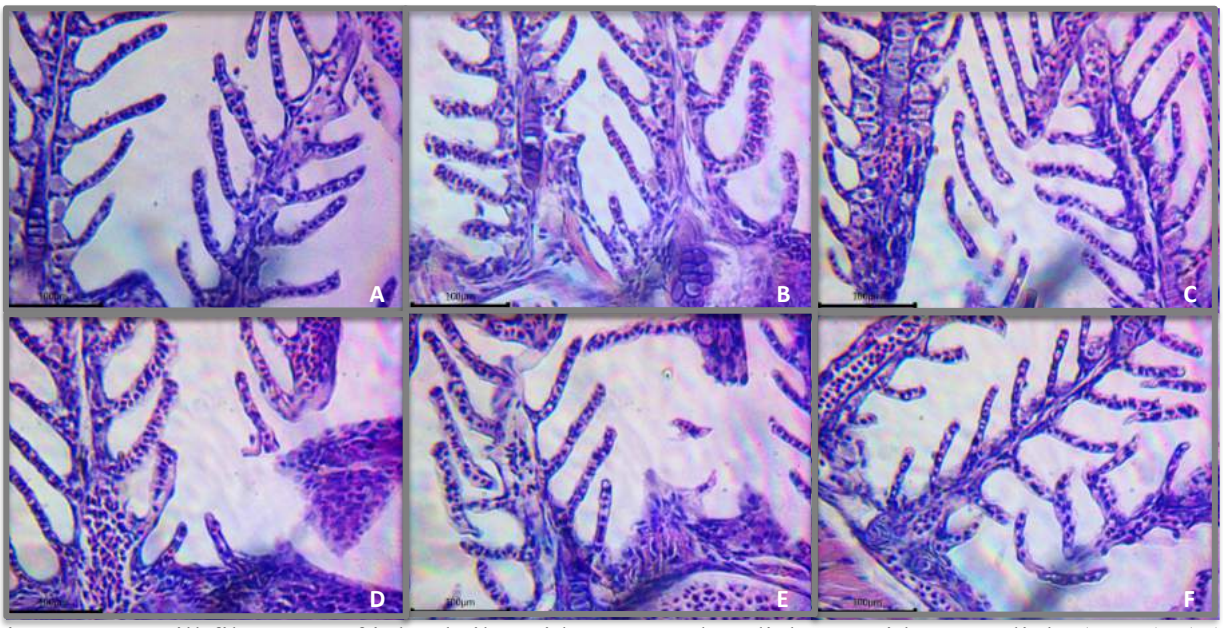

Figure 5.32. Gill filaments of inland silversides exposed to dicloran without sunlight (2.5%). (A) Control, (B) 0.10 mg/L, (C) 0.17 mg/L, (D) 0.25 mg/L, (E) 0.50 mg/L, and (F) 0.75 mg/L.

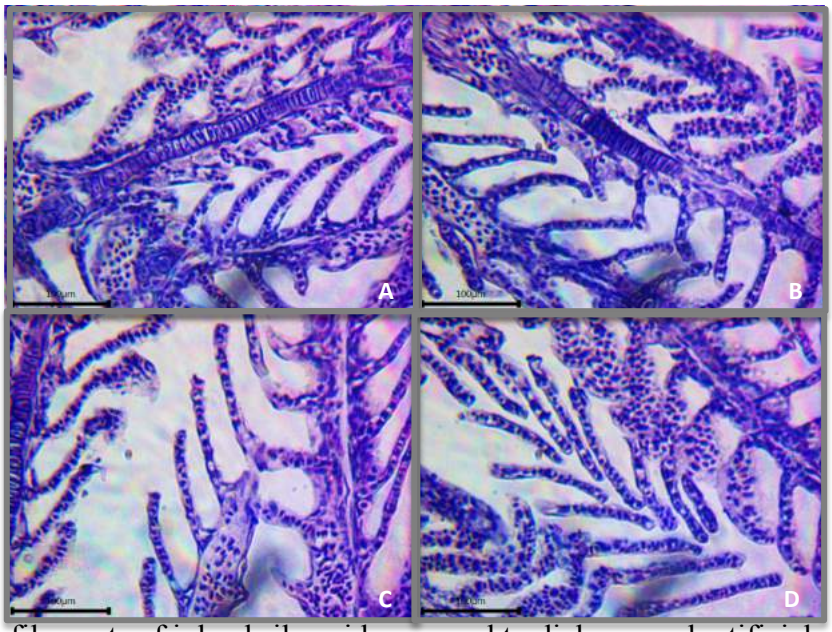

Figure 5.33. Gill filaments of inland silverside exposed to dicloran and artificial sunlight (2.5%). (A) Control, (B) 0.10 mg/L, (C) 0.17 mg/L, and (D) 0.25 mg/L.

Figure 5.32(A) is the healthy gill of a control fish. Responses within the gills appear progressively worse as the concentration of dicloran increases in both dark and light treatments. The lowest concentrations of dicloran exposures in 2.5% ASW were 0.10 mg/L (B) and 0.17 mg/L (C), where cell proliferation can be observed. At the higher concentrations, 0.25-0.75

mg/L (D-E), minor apoptosis is apparent as a defense mechanism to increased concentrations of chemical exposure. Figure 5.33(A) is the gill filament of the light control; although the fish was not exposed to dicloran, the intensity of light was enough to cause cell proliferation within the gills. Cell proliferation and apoptosis can be observed in 0.10 mg/L and light (B), while apoptosis and minimal cell proliferation is observed in fish exposed to 0.17 mg/L dicloran (C). The estimated $LC_{50}$ of silversides exposed to light and dicloran (in 2.5% ASW) was 0.18 mg/L, therefore (D) 0.25 mg/L is above the $LC_{50}$ and extensive damage to the gill can be observed. Aneurisms appear to be present as a result to the increased stress, and potential necrosis consequently is apparent. Comparable to 2.5% ASW, Figures 5.34 and 5.35 show the response within the gills of inland silversides in 1.2% ASW exposed to dicloran in dark and irradiated trials.

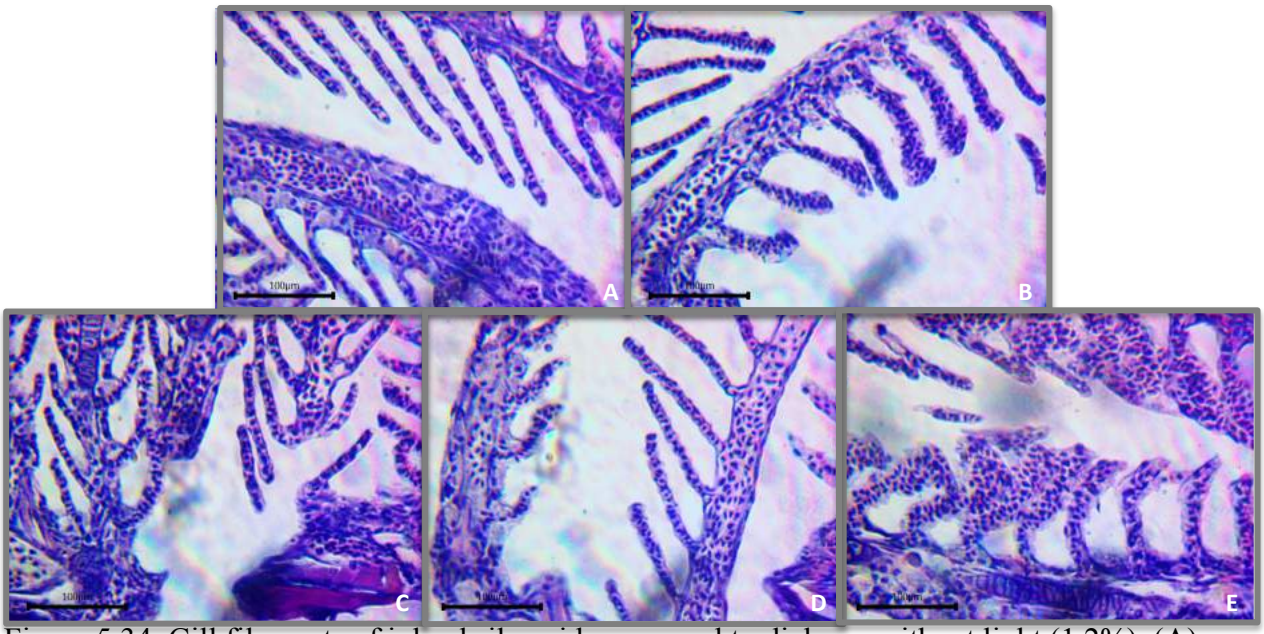

Figure 5.34. Gill filaments of inland silversides exposed to dicloran without light (1.2%). (A) Control, (B) 0.17 mg/L, (C) 0.25 mg/L, (D) 0.50 mg/L, and (E) 0.75 mg/L).

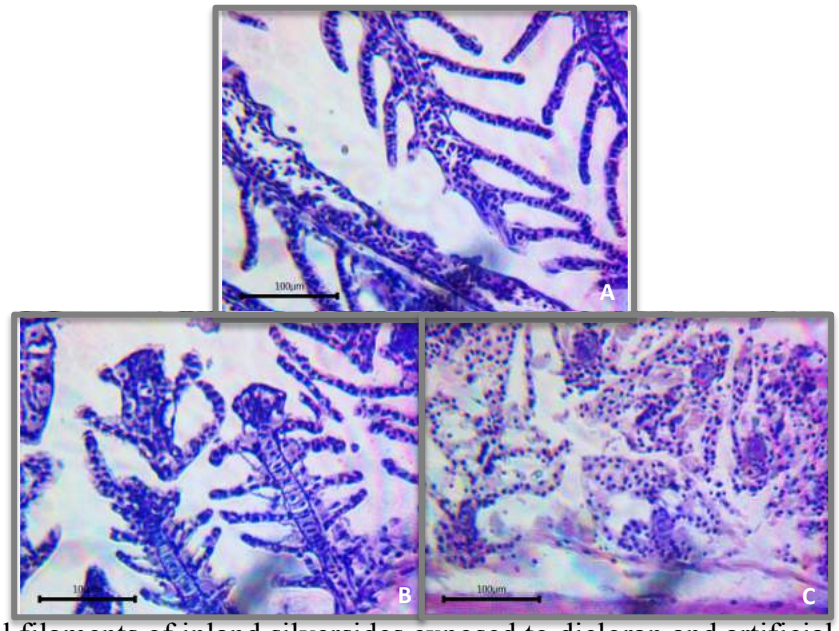

Figure 5.35. Gill filaments of inland silversides exposed to dicloran and artificial sunlight (1.2%). (A) Control, (B) 0.17 mg/L, and (C) 0.25 mg/L.

Figure 5.4(A) shows the gills of a dark control fish at 1.2% ASW. Similar to the response of silversides in 2.5% ASW exposed to 0.17 mg/L dicloran without light, (B) shows cell proliferation within the filament. Increased concentrations (0.25 and 0.50 mg/L; C and D) result in apoptosis and minor cell proliferation. Fish exposed to 0.75 mg/L dicloran shows signs of aneurisms in their gill filaments, a response that was not observed in the gill of fishes from dark treatments at 2.5% ASW. While inland silversides are found in estuarine bodies of water where the salinity is often 1.2%, it appears that the lower salinity results in an increased response to environmental stressors. When light is included in the treatments, the response of gill filaments increases in severity. Cell proliferation is observed in the light control for fishes at 1.2% ASW, therefore the absence of chemical but inclusion of sunlight is a great enough contributing factor to cause a defensive response within the gills. Apoptosis and aneurisms can be observed in the gills exposed to 0.17 mg/L; while necrosis and severe gill damage is observed for 0.25 mg/L. The fish used for analysis at 0.25 mg/L dicloran and light in 1.2% ASW was

harvested prior to death (24-hr post-exposure). Figures 5.36 and 5.37 show the response of fishes exposed to dicloran at 0.5% ASW.

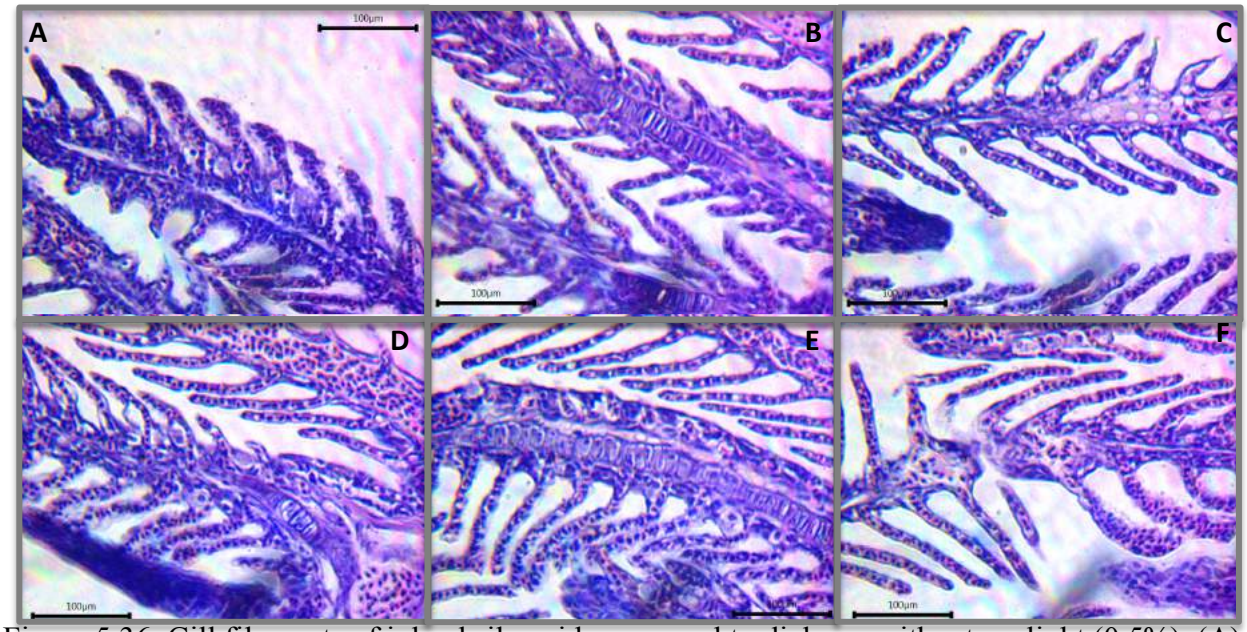

Figure 5.36. Gill filaments of inland silversides exposed to dicloran without sunlight (0.5%). (A) Dark control, (B) 0.10 mg/L, (C) 0.17 mg/L, (D) 0.25 mg/L, (E) 0.50 mg/L, and (F) 0.75 mg/L.

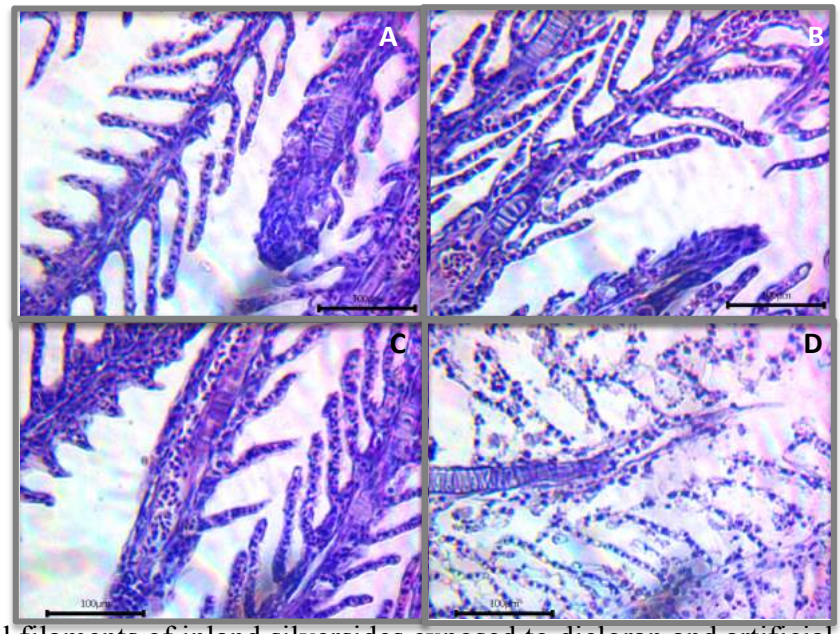

Figure 5.37. Gill filaments of inland silversides exposed to dicloran and artificial sunlight (0.5%). (A) Light control, (B) 0.05 mg/L, (C) 0.10 mg/L, and (D) 0.17 mg/L.

Cell proliferation can be observed in the dark control (5.36A) for at 0.5% ASW. The low salinity appears to result in sub-lethal stresses without the present of chemical. No mortality was

observed in dark or light control fishes; therefore, the decreased salinity alone did not influence lethal responses for the fish. Dark exposures appear consistent with the observations for 1.2 and 2.5% ASW. Cell proliferation increases with increased dicloran (B-D); as concentration continues to increase, cell proliferation becomes minimal as apoptosis appears to be the major defense mechanism (E-F). Light significantly impacts the response of fish as a result of exposure to dicloran. Without light, lethality is minimal and sub-lethal impacts (to gills) extend as far as apoptosis, where the death of the cells is by mechanisms within the fish to prevent death. Cell proliferation and apoptosis is again observed in the light control (5.37A). Significant damage to the gills is observed at 0.17 mg/L exposure; necrosis of the gill is highly likely.

## 5.4. Discussion and conclusions

Salinity appears to impact the behavior of dicloran chemically and toxicologically. While statistically insignificant, the half-life of dicloran varies between 0.5, 1.2, and 2.5% salinity, estimated to be 12.6, 13.4, and 14.8 hours respectively. While dicloran appears most phototoxic to inland silversides at 1.2% salinity, the estimated $LC_{50}$ for 0.5% was 0.11 mg/L; results for 0.5% may be skewed due to the fish used for this analysis were obtained from two different suppliers. Fish appeared more resilient at higher doses in the first trial, compared to the same doses for the second and third trials where fish were obtained from a separate supplier due to unforeseen circumstances (i.e. the first supplier went out of business).

Quinones are naturally occurring and found in plants and bacteria. They are involved in biological functions such as oxidation phosphorylation and electron transfer (primary metabolism for respiration) in many organisms (Abraham et al., 2011; Alegría et al., 1999). Benzoquinones and hydroquinones are degradation products of many pesticides including dicloran and 2,4-D; they are also known to be derivatives in the synthetization of pesticides and

pharmaceuticals. They are photochemically active, typically with short half-lives, and can generate radicals as a result of photolytic activation (Gan et al., 2008; von Sonntag et al., 2004). The potential for phototoxicity of benzoquinones and hydroquinones may be a resultant of the activation of the chemicals by UV-light within the photodegradation of a parent compound. BQ and CBQ exhibited toxic effects to silversides at 1.2 and 2.5% salinities without sunlight; and resulted in nearly 50% mortality to silversides exposed to 0.12 mg/L at 1.2% salinity.

Euryhaline fishes have biological adaptations that allow them to inhabit waters of various salinities and osmoregulation is a major factor allowing fish to maintain homeostasis across different salinities. Inland silversides acclimated to 0.5% salinity showed no obvious stresses in terms of behavior; although their gill filaments and gene expression for ATP1A1a (osmoregulation) appears to show indications of stress. An upregulation in gene expression was observed between controls and 0.17 mg/L in dark and light treatments; 0.10 mg/L resulted in very low expression levels. This may be, in part, due to the estimated $LC_{50}$ of 0.11mg/L for inland silversides exposed to irradiated dicloran at 0.5% salinity. Fish exposed to dicloran from Aquatic Biosystems resulted in 100% mortality at 0.17 mg/L irradiated dicloran, while fish from C. K. Associates did not; this variation in response may skew the $LC_{50}$ and qPCR data. The gill filaments of inland silversides (0.5%) in dark controls show indications of cell proliferation, therefore the low salinity alone is a sub-lethal stressor. Longer acclimation periods may result in less stresses to the fish.

Inland silversides are often found in estuarine environments where salinities average 1.2%, this also is where the lowest $LC_{50}$ value was calculated (0.07 mg/L dicloran and light). The highest levels of gene expression for 1.2% salinity of ATP1A1a, BAIAP212, CCL28, and GSR were at 0.10 mg/L irradiated dicloran, just above the $LC_{50}$. This indicates an elevated

response from genes responsible for cell structure, immune function, osmoregulation, and stress defense. The gene expressions correlate with increased cell proliferation and apoptosis leading to aneurisms and necrosis in the gill filaments for the fishes at 1.2% salinity. Therefore, although inland silversides may inhabit estuarine waters frequently they may also be most at risk for [photo]toxic stressors (lethal and sub-lethal) in these regions. Juvenile silversides typically inhabit shallow-water estuaries with seagrasses, therefore the potential for direct light exposure is relatively low at the rates used in this experiment although, the potential for exposure and uptake of irradiated chemicals in these regions is likely.

Relative gene expressions were typically the least at 2.5% salinity. Multiple reasons may account for this observation including the fish not having to be acclimated to this salinity prior to exposure to dicloran. Although limited mortality (<5%) was observed in fishes acclimated to lower salinities prior to chemical exposure, this may account for initial increased stresses. The $LC_{50}$ at 2.5% salinity was the highest concentration of dicloran, 0.18 mg/L. An obvious variation between salinity and exposure treatments was observed for dicloran and inland silversides, and that may indicate the potential for other pesticides to impact the fish in similar ways. The need for toxicity testing to include light and salinity in regions where [euryhaline] fishes are at risk for exposure may be necessary and beneficial in regulatory studies to understand the risk potential.

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# CHAPTER 6. POTENTIAL PHOTOTOXICITY OF HYDROXYCHLOROTHALONIL TO INLAND SILVERSIDES, *MENIDIA BERYLLINA*

## 6.1. Introduction

Chlorothalonil is a broad-spectrum fungicide that has been registered in the United States since 1966. Since then, it has become one of the most highly used fungicides as it is labeled for use on over 65 crops and is effective against four fungi classes, fungi imperfecti, ascomycetes, oomycetes, and basidiomycetes by influencing physiological processes such as disturbing cell metabolism (Kelly, 2012; Roberts et al., USEPA, 1998). Chlorothalonil is registered for use throughout the United States on a wide range of crops including peanuts, tomatoes, and turf, specifically golf course turf. Regions in which chlorothalonil is applied vary from inland terrestrial to coastal estuarine, and therefore multiple ecosystems are at risk for runoff and spray drift (Tomlin, 1994; USEPA, 1998). Chlorothalonil is typically combined with other fungicides when applied to golf course turf; the application rate for fairways is 26 lbs ai/acre and 72 lbs ai/acre on the greens (Kelly et al., 2012).

Chlorothalonil (CHT) is fairly hydrophobic and binds to soils and sediments in systems where it is applied or is transported as a result of runoff; due to its unlikely nature to bind in water, it is stable to hydrolysis and photolysis (USEPA, 1998). When CHT binds to sediments, its degradation in the sediment is principally microbially mediated where a primary soil degradation product is 4-hydroxychlorothalonil (4-OH-CHT) is formed (Figure 6.1). Low concentrations of CHT have been detected in golf course leachate and receiving waterways, 0.12 mg/L in golf course runoff and 0.031 μg/L in streams (Armbrust, 2001a,b; Battaglin et al., 2011). Contradictory reports state hydroxychlorothalonil preferably is desorbed from soils and sediment and resuspended into the water column, while others state it can persist in sediment where it is mobile and takes over 6 months to degrade (Armbrust, 2001b; Chaves et al., 2008; Wang et al.,

2011). When 4-OH-CHT enters waters, it has been shown to undergo rapid photolysis (Armbrust, 2001a, b; Gamble et al., 2001).

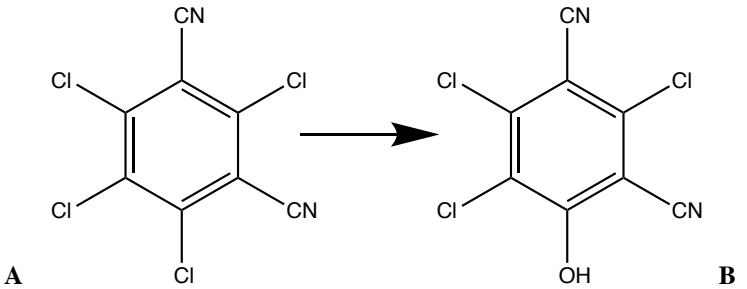


Figure 6.1. The chemical structures of chlorothalonil (A) and 4-hydroxychlorothalonil (B).

The photodegradation of 4-OH-CHT has been reported in distilled water (32 min and 35 min), in 0.5 M NaCl (42 min) and in 2.0 M NaCl (301 min). The proposed primary degradation mechanism is photonucleophilic substitution, and the degradation pathway consists of quinone-semiquinone intermediates until ultimately breaking down into small acids (Armbrust, 2001a,b). This pathway is very similar to that of dicloran which has been shown to be phototoxic to fish and invertebrates (Vebrosky et al., 2018).

CHT is listed as "highly toxic to very highly toxic" to aquatic organisms including bluegill (*Lepomis macrochirus*), rainbow trout (*Oncorhynchus mykiss*), and fathead minnow (*Pimephales promelas*) with $LC_{50}$ concentrations ranging 51-84, 42.3, and 23 μg/L respectively; while 4-OH-CHT is listed as "slightly toxic" to bluegill with $LC_{50}$ concentrations of 15 and 45 mg/L (USEPA, 1998). However, no experiments to date have investigated its potential for phototoxicity. Given its similar degradation mechanism to dicloran, it is conceivable that 4-OH-CHT may also be phototoxic to aquatic organisms as well (Armbrust, 2001a).

The purpose of this investigation was to determine if similar toxicological responses would be observed with inland silversides exposed to 4-hydroxychlorothalonil in comparison to dicloran.

## 6.2. Materials and methods

### 6.2.1. Chemical reagents and materials

Analytical grade 4-hydroxychlorothalonil was obtained from LCG Standards (Middlesex, UK). Standards of 4-OH-CHT in acetonitrile (ACN; VWR, Radnor, PA) were used in photodegradation and toxicity experiments. Artificial seawater (ASW) for photodegradation experiments was made with Instant Ocean® (Blacksburg, VA) in distilled water (VWR; Radnor, PA) and Crystal Sea (Marine Enterprises International, LLC, Baltimore, MD) in filtered well water for toxicity experiments; the salinity was monitored using a YSI Model 30 salinity, conductivity, and temperature meter (YSI; Yellow Springs, OH).

### 6.2.2. Juvenile inland silversides

Juvenile inland silversides (*Menidia beryllina*) were obtained from C. K. Associates (Baton Rouge, LA) at 1.5 months post-hatch and transported to the Aquaculture Research Station at Louisiana State University (Baton Rouge, LA). The silversides were maintained at 2.5% salinity, fed dried *Artemia*, and acclimated to experimental conditions for a minimum of 24 hours prior to exposure.

### 6.2.3. Photodegradation of hydroxychlorothalonil in seawater

The photodegradation of 4-hydroxychlorothalonil was monitored in four aqueous phases; distilled water, 0.5% artificial seawater, 1.2% artificial seawater, and 2.5% artificial seawater. Borosilicate glass vials (Agilent Technologies, Santa Clara, CA) containing 1 mL of 1 mg/L 4-OH-CHT (1,000 mg/L in ACN) in each aqueous phase were exposed to artificial sunlight in an ATLAS SUNTEST XXL+ environmental chamber outfitted with a daylight filter at 40 $W/m^2$ and analyzed by an Agilent 1260 Infinity HPLC (Agilent Technologies) with a water, ACN, and 0.1% formic acid in water mobile phase and a ZORBAX C-8 Eclipse Plus Analytical 4.6 x 150

mm 5 μm column, monitoring the 280 nm wavelength with photodiode array. Vials were removed from the environmental chamber for analysis at 0, 15, 30, 60, and 180 min (n = 3). One-way ANOVA with Tukey's multiple comparisons test ($\alpha = 0.05$) was used to determine statistical differences between the degradation rate of 4-OH-CHT in the four treatments (Prism 7, GraphPad, Inc., La Jolla, CA).

### 6.2.4. Toxicity experimental design

Silversides were separated into 2.5-gallon glass fish tanks (Carolina Biological, Burlington, NC), 20-25 per tank, with an air stone. Tanks were dosed with concentrations of 4-OH-CHT (10,000 mg/L in ACN) ranging from 0.10-1.5 mg/L. Hydroxychlorothalonil has been measured at concentrations as high as 2.0 mg/L (0.5 mg/L mean measurement) in golf course leachate in Georgia, but not in the receiving ponds and 0.05 μg/L in US streams (Armbrust, 2001; Battaglin et al., 2011). Both dark and light trials were completed in triplicate, with non-dosed controls for each treatment, for a 48-hour experimental design. Preliminary analysis of the effects of prolonged exposure in the dark were completed with 3- and 10-day dark exposure periods, followed by the transfer of fish to clean water, and 48 hours of light exposure. A combination of UVA 340 fluorescent UV bulbs (Q-Lab Corporation, Westlake, OH) and 40-Watt Linear Fluorescent Lamps (Grainger®, Lake Forest, IL) were housed in 48-inch shop light fixtures (Grainger®, Lake Forest, IL) to simulate natural sunlight.

### 6.2.5. Sub-lethal analysis

Surviving silversides were preserved in TRIzol® solution (Fisher Scientific, Hampton, NH) for analysis of variation in relative gene expression (mRNA) after exposure to 4-OH-CHT; controls, 1.0, and 1.5 mg/L dark and light treatments were preserved for analysis. Whole fish were homogenized in 1.5 mL Eppendorf tubes (Fisher Scientific, Hampton, NH) with 800 μm

glass beads (VWR, Radnor, PA) for 5 min then centrifuged for 20 min with an additional 200 µL chloroform in each tube (VWR, Radnor, PA); 400 µL of the colorless supernatant was transferred to a 1.5 mL centrifuge tube (Fisher Scientific, Hampton, NH) with 400 µL isopropyl alcohol VWR, Radnor, PA) and placed in a -20ºC freezer for 30 min. The samples were centrifuged, the supernatant was removed, 1 mL 70% ethanol (VWR, Radnor, PA) in RNase Free water (Fisher Scientific, Hampton, NH) was added and centrifuged for another 10 min. The supernatant was removed and the small pellet remaining in the tube was allowed to air dry for 5 min; 25 µL of a mixture of RNase free water, 10x DNase buffer, and TURBO DNase (Invitrogen, Carlsbad, CA) was added and samples were incubated in a 37°C water bath for 30min. The tubes were removed, 5 µL of DNase Inactivation Reagent (Invitrogen, Carlsbad, CA) was added and centrifuged. The supernatant was transferred to a new centrifuge tube and kept in a -80°C freezer until cDNA synthesis (Xu et al., 2018).

RNA concentration, 260/230, and 280/260 values, ranging 394-804, 0.50-0.70, and 1.52-1.67 respectively, of each samples were measured with a GeneQuant Pro (Amersham BioSciences, Little Chalfont, UK). The RNA was combined with deoxynucleotide mix [10 mM] (dNTP; G-Biosciences, St. Louis, MO) and oligo DT (Integrated DNA Technologies, Coralville, IA) in PCR tube strips (VWR, Radnor, PA). The samples were set to follow protocol using a T100™ Thermal Cycler (Bio-Rad, Hercules, CA). The protocol included a 5 min cycle at 65°C, 10 min at 4°C, and completed after 30 min; after the first 10 min, the tubes were removed and 5x SSIV (SuperScript IV) buffer, DTT, SSIV (SuperScript IV) Reverse Transcriptase (Invitrogen, Carlsbad, CA), and RNase free water was added, and allowed to finish the sequence. The cDNA was diluted with RNase free water and stored until qPCR analysis (Xu et al., 2018).

Genes were chosen based off previously reported qPCR studies of silversides in response to toxicants (Brander et al., 2016; Jeffries et al., 2015). The primers were obtained from Integrated DNA Technologies (IDT, Coralville, IA). GAPDH was chosen as the reference gene for analysis. Gene functions including hormone receptor, immune function, cell structure, and stress defense were chosen for analysis (Table 6.1).

Table 6.1. Genes for qPCR analysis of inland silversides exposed to 4-OH-CHT.

| Function | Gene | Identity | Sequence |
|---|---|---|---|
| Reference | GAPDH | Glyceraldehyde 3-phosphate dehydrogenase | CAGTGGTGGACCTGACATGC TTCTTGACGGCGTCCTTGAT |
| Hormone receptor | GPR30 | G-protein coupled estrogen receptor | CGTCCTCTCCGGCCTCTAC TGAGGATGTTCCCAATGAAGC |
| Immune function | MMP9 | matrix metalloproteinase | CAACCAGCAGCTTTGACAGTG TCCGATTACAGCAGTGTCACG |
| Immune function | CCL28 | c-c motif chemokine 28 precursor | GCTCTGGCCATCACCTTCAC AAATTAACGGGCCTTATGCTGA |
| Cell structure | BAIAP212 | Brain-specific angiogenesis inhibitor 1-associated protein | GAGGGGCACCAATCCATTC GCCGATCTGTCATTGGTGGT |
| Stress defense | GSR | Glutathione reductase | CTCCAATGTATCACGCCATCAC TTCTCCTCTTTGCCAACACACA |

Six fish per treatment were used for gene expression analysis. 384-well PCR plates (VWR, Radnor, PA) were prepared by combining the cDNA with RNase free water, 5x GoTaq polymerase colorless reaction buffer, GoTaq polymerase (Promega, Madison, WI), dNTP, Sybr Green 10x, ROX reference dye, (Fisher Scientific, Hampton, NH), and the primer pairs for each gene (IDT, Coralville, IA). PCR plates were set to follow protocol using a 7900HT Fast Real-Time PCR System using sds2.4 software (Applied BioSystems, Foster City, CA). Results were analyzed and generated with RQ Manager software (Applied BioSystems, Foster City, CA).

Normalized $C_t$ values were used to calculate relative gene expressions (mRNA) using $\Delta\Delta C_t$ result values. One-way ANOVA analysis of variance with Tukey's multiple comparison

test ($\alpha = 0.05$) was used to determine significant differences between relative gene expressions (Prism 7, GraphPad, Inc., La Jolla, CA) (Brander et al., 2016; Livak et al, 2001; Xu et al., 2018).

## 6.3. Results and discussion

### 6.3.1. Chemical analysis

The photodegradation of 4-hydroxychlorothalonil was monitored over 180 min in distilled water, 0.5% ASW, 1.2% ASW, and 2.5% ASW and analyzed by HPLC in triplicate. Trials were irradiated for 3-hours total, but no 4-OH-CHT was detectable in any of the aqueous phases after 60 min of irradiated therefore the 180 min samples were not considered for degradation rate and half-life calculations or statistical analysis (Table 6.2). The degradation trend did not vary between salinity, but the rate of degradation was slightly slower (Figure 6.2).

Table 6.2. The estimated half-lives of 4-hydroxychlorothalonil in various aqueous media.

| | Distilled water | 0.5% ASW | 1.2% ASW | 2.5% ASW |
|---|---|---|---|---|
| Half-life (min) | 14.8 ± 0.5462 | 15.5 ± 1.604 | 16.6 ± 0.7846 | 19.3 ± 0.6148 |

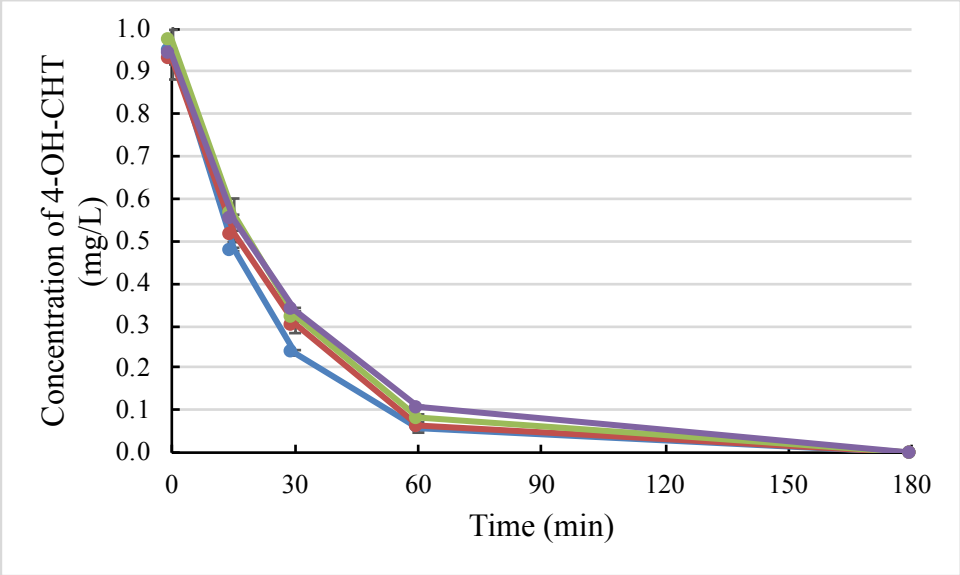


Figure 6.2. The photodegradation of 4-hydroxychlorothalonil in distilled water (blue), 0.5% ASW (red), 1.2% ASW (green), and 2.5% ASW (purple); error bars indicated standard deviation.

One-way ANOVA determined no significant differences between degradation rates in each salinity. Unlike previously reported data, 4-OH-CHT does not appear to be significantly

impacted by salinity; Armbrust used 2.0 M NaCl and observed a slower degradation in comparison to the distilled water half-life however there was only a slightly slower rate observed in 0.5 M NaCl (Armbrust, 2001b).

### 6.3.2. Toxicological analysis

No mortality was observed for fish exposed to 4-hydroxychlorothalonil in dark or light treatments. It was hypothesized that 4-OH-CHT would impact silversides similar to the responses of dicloran exposure. Dicloran and 4-OH-CHT have both been reported to photodegrade through a series of quinone-semiquinone intermediate degradation products (Armbrust, 2001b; Vebrosky et al., 2018). As stated in Chapter 5, dicloran is phototoxic to inland silversides at concentrations ranging 0.10-0.50 mg/L in 0.5, 1.2, and 2.5% ASW. While dicloran and 4-OH-CHT chemically behave similarly, they do not behave toxicologically similar to inland silversides. Although lethal responses were not observed, there is potential for sub-lethal responses influenced by chemical exposure.

Six genes were chosen for the analysis of potential variation in relative expression levels as a result of 4-OH-CHT, GAPDH was chosen as the reference gene. An upregulation in expression level can be observed for BAIAP212 in dark treatments and lower expression levels in light treatments in comparison to dark, with the exception of light control (Figure 6.3). CCL28 resulted in a downregulation in expression level for light treatments, while an upregulation in expression level from 1.0 to 1.5 mg/L exposures in dark treatment (Figure 6.4). Trends vary in expression level for GPR30; due to limited treatments analyzed, a trend could not be determined. The expression level from light and dark controls appears to upregulate when compared to 1.5 mg/L exposure of 4-OH-CHT (Figure 6.5). An upregulation in expression level for GSR in dark treatments can be observed, as well as an upregulation from 1.0 to 1.5 mg/L 4-

OH-CHT in light treatments (Figure 6.6). MMP9 results show a lower gene expression for each treatment in comparison to the dark control; an upregulation in expression level can be observed between 1.0 and 1.5 mg/L in both dark and light treatments, with dark treatments consisting of higher expression levels overall (Figure 6.7).

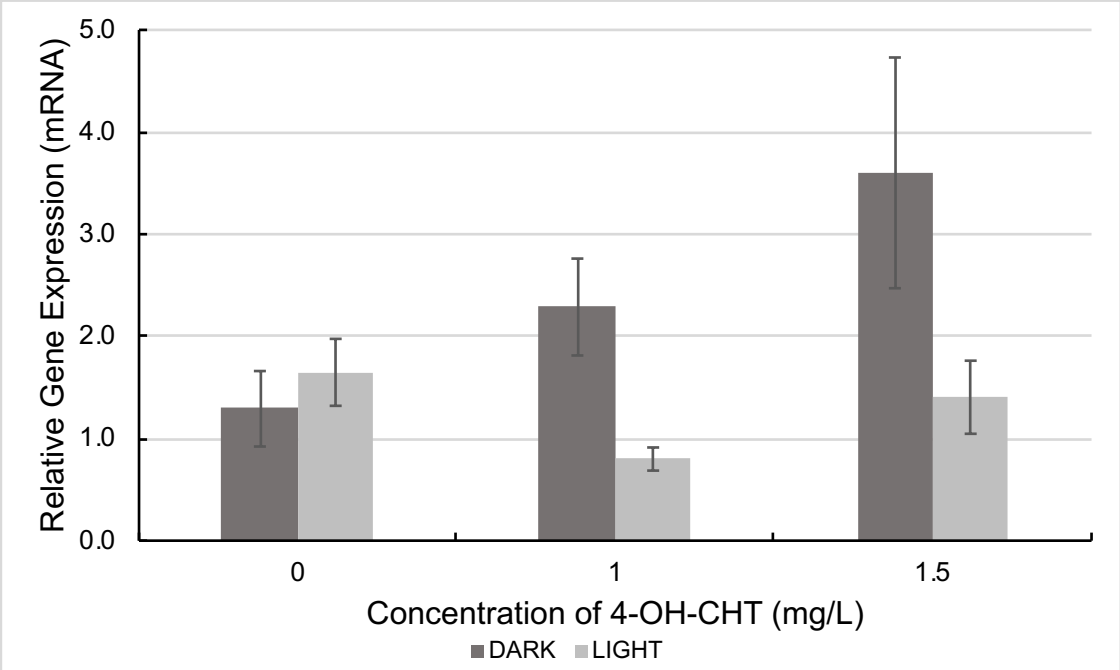


Figure 6.3. The relative gene expression for BAIAP212; error bars indicate standard error.

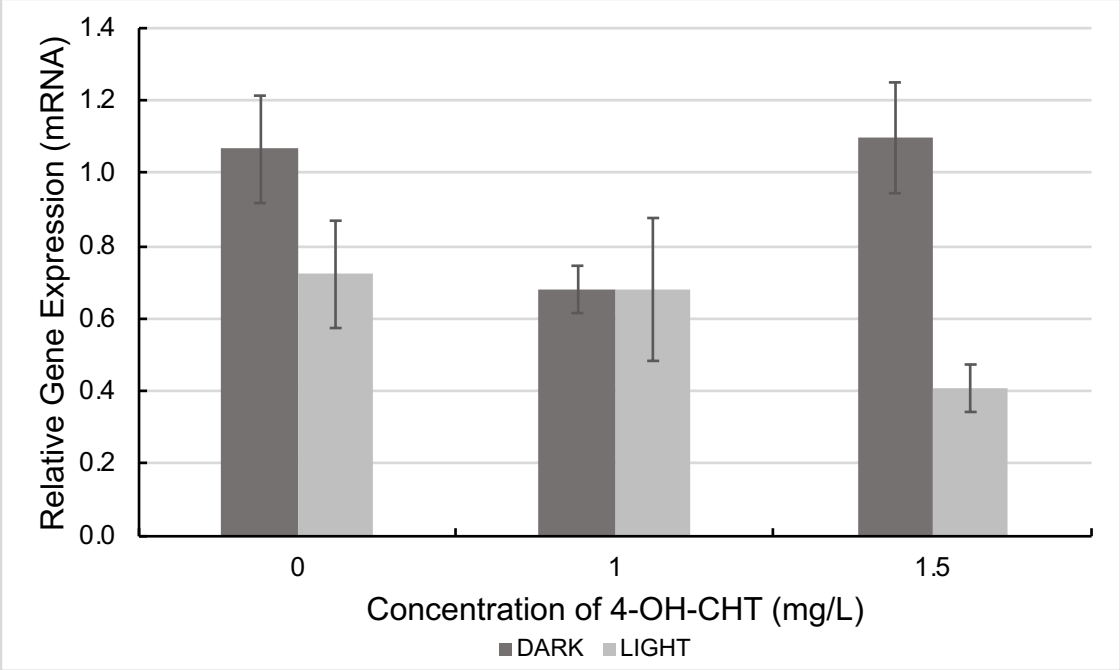

Figure 6.4. The relative gene expression for CCL28; error bars indicate standard error.

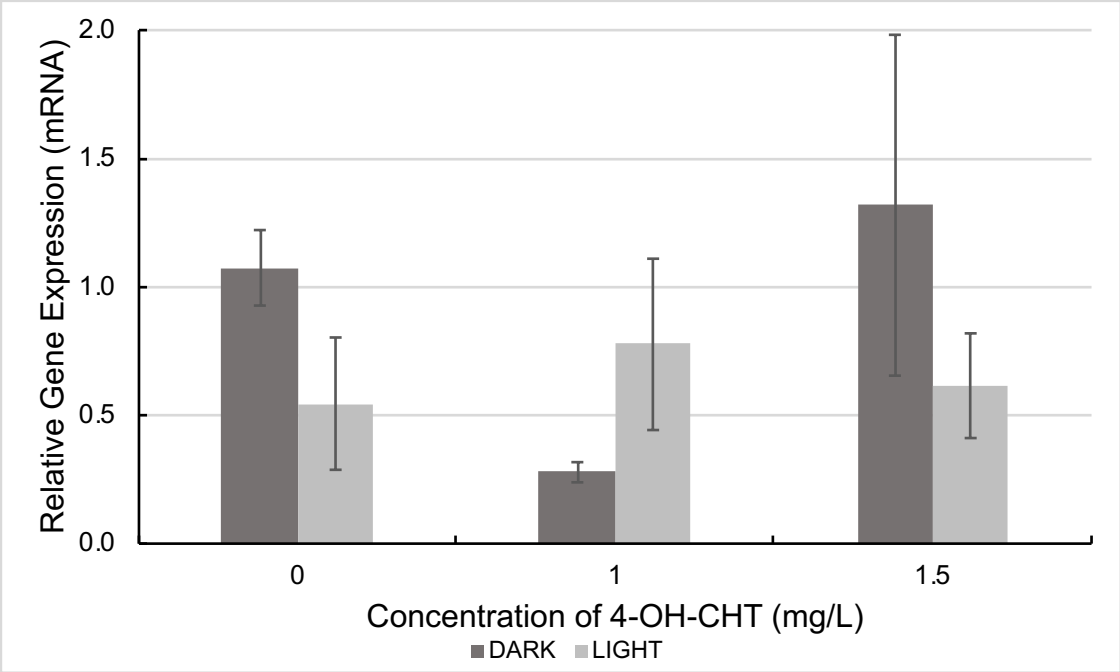


Figure 6.5. The relative gene expression for GPR30; bars indicate standard error.

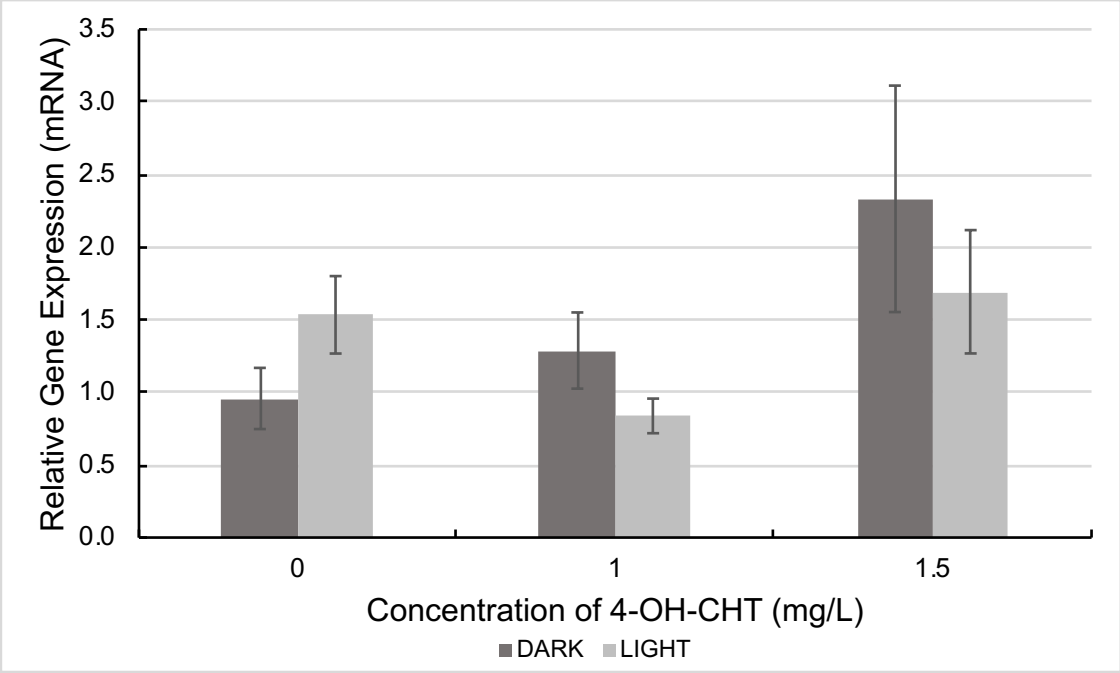


Figure 6.6. The relative gene expression for GSR; error bars indicate standard error.

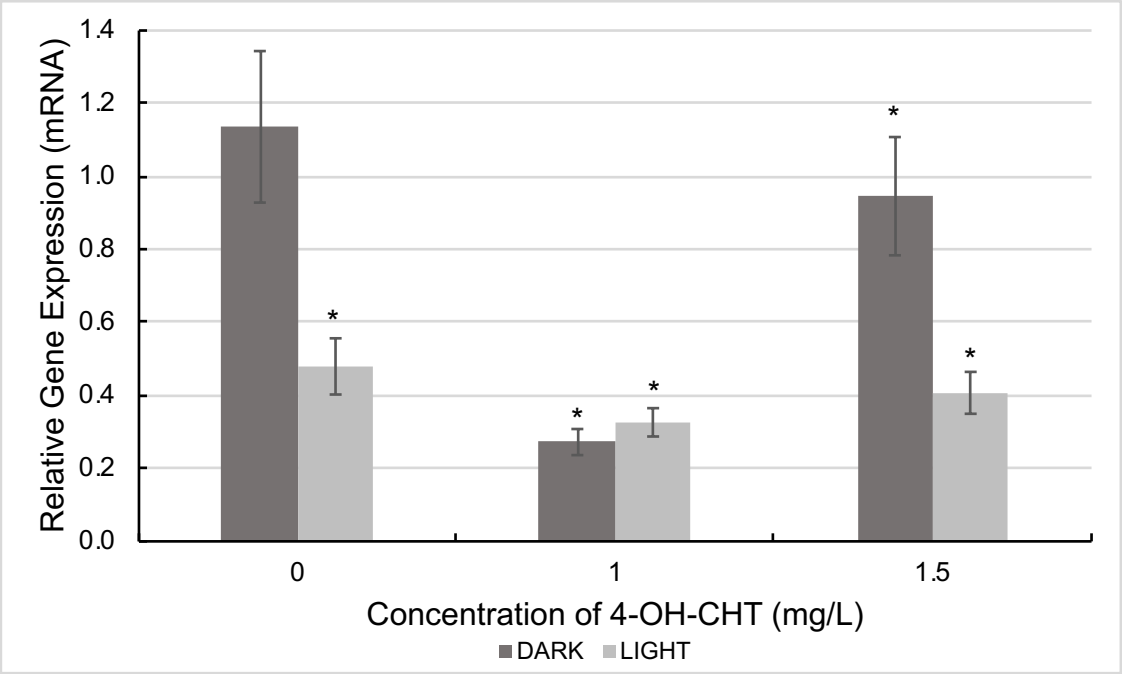

Figure 6.7. The relative gene expression of MMP9; error bars indicate standard error and (*) indicates a significant difference.

Upregulation in gene expression can be observed more frequently in dark treatments in comparison to light treatments in the genes analyzed. A contributing factor to the limited elevated expression level may be the short photolysis half-life of 4-OH-CHT. Hydroxychlorothalonil has a half-life of 19.3 min in 2.5% ASW exposed to artificial sunlight at 40 W/m$^2$ and 301 min in 2.0 M NaCl in a growth chamber (Armbrust, 2001b). Therefore, the rapid degradation may occur before the fish have the opportunity to uptake the activated chemical.

Significant differences between treatments was determined by one-way ANOVA analysis of variance with comparison to dark controls and Tukey' multiple comparisons test. No significant results were observed for BAIAP212, CCL28, GPR30, or GSR. MMP9 expression levels were significantly different; with p-values 0.0038, <0.0001, 0.002, <0.0001, and 0.0007 for light control, 1.0 mg/L dark, 1.0 mg/L light, 1.5 mg/L dark, and 1.5 mg/L light in comparison with dark controls ($\alpha = 0.05$).

Delayed responses were observed for 4-OH-CHT treatments in inland silversides. Fish were exposed in the dark for 3 days to various concentrations of 4-OH-CHT to allow uptake and accumulation of the chemical, transferred to clean water, and exposed to light for 48 hours. No mortality was observed for fish after 48 hours of light exposure. It appears 3 days is not a sufficient amount of time for fish to accumulate enough 4-OH-CHT to result in lethal effects.

Silversides were exposed to low concentrations of 4-OH-CHT, in comparison to the concentrations of the previously mentioned trials, in the dark for 10 days (0.05, 0.10, and 0.50 mg/L) then transferred to clean water and exposed to light for 48 hours. The prolonged exposure period was a sufficient amount of time for enough uptake of the chemical to result in a lethal response. While 4-OH-CHT has been detected in golf course leachate at concentrations reaching 2.0 mg/L, lower concentrations, such as those chosen, are likely to be found downstream or as a result of runoff and/or mixing (Armbrust, 2001b). Fish exposed to 0.05 mg/L resulted in 42.9% mortality, 0.10 mg/L resulted in 38.7% mortality, and 0.50 mg/L resulted in 36.4% mortality. No mortality was observed prior to transferring fish to clean water and exposing them to light.

A 10-day dark exposure period is highly unlikely in an environmental scenario, therefore the potential for 4-OH-CHT to generate phototoxic impacts (i.e. mortality) appears unrealistic.

### 6.4. Discussion and conclusions

Based upon the experimental data, dicloran is chemically a "model" fungicide for degradation behaviors of similarly structures pesticides. Both dicloran and hydroxychlorothalonil photodegrade in the presence of sunlight, and both are impacted by the salinity of the aqueous phase in which the chemical is being analyzed, although the degradation rates are dependent upon light intensity. The influence of both light intensity and salinity (ASW vs. specific ions in seawater) result in different responses for the degradation of both dicloran

and hydroxychlorothalonil. At 60 W/m$^2$, dicloran was previously reported to photodegrade with no significant difference between the degradation rate in distilled water or 3.2% ASW; when the light intensity and salinity were decreased, 40 W/m$^2$ and 2.5% ASW, the difference between media was significant (Vebrosky et al., 2018). Hydroxychlorothalonil appears to behave in similar responses; Armbrust reported a significant difference between distilled water and 2.0 M NaCl which may be an ion-specific result and also reported a slightly slower half-life of 35 min in distilled water in a separate paper. Vebrosky used an ATLAS environmental chamber, and Armbrust used a growth chamber which would have produced a lower light intensity in comparison to the environmental chamber. Therefore, experimental design is influential on the results that are generated for photodegradation experiments (Armbrust, 2001a,b; Vebrosky et al., 2018).

It was hypothesized that dicloran could toxicologically be a model for the phototoxic effects of a chemical to fishes, such as inland silversides. Unlike dicloran, 4-OH-CHT did not appear to be phototoxic to inland silversides at environmentally relevant concentrations or conditions. Prolonged dark exposure and transfer to clean water resulted in what could be assumed as internal phototoxicity of 4-OH-CHT that had been up-taken by the inland silversides where mortality was observed. Treatments where fish were exposed to chemical and light immediately resulted in no mortality or obvious behavioral differences (i.e. limited mobility) were observed. Further analysis using multiple salinities was unnecessary as no lethal phototoxic effects were observed at the high salinity (2.5%) at the highest dose (2.0 mg/L 4-OH-CHT). The rapid photodegradation of 4-OH-CHT is a possible contributing factor as to why phototoxic effects were not observed; the chemical was likely to fully degrade before phototoxicity could be initiated.

Sub-lethal effects were observed for relative gene expression levels. All genes analyzed showed no significant differences between treatments with the exception of MMP9. MMP9 is a gene related to immune function and therefore the exposure of 4-OH-CHT appears to impact the immune function of inland silversides. Both chlorothalonil and hydroxychlorothalonil have previously been reported to sub-lethally impact fishes such as zebrafish (*Danio rerio*). RNA was obtained from the livers of zebrafish and real-time PCR was used to monitor the expression levels of β-actin, cytochrome P450 (cyp1a), estrogen receptor 1 (ESR1), ribosomal subunit 18 (rps18), vitellogenin 1 (vtg1), glutathione reductase (GSR), and hypoxia induced gene 1 (hif1a). Cyp1a expression appeared to decrease with increasing concentrations of CHT, while no significant differences were observed for ESR1, vtg1, GSR, and hif1a (Sánchez Garayzar et al., 2011). Similar responses were observed for inland silversides and GSR, where there were no significant differences between treatments for relative gene expression levels. Zhang *et al* used both CHT and 4-OH-CHT for gene expression analysis for zebrafish embryos. $LC_{50}$ values were also estimated for CHT and 4-OH-CHT; at 96 hr, the % mortality for zebrafish embryos was nearly 60% at 50 μg/L exposure and <20% for 5 μg/L and mortality rates for CHT at concentrations ranging 5-50 μg/L did not exceed 20%. Estrogen receptor α (ERα) and thyroid receptor β (TRβ) were monitored for impacts; luciferase activity was influenced by 4-OH-CHT but was not significant (ERα) while an increase and significant difference was observed for TRβ (Zhang et al., 2016). While the toxic potential of intermediate degradation products is of concern, it appears that 4-hydroxychlorothalonil is not a highly toxic or phototoxic compound to estuarine fishes.

## 6.5. References

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# CHAPTER 7. PHOTOTOXIC IMPACTS OF THE FUNGICIDE, DICLORAN, TO NATIVE *PROCAMBARUS CLARKII*

## 7.1. Introduction

Fungicides are widely used to prevent the spread of fungal diseases on agricultural crops, golf courses, and home gardens. Dicloran (2,6-dichloro-4-nitroanline) is a substituted aniline fungicide registered throughout the southern and western United States on a variety of crops including sweet potatoes, tomatoes, and specialty crops (Figure 7.1).

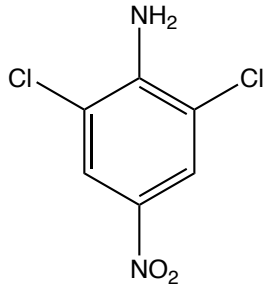


Figure 7.1. The chemical structure of dicloran.

Dicloran has a reported photodegradation half-life between 7.6 and 12.9 hours in distilled water, dependent upon light intensity (Vebrosky et al, 2018). It has also been shown to partition into sediment and is reported by EPA to have low mobility once in the sediment (USEPA, 2006). Benthic macroinvertebrates, such as crayfish, may be at a higher risk for exposure to chemicals when bound to sediment or in the sediment porewater, as they dwell and feed at the sediment-water interface in shallow water systems. Sediment has the potential to alter chemical behavior, and like salinity, it can enhance or decrease toxic impacts of chemicals to aquatic organisms. Dicloran photodegrades slower in the presence of sediment, as opposed to a non-sediment aquatic system. Laboratory, or regulatory, designed experiments do not typically take external environmental factors such as sediment into account in toxicity experiments.

Crayfish are benthic macroinvertebrates and are opportunistic feeders; often detritivores feeding off of organic material in their habitats, they have also been known to eat plants, small

fishes, macroinvertebrates, and even exhibit cannibalistic behaviors and consume one another (Hobbs, 1993; Momet et al., 1987; McClain et al., 2007). Both aquatic and terrestrial organisms use crayfish as a major food source; therefore, the potential for a chemical to bioaccumulate (or biomagnify) through a food web as a result of consumption of crayfish that are exposed to and uptake the chemical(s) is likely. Crayfish are tolerant of a wide range of environments, including areas with high turbidity and low levels of dissolved oxygen (DO) (Nyström, 2002). Certain crayfish such as *Procambarus clarkii* are burrowing species while others inhabit rocky bottom streams (McClain et al., 2007).

In southern states, particularly Louisiana, crayfish are a culturally, economically, and ecologically important invertebrate. Red swamp crayfish (*Procambarus clarkii*) are the most abundant crayfish species in Louisiana, resulting in over $45 million (2003) to $120 million annual revenue (2008) to the state (Issacs et al., 2010). Aquaculture and harvest from agriculture fields, specifically rice fields, are the most common; wild-caught crayfish are less abundant and available for consumer purchase (McClain et al., 2007). Therefore, the risk of exposure to pesticides of crayfish harvested in agriculture regions is of relatively greater important and concern to both consumers as well as rice growers in Louisiana.

Pentachlorophenol (PCP), a pesticide reported to have a similar photodegradation pathway as dicloran, has been shown to be toxic to the European crayfish (*Astacus fluviatilis L.*) with an $LD_{50}$ (8 days) of 26 mg/L (Kaika et al., 1977; Miille et al., 1983; Vebrosky et al., 2018; Wong et al., 1981). Nontraditional assays using red swamp crayfish (*P. clarkii*) have reported an $LC_{50}$ (96 hr) of 0.39 mg/L for exposure to permethrin (Jolly Jr et al., 1978). Benli *et al.* reported the toxicity of 2,4-D to Danube crayfish (*Astacus leptodactylus*) with a 96 hr $LC_{50}$ of 32.6 mg/L (Benli et al., 2007). Red swamp crayfish have previously been used to analyze their response to

insecticides including phosphamidon, dibrom, DDT, methyl parathion, and endrin and Muncy *et al.* reported 72 hr $LC_{50}$ values of 5.5, 4.0, 0.6, 0.04, and 0.3 mg/L respectively. The response varied amongst the insecticides. An interesting observation included the that $LC_{50}$ for DDT has been reported to be highly toxic to fishes while it did not appear to impact crayfish the same way; DDT has an $LC_{50}$ of 8.7 μg/L to rainbow trout (*Oncorhynchus mykiss*) and 21.5 μg/L for channel catfish (*Ictalurus punctatus*) (Johnson et al., 1980; Muncy et al., 1963). Therefore, the risk for invertebrates compared to vertebrates for pesticides exposure is highly variable and individual analyses are necessary for risk assessment.

Crayfish are not a standard freshwater toxicity invertebrate species, but in regions where they are ecologically (and economically) important, the use of a nontraditional organism is beneficial. Therefore, previous analyses have used crayfish as a toxicity study and shown negative impacts with exposures to various pesticides. Dicloran has previously shown to be phototoxic to aquatic vertebrates, including fathead minnows (*Pimephales promelas*) and inland silversides (*Menidia beryllina*), and to the cardiomyocyte cell culture of eastern oyster (*Crassostrea virginica*) (Xu et al., 2018). The impacts of dicloran to two fish species have previously been discussed in chapters 4 and 5, therefore the purpose of this investigation was to determine the differences in response to dicloran phototoxicity between a freshwater vertebrate and invertebrate.

## 7.2. Materials and methods

Methods and materials are similar to those described in previous chapters for phototoxicity exposures but have been modified for this chapter.

### 7.2.1. Chemical reagents

Analytical grade dicloran (2,6-dichloro-4-nitroaniline) was obtained from Sigma Aldrich (St. Louis, MO). A stock solution of 10,000-mg/L dicloran in ACN (VWR; Radnor, PA) was used to dose crayfish for toxicological analysis at concentrations: 0.25, 0.50, 0.75, 1.0, and 1.5 mg/L.

### 7.2.2. Red swamp crayfish collection

Red swamp crayfish were collected by dip net (Forestry Suppliers, Inc., Jackson, MS) in agricultural ditches (Figure 7.2) surrounding the Aquaculture Research Station (ARS; Louisiana State University, Baton Rouge, LA) during February and March of 2017 and 2018. Crayfish measuring 3-6 cm were collected and transferred to 20-gallon fish tanks until experimental testing; because they were collected in the wild and not spawned in the lab, the exact age of the crayfish is unknown therefore crayfish larger than 6 cm were not used in toxicity trials. Tanks contained plastic mesh to help to prevent injury or cannibalism prior to experiments.

Figure 7.2. Crayfish collection using dip nets from the agriculture ditches at the Aquaculture Research Station (Baton Rouge, LA).

### 7.2.3. Phototoxicity experimental design

To prevent mortality as a result of cannibalism and fighting, individual crayfish were placed in 250 mL glass jars (Fisher Scientific, Hampton, NH) for experimental analysis. For each treatment, 20-25 crayfish were individually analyzed. Crayfish were exposed to concentrations of dicloran (0.25, 0.50, 0.75, 1.0, and 1.5 mg/L) in 150 mL of well water (Baton Rouge, LA) with and without sunlight for 48 hours; preliminary analyses to include silt-loam rice field sediment (Crowley, LA) as an impacting factor were also examined.

Artificial sunlight mimicked the output of natural sunlight using a combination of 40-Watt Linear Fluorescent Lamps (Grainger®, Lake Forest, IL) and UVA 340 fluorescent UV bulbs (Q-Lab Corporation, Westlake, OH) that were contained in 48-inch shop light fixtures (Grainger®, Lake Forest, IL). The light intensity and spectra were monitored with a Black Comet Concave Grating UV-VIS spectrometer (Stellar Net, Inc., Tampa, FL).

### 7.2.4. Sub-lethal analysis

Surviving crayfish were harvested and preserved in TRIzol® solution (Fisher Scientific, Hampton, NH) for qPCR analysis. Crayfish tissue was removed from the shell and carapace, then homogenized in 1 mL TRIzol® and 800 μm glass beads (VWR; Radnor, PA) for 10 min in a 1.5 mL centrifuge tube (Fisher Scientific, Hampton, NH) using a Vortex-Genie 2 (Scientific Industries, Inc., Bohemia, NY) and 200 μL of chloroform (VWR, Radnor, PA) was added. The samples were centrifuged for 20 min and 400 μL of the supernatant was transferred to a new Eppendorf tube (Fisher Scientific, Hampton, NH) with 400 μL isopropyl alcohol (VWR, Radnor, PA) and kept in a -20°C freezer for a minimum of 30 min (Xu et al., 2018).

Samples were centrifuged and the supernatant was discarded; 1 mL of 70% ethanol (VWR, Radnor, PA) prepared with RNase free water (Fisher Scientific, Hampton, NH) was

added and centrifuged for another 10 min. The supernatant was again removed, the pellet was allowed to air dry, and a mixture of RNase free water, 10x DNase buffer, and TURBNO DNase (Invitrogen, Carlsbad, CA) was added to each tube prior to a 30 min incubation period at 37°C. DNase Inactivation Reagent (Invitrogen, Carlsbad, CA) was added to each tube, centrifuged, and 25 μL of the solution was removed and stored at -80°C until cDNA synthesis (Xu et al., 2018).

A GeneQuant Pro (Amersham BioSciences, Little Chalfont, UK) was used to measure the concentration of RNA, 260/230, and 280/260 values. RNAs were transferred to PCR strips (VWR, Radnor, PA) and a mixture of deoxynucleotide mix [10 mM] (dTNP; G-Biosciences, St. Louis, MO), RNase free water, and oligo DT (Integrated DNA Technologies, Coralville, IA) was added. cDNA was synthesized using a T100™ Thermal Cycler (Bio-Rad, Hercules, CA) and set to follow protocol. A mixture of 0.1 M DTT, SSIV reverse transcriptase, and 5x SSIV buffer (Invitrogen, Carlsbad, CA) were added after about 10 min of the sequence; cDNA was diluted with RNase free water and stored at -20°C until qPCR analysis (Xu et al., 2018).

Genes (Table 7.1) were chosen based upon previous literature monitoring the gene expression of red swamp crayfish in accordance to immune function (Chen et al., 2013; Jiang et al., 2015; Liu et al., 2017; Meng et al., 2013; Wang et al., 2011; Yi et al., 2017; Zhang et al., 2011; Zhang et al., 2013). Primers were obtained from Integrated DNA Technologies (IDT, Coralville, IA). Six individuals from each treatment (0.50, 0.75, and 1.0 mg/L dark and light) were used for qPCR analysis; the cDNA was combined with primer pairs (IDT, Coralville, IA), DNase free water, dNTP, ROX reference dye, 10x Sybr Green (Fisher Scientific, Hampton, NH), GoTaq polymerase, and 5x GoTaq polymerase colorless reaction buffer (Promega, Madison, WI). The samples were analyzed in 384-well plates on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster, CA) using sds2.4 software, results data was exported with RDS

1.2.1. software, and analyzed in Prism 7.0 (GraphPad Software, Inc., La Jolla, CA) to calculate relative gene expression of mRNA (Xu et al., 2018).

Table 7.1. Genes chosen for analysis.

| Function | Gene | Identity | Sequence |
|---|---|---|---|
| Reference | GAPDH | GAPDH | AGGCTGTCGGAAAGGTTATTC<br>CCAGCCTTAGCGTCAAAGAT |
| Reference | ACTB | β-Actin | TGCGACTCTGGTGATGGTGT<br>AGCGGTGGTGGTGAAGGAAT |
| Immune function | Ast | Astacidin | ATGCGTCTTCTCCATCTCC<br>TTACTTGCCTGGACGGTA |
| Immune function | Fer | Ferritin | GAGTCAAGTGATGAAGAGCG<br>ATGAAGAGTACCCCATTCTTG |

## 7.3. Results of crayfish exposures

### 7.3.1. Lethal impacts for crayfish exposed to dicloran

Mortality was observed for crayfish exposed to dicloran. Low mortality rates were observed for crayfish exposed without sunlight present, <10% in treatments exceeding 0.75 mg/L. When light is an added factor, the mortality rates increase dramatically; 100% mortality was observed for crayfish exposed to 1.5 mg/L dicloran and sunlight (Figure 7.3). About 50% mortality was observed for crayfish exposed to light and 0.75 mg/L of dicloran. Standard deviations could not be generated because crayfish were treated as individuals and not grouped for trials. Control crayfish exposed to only light did not show any obvious behavioral changes, and no mortality. Crayfish exposed to lower concentrations (0.25 and 0.50 mg/L) of dicloran and light were severely lethargic. Many appeared to be on their sides and unresponsive, though their gills remained functioning.

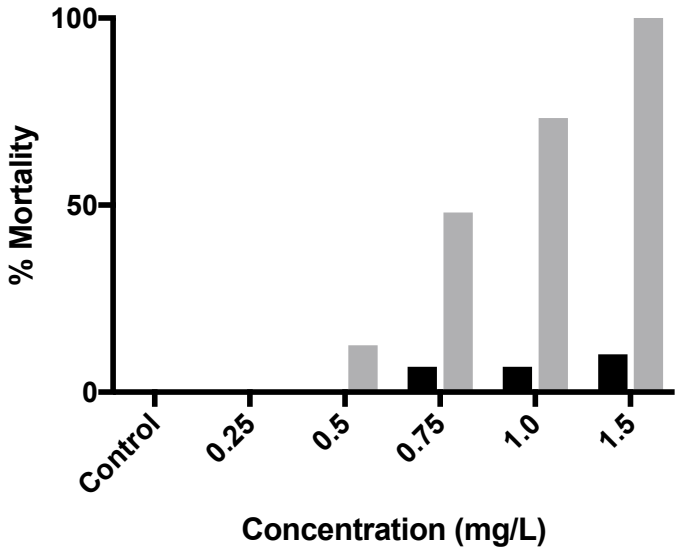


Figure 7.3. The % mortality for crayfish exposed to dicloran in the dark (black) and light (gray); 100% mortality was observed for crayfish exposed to 1.5 mg/L dicloran and light.

Crayfish appear less sensitive to dicloran exposure compared to fishes such as fathead minnows and inland silversides. This may be a result of physiological influences and has previously been reported by Muncy *et al.* when comparing DDT exposure to fish and red swamp crayfish (Muncy et al., 1963). While juvenile red swamp crayfish are less pigmented (gray, slightly translucent) than their adult, deep red counterparts, they have a carapace to help to protect their internal organs from chemicals absorbed through the skin and light penetrating and causing internal photodegradation/activation of chemicals.

Juvenile crayfish are a much more sensitive population as opposed to adult crayfish. They molt 7-11 times in their first year and the carapace is softer directly before and after a molt. Molting is a result of crayfish shedding their exoskeleton to grow; after molting, they have a soft shell until it finishes calcifying. This soft shell can present a risk for crayfish to have a new exposure route for chemicals (i.e. absorption).

### 7.3.2. Sub-lethal impacts for crayfish exposed to dicloran

Nine genes in total were chosen for qPCR analysis; the best depiction of the results are shown in asticidin (Figure 7.4), c-type lectin (Figure 7.5), and ferritin (Figure 7.6).

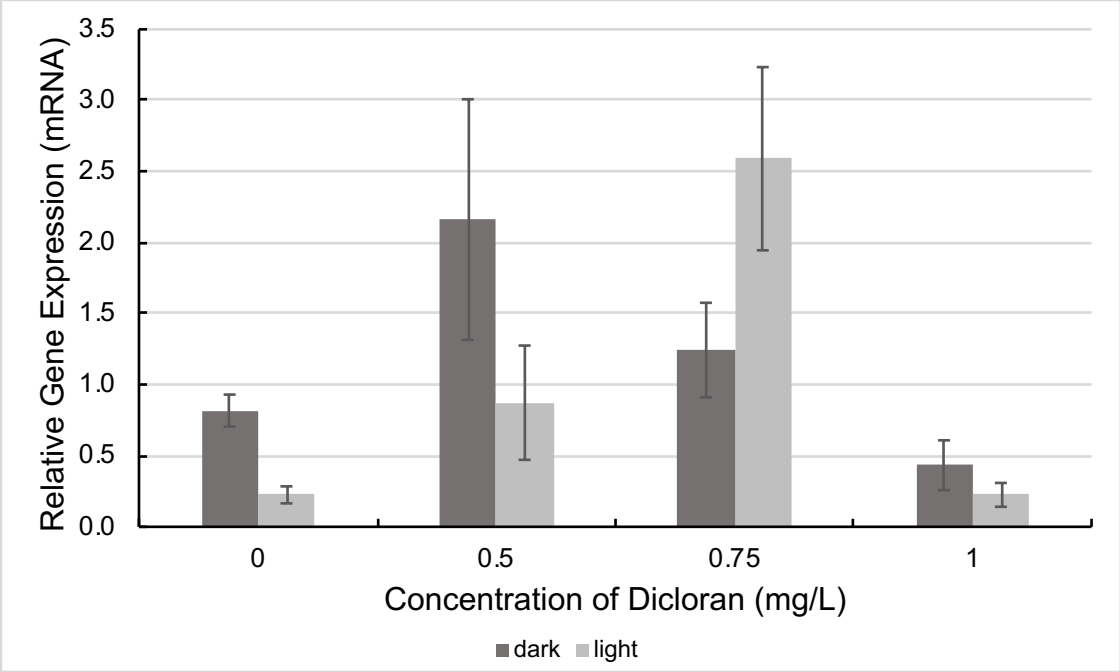


Figure 7.4. The relative gene expression for asticidin.

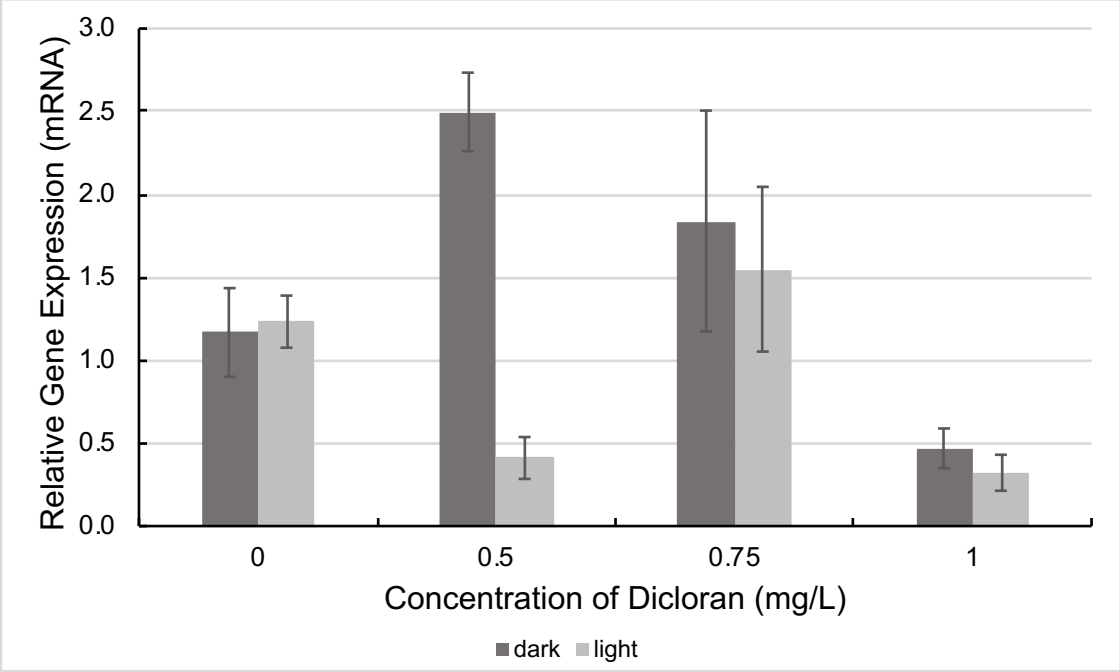


Figure 7.5. The relative gene expression of c-type lectin.

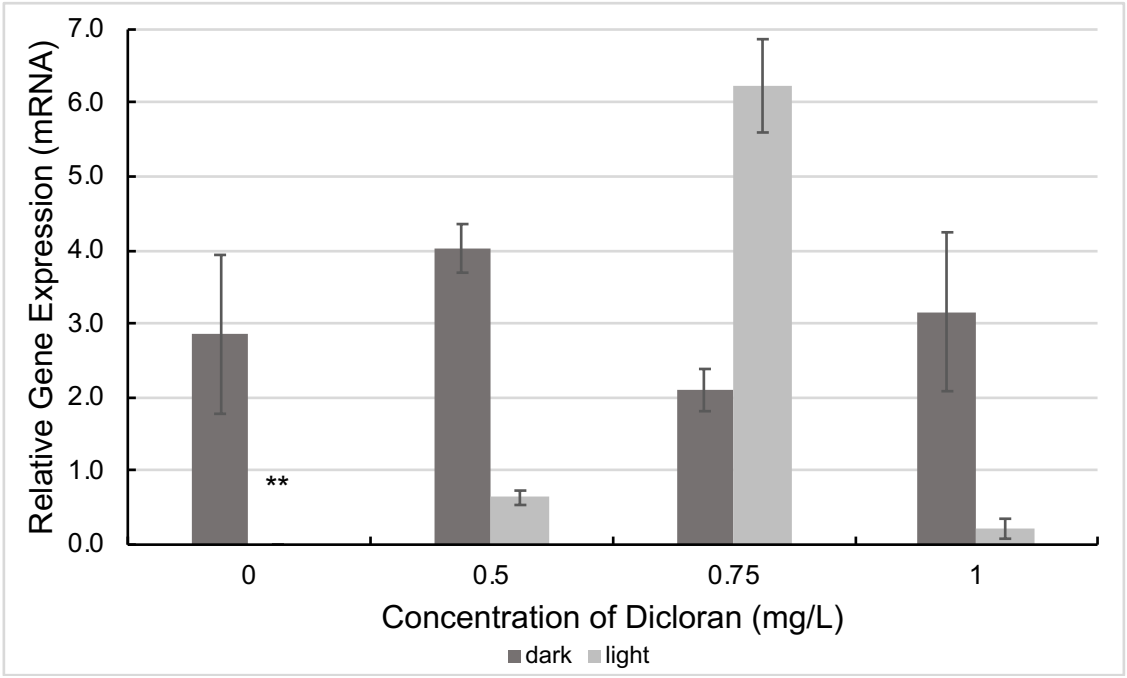

Figure 7.6. The relative gene expression ferritin. Asterisks (**) indicates no data from that time point.

Gene expressions for c-type lectin and ferritin were calculated using GAPDH as the reference gene, while asticidin used β-actin as the reference. C-type lectin and asticidin show similar trends for dark treatments where there is an initial increase in gene expression from dark control to 0.50 mg/L dicloran followed by a downregulation in expressions for the higher concentrations. Asticidin and ferritin have the highest expression level at 0.75 mg/L light treatment, which is also the closest concentration to the $LC_{50}$ for crayfish exposed to irradiated dicloran; c-type lectin has the highest light expression at 0.75 mg/L but the highest expression for the gene is at 0.50 mg/L dicloran in the dark.

No significant differences were observed for asticidin between any treatment, with *p*-values ranging 0.3250 to 0.9997 or c-type lectin with *p*-values ranging 0.1601 to 0.9999. No significant differences were observed for ferritin between dark controls and treatments; significant differences were calculated between 0.75 light compared to 0.50 light (*p*-value 0.0139) and 1.0 mg/L light (*p*-value 0.0190). While differences in expression levels can be

observed for each of the three genes analyzed, minimal statistical differences were calculated. This could be due to the general variation in DNA for wild-caught crayfish or the small size of the samples used for analysis.

### 7.4. Discussion and conclusions

Dicloran is phototoxic to crayfish at elevated concentrations, compared to those that are environmentally relevant. Data reported by the EPA and supported by the irradiated water-sediment analysis in Chapter 3 suggests that dicloran will partition into sediment if it runs off into non-target aquatic ecosystems. EPA reports low mobility in sediments, but also the ability to persist (USEPA, 2006). Persistence was not a factor considered for Chapter 3, as the latest sampling timepoint was 24-hr post-dosage exposure. The data suggests dicloran partitions into sediment after less than 24 hours after entering the water-sediment system. Photodegradation appears to degrade the dicloran in the water, but dicloran remains in the sediment. The potential for dicloran to resuspend into the water column exists, though is unsupported by EPA reports.

Preliminary analysis of the phototoxicity of dicloran to red swamp crayfish with the inclusion of sediment determined sediment lessened the degree of phototoxicity. Crayfish (n = 4) were exposed to dicloran at either 0.50, 0.75, 1.0, or 1.5 mg/L with light and sediment. Without sediment, 100% mortality was observed for all crayfish exposed to dicloran and light; with sediment, 75% mortality was observed. Additional analyses to include sediment as a factor in phototoxicity would be necessary to make further conclusions.

Similar to the results reported for DDT toxicity, dicloran is less toxic to red swamp crayfish compared to fishes such as fathead minnows. While fathead minnows and red swamp crayfish are both freshwater organisms, crayfish appear more tolerant of pesticide exposure.

Therefore, it is beneficial to include both invertebrates and vertebrates in toxicity and phototoxicity experiments to correlate the differences between responses of various organisms.

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# CHAPTER 8. PHOTODEGRADATION AND POTENTIAL PHOTOTOXICITY OF BENZOBICYCLON [HYDROLYSATE] TO RED SWAMP CRAYFISH (*PROCAMBARUS CLARKII*)

## 8.1. Introduction

Benzobicyclon (BZB) is a recently approved pro-herbicide for application on rice in California and is undergoing research with the anticipation of registration in states such as Louisiana, Texas, Mississippi, and Arkansas. It has been used in countries such as Japan for >10 years (Komatsubara et al., 2009). Benzobicyclon is registered under the tradename BUTTE®, and will likely be registered as Rouge® or RougePlus® in Louisiana and throughout the southeastern United States. The compound rapidly hydrolyzes in waters such as flooded rice fields, to form the active ingredient, benzobicyclon hydrolysate (BH) (Figure 8.1). BZB has a reported hydrolysis half-life of 16 hours in rice water at 25°C (Williams et al., 2016).

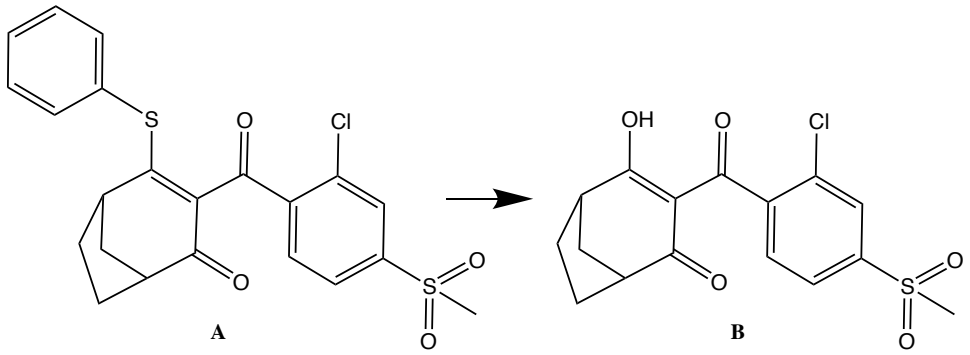


Figure 8.1. The chemicals structures of benzobicyclon (A) and benzobicyclon hydrolysate (B).

There is little to no risk for spray drift or runoff as BUTTE® is applied in the granular form, although there is risk of transport of the herbicide to non-target aquatic systems after flooded fields are drained if BZB or BH remains in the water column. In Louisiana it is assumed that BZB will be applied to fields post-flood as indicated on the label. BZB has a different mode of action than previously registered herbicides; it inhibits 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) by bleaching the plants after uptake (USEPA, 2017).

Rice fields in Louisiana are a unique ecosystem, as farmers often harvest both rice in rotation with crayfish from the same fields. Red swamp crayfish (*Procambarus clarkii*) are the most abundant species of crayfish in Louisiana, with white river crayfish (*Procambarus acutus* or *zonagulus*) the second most abundant. In other regions of the United States, red swamp crayfish are considered an invasive species but in Louisiana they are a cultural food staple. Red swamp crayfish are ecologically and economically important in Louisiana; they are a multi-million-dollar commercial industry, often exceeding $45 million annually, and are also a food source for many migratory and native bird species. Anglers often use crayfish as bait when fishing for large and smallmouth bass and this may account for the introduction of red swamp crayfish into non-native waters such as Michigan. Crayfish, specifically *P. clarkii*, are grown and harvested not only in rice fields but also in aquaculture. Prior to the 1960s, commercial harvest of crayfish was limited and nearly all crayfish were wild-caught in bayous, ditches, or other shallow-water systems. Currently, nearly 90% of harvest is no longer wild-caught; the majority of the crayfish harvest occurs in agriculture fields, thus the risk for exposure to agrochemicals is of particular concern (McClain et al., 2007). The insecticide fipronil was implicated in declines in crayfish harvest in Louisiana following its use to control rice water weevil after carbofuran was banned for this use (Bedient et al., 2005).

The behavior of agrochemicals in shallow-water systems where crayfish are likely to be found is unique. The sediment-water interface is responsible for a series of exchanges of chemicals, nutrients, and oxygen which all impact a chemical's persistence and degradation. UV-light has been reported to penetrate the first 2 m of a waterbody and can degrade chemical present within shallow-water systems. BH absorption spectrum illustrates that it is capable of absorbing the energy of sunlight greater than 290 nm and therefore the potential for light-

activated degradation or toxicity may exist in a shallow rice field. Williams *et al.* previously reported a half-life of BH in high-purity water as 320 hr when exposed to natural sunligh and 12 hr in simulated sunlight with an intensity equivalent to fall equinox in filtered California rice water, the half-life was 80 hr in natural sunlight and 46 hr in simulated sunlight (Williams et al., 2018). Williams *et al.* also reported the potential for BH to dissipate into California rice field sediment based upon its measured soil adsorption coefficient. The solubility of BH in water at pH 7 was reported to be 146 mg/L, with a log $K_f$ (adsorption) of 1.15 and 1.27 (desorption) at 25°C. At 24°C, the $DT_{50}$ for BH in anaerobic soils was 326 days ($K_{deg} = 2.124 \times 10^{-3}$) and 89.7 days in aerobic soils ($K_{deg} = 7.732 \times 10^{-3}$). Williams was able to detect BH in soil mesocosms over 300 days post-application (Williams et al., 2017).

Previous analyses of toxicity and phototoxicity of herbicides and fungicides to crayfish indicated negative responses as a result of exposure. Exposure to the fungicide dicloran has been shown to negatively impact red swamp crayfish, and while dicloran is unlikely to enter the bayous of Louisiana due to its method of application to sweet potatoes (dip tank or waterfall post-harvest application within a recirculating system), the possibility of an emerging rice herbicide to cause negative impacts to crayfish raises concern (EPA, 2006). Previous aquatic ecotoxicological analysis has shown no acute effects on freshwater fish, including fathead minnows (*P. promelas*) and rainbow trout (*O. mykiss*) or estuarine/marine fish, sheepshead minnow (*C. variegatus*). Eastern oysters (*C. virginica*) and *Daphnia magna* were used as invertebrate test species and no acute effects from exposure was observed. Chronic exposure resulted in reduced numbers of offspring and stunted growth for freshwater and estuarine/marine species (USEPA, 2017).

The purpose of this investigation was to monitor the behavior of BH in different aqueous phases in the presence of sunlight, the behavior and dissipation of BZB and BH in the presence of sediment and/or sunlight. An additional purpose was to assess the potential toxicological impacts that exposure and degradation of BZB and BH may have on red swamp crayfish.

## 8.2. Materials and methods

Methods have previously been described in Chapters 3 and 7 but have been updated here to consider variations in the analysis benzobicyclon and benzobicyclon hydrolysate.

### 8.2.1. Chemical reagents and materials

Standards of benzobicyclon used to dose crayfish were obtained from LGC Standards (Middlesex, UK); standards of benzobicyclon and benzobicyclon hydrolysate used in photodegradation and irradiated water-sediment analyses were generous gifts from the Gowan Company, LLC (Yuma, AZ). Solvents included high performance liquid chromatography (HPLC) grade distilled water and acetonitrile (ACN) (VWR, Radnor, PA). Instant Ocean® (Blacksburg, VA) was mixed in distilled water to prepare artificial seawater (ASW) and salinity was monitored using a YSI Model 30 salinity, conductivity, and temperature meter (Yellow Springs, OH) to maintain 2.5% salinity for analysis.

### 8.2.2. Rice water and sediment collection

Rice water and sediment were collected from fields not treated with rice herbicides in 2017 or 2018 in Crowley, LA at Louisiana State University's H. Rouse Caffey Rice Research Station (Figure 8.2). Water pH was measured with a Mettler Toledo SevenCompact Benchtop pH meter (Columbus, OH) and stored in a refrigerator for no more than 30 days before use. Filtered and unfiltered rice waters were used in photodegradation analyses; filtered rice water was filter-sterilized with 0.22 μm filter (Fisher Scientific, Hampton NH).

Figure 8.2. Flooded rice fields where sediment and water were collected for photolysis and irradiated water-sediment experiments in Crowley, LA. Photo credit: Kevin Armbrust.

The sediment was characterized by a Series 1 Characterization by AgVise Laboratories (Table 8.1); it was stored in a -80°C freezer, ball-milled to a fine consistency, and dried in a Heratherm Oven (Fisher Scientific, Hampton, NH) at 105°C for a minimum of 24 hours.

Table 8.1. Characterization of rice field sediment collected in Crowley, LA. Sediment was analyzed by AgVise Laboratories according to the Series 1 Characterization.

| Sediment Characterization (Crowley, LA) | |
|---|---|
| % Sand | 16 |
| % Silt | 60 |
| % Clay | 24 |
| USDA Textural Class | Silt Loam |
| Bulk Density (gm/cc) | 1.10 |
| Cation Exchange Capacity (meq/100 g) | 15.6 |
| % Moisture at 1/3 Bar | 32.8 |
| % Organic Matter (Walkley-Black) | 2.1 |
| pH (water) | 6.5 |
| % Total Nitrogen | 0.119 |
| Olsen Phosphorus (ppm) | 13 |
| Cation %: Calcium | 59.4 |
| Cation %: Magnesium | 22.0 |
| Cation %: Sodium | 1.9 |

### 8.2.3. Analytical methods

Water and sediment samples were extracted by methods described in 8.2.4. and 8.2.5., and the extract was analyzed using an Agilent 1260 Infinity HPLC (Santa Clara, CA). The method included a gradient mobile phase comprised of water, 0.1% formic acid in water, and 0.1% formic acid in ACN using a ZORBAX C-8 Eclipse Plus Analytical 4.6 x 150 mm 5 μm column, monitoring 280 nm and 340 nm wavelengths with photodiode array detection, and a 20 μL injection volume.

### 8.2.4. Photodegradation of benzobicyclon hydrolysate

Benzobicyclon hydrolysate in assorted aqueous media was exposed to artificial sunlight to monitor photodegradation and calculate degradation rates and half-lives. Clear, 2 mL borosilicate glass vials (Agilent Technologies, Santa Clara, CA) containing 1 mL of 1.0 mg/L solutions of BH in distilled water, artificial seawater (2.5%), filter-sterilized rice field water, and unfiltered rice field water were irradiated in an ATLAS SUNTEST XXL+ environmental chamber fitted with a daylight filter with a light intensity of 40 $W/m^2$ for a total of 96 hours (Figure 8.3); amber vials were run simultaneously to monitor potential degradation in dark controls (96 hr). Photodegradation vials were completed in quintuplicate. Samples were removed from the chamber at selected time points and analyzed using an Agilent 1260 Infinity HPLC (Santa Clara, CA).


Figure 8.3. ATLAS XXL+ environmental chamber outfitted with a daylight filter used to simulate natural sunlight for photodegradation.

The UV absorbance spectrum of a solution of 0.01 mM BZB and BH in water was measured between 250 and 400 nm using a Varian Cary 50 UV−vis spectrophotometer (Agilent Technologies, Santa Clara, CA) (Figure 8.4). BZB and BH have measured absorbances exceeding 290 nm and therefore as capable of absorbing the energy of sunlight reaching the surface of the earth, and also capable of being degraded by direct photolysis processes.

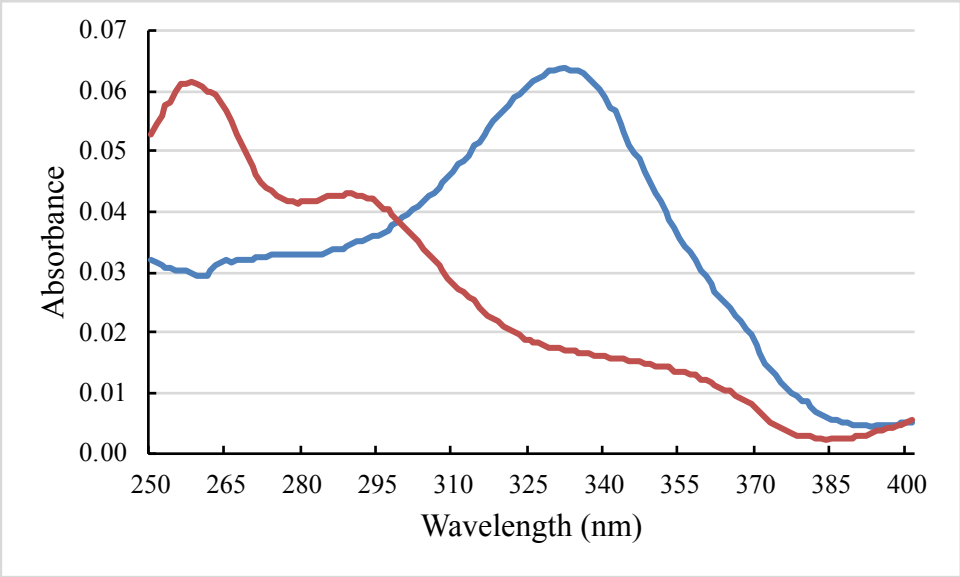

Figure 8.4. The UV absorption spectra of BZB (blue) and BH (red); 250-400 nm.

### 8.2.5. Irradiated water-sediment experimental design

Savillex Teflon jars (240 mL standard jar; Savillex Corporation, Eden Prairie, MN) containing 25 g (dry weight) of characterized (AgVise Laboratories, Northwood, ND), dried, and ball-milled rice field sediment from Crowley, LA and 150 mL of distilled water or 2.5% artificial seawater were placed in a refrigerator for a minimum of 24 hours to allow sediment to settle and prevent turbidity from impacting results. Sediment measured ~1 cm and water measured ~5 cm in depth in each jar. The jars were dosed with 1 mg/L benzobicyclon or benzobicyclon hydrolysate (Figure 8.5) and immediately placed in the SUNTEST environmental chamber outfitted with a recirculating water system (Fisher Scientific, Hampton, NH) for temperature control (72 hr) or in a dark 40°C incubator (96 hr). Irradiated water-sediment mesocosms for BH in seawater were sampled over a 24 hr period due to the short half-life of BH in seawater.

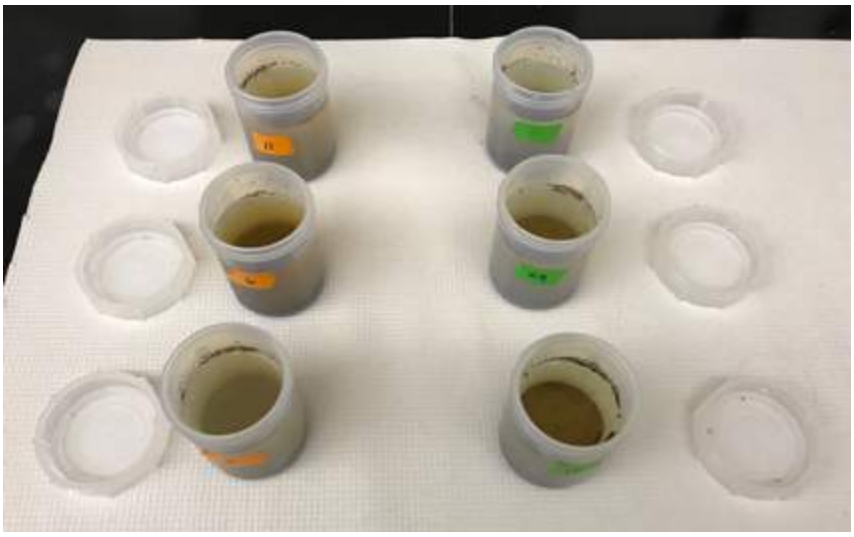

Figure 8.5. Teflon jars containing 150 mL water and 25 g rice sediment for irradiated water-sediment analyses. Photo credit: Jessica Landry.

Water samples were filtered through a 0.45 μm filter (Fisher Scientific, Hampton NH) and analyzed directly by HPLC. To measure BZB and BH in sediment, the sediment (25 g) from the jars was transferred to a sterile polypropylene centrifuge tube (Fisher Scientific, Hampton, NH) and 25 mL of a 70:30 mixture of ACN and 0.1% formic acid in ACN was added to each tube. The tubes were shaken on a Pilot-Shake® rack shaker (Kühner AG; Birsfelden, Switzerland) for 2 hours, centrifuged, and the extract was decanted into a clean beaker; a second 25 mL volume of the 70:30 solution was added to the original tube and shaken for 2 more hours and centrifuged. The final extract was decanted, and 1 mL of the total solution was filtered through a 0.45 μm syringe filter, transferred to a vial, and analyzed by HPLC following the same method previously described in 8.2.3.

To compare the impact of sediment on photodegradation and the effect of partitioning for persistence and degradation of BZB and BH, 150 mL of distilled water or 2.5% seawater was added to the Savillex jars without sediment. The jars were placed in the ATLAS chamber or in the incubator following the same methods as previously described for the irradiated water-

sediment analysis and analyzed by HPLC. All water-sediment experiments were completed in triplicate, with individual samples analyzed in duplicate.

### 8.2.6. Phototoxicity experimental design

Dr. Greg Lutz along with his undergraduate and graduate students spawned red swamp crayfish (*P. clarkii*) at Louisiana State University's Aquaculture Research Station (ARS). One male and one female were paired in a 20-gallon fish tank for roughly 3 weeks before the female was removed, placed in a dark, mud-filled container, and remained until her eggs hatched and the juveniles were released from the carapace. The juvenile crayfish were released from the female carapace and were transferred (by "family") to separate 20-gallon fish tanks until they grew to 3-6 cm in length for experimental analysis.

Juvenile crayfish, measuring between 3-6 cm and aged roughly 9 months were put in individual 250 mL glass jars (Fisher Scientific, Hampton, NH) with an air stone; 150 mL of filtered well water (Baton Rouge, LA) was added to each jar (Figure 8.6). The jars were dosed with various concentrations of a 10,000-mg/L benzobicyclon in ACN stock solution ranging from 0.10-2.0 mg/L and untreated "control" crayfish were monitored simultaneously. The highest concentration of ACN (based off of BZB dosage) was used in controls to ensure the solvent did not cause negative effects on the crayfish. Both light and dark trials were completed, and crayfish were exposed to chemicals in solution for a total of 48 hours. A series of UVA 340 fluorescent UV bulbs (Q-Lab Corporation, Westlake, OH) and 40-Watt Linear Fluorescent Lamps (Grainger®, Lake Forest, IL) were housed in 48-inch shop light fixtures (Grainger®, Lake Forest, IL) and used to simulate the output of natural sunlight for phototoxicity experiments. The output of light was monitored using a Black Comet Concave Grating UV-VIS spectrometer (Stellar Net, Inc., Tampa, FL).


Figure 8.6. Experimental setup for phototoxicity experiments.

The top two shelves were blacked out for dark controls and the bottom two shelves are fitted with a series of lights that mimic natural sunlight, equipped with air-stones for air supply to crayfish. Each crayfish was placed in an individual 250-mL glass jar to rule out fighting, injury, and cannibalism as a possible reason for mortality.

**8.2.6.1. Quantitative PCR analysis**

Surviving crayfish were preserved in TRIzol® solution (Fisher Scientific, Hampton, NH) for qPCR gene expression analysis. The shells were removed, and tail tissue and carapace tissue were homogenized in a 1.5 mL centrifuge tube (Fisher Scientific, Hampton, NH) with 1 mL TRIzol® and 800 μm glass beads (VWR, Radnor, PA) for 10 min using a Vortex-Genie 2 (Scientific Industries, Inc., Bohemia, NY). After homogenization, 200 μL of chloroform (VWR, Radnor, PA) was added to each tube, shaken and left to sit at room temperature for 5 min, then centrifuged for 20 min. The supernatant (400 μL) was removed and added to an Eppendorf tube

(Fisher Scientific, Hampton, NH) with 400 μL of isopropanol (VWR, Radnor, PA) and placed in a -20°C freezer for a minimum of 30 min (Xu et al., 2018).

After 20 min of centrifuging, the supernatant was discarded and 1 mL of 70% ethanol (VWR, Radnor, PA) in RNase free water (Fisher Scientific, Hampton, NH) was added and centrifuged. The supernatant was removed and 25 μL of a mixture containing RNase free water, 10x DNase buffer, and TURBO DNase (Invitrogen, Carlsbad, CA) were aliquoted to each tube and incubated in a 37°C water bath. DNase Inactivation Reagent (Invitrogen, Carlsbad, CA) was added and centrifuged; the supernatant was transferred to a new Eppendorf tube in a -80°C freezer until cDNA synthesis (Xu et al., 2018).

The concentration, 260/230, and 280/260 values were measured using a GeneQuant Pro (Amersham BioSciences, Little Chalfont, UK). RNAs were transferred to PCR strips (VWR, Radnor, PA) with deoxynucleotide mix [10 mM] (dTNP; G-Biosciences, St. Louis, MO), RNase free water, and oligo DT (Integrated DNA Technologies, Coralville, IA). The cDNA was synthesized in a T100™ Thermal Cycler (Bio-Rad, Hercules, CA) and set to follow protocol. DTT, SSIV reverse transcriptase, and 5x SSIV buffer (Invitrogen, Carlsbad, CA) were added after ~10 min into the sequence; cDNA was diluted with RNase free water once the sequence finished and stored at -20°C until qPCR analysis (Xu et al., 2018).

Genes were chosen based off of previously published literature, and primers for those genes were obtained from Integrated DNA Technologies (IDT, Coralville, IA). Gene functions including immune function (Chen et al., 2013; Jiang et al., 2015; Li et al., 2010; Liu et al., 2017; Meng et al., 2013; Wang et al., 2011; Yi et al., 2017; Zhang et al., 2011; Zhang et al., 2013). Five individual cDNAs for each treatment were combined with primer pairs, DNase Free water, dNTP, ROX reference dye, Sybr Green 10x (Fisher Scientific, Hampton, NH), 5x GoTaq

polymerase colorless reaction buffer, and GoTaq polymerase (Promega, Madison, WI). Samples were prepared for and analyzed in 384-well plates using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster, CA) using sds2.4 software and analyzed in Prism 7.0 (GraphPad Software, Inc., La Jolla, CA) to calculate relative gene expression of mRNA (Xu et al., 2018).

## 8.3. Results and discussion

### 8.3.1. Photodegradation of benzobicyclon hydrolysate

Benzobicyclon hydrolysate was aliquoted into 1 mL samples in four media including distilled water, 2.5% artificial seawater, filter-sterilized rice field water (pH 7.8), and unfiltered rice field water (pH 7.9) and exposed to artificial sunlight to monitor photodegradation and calculate the photodegradation rates and half-lives in each media (Table 8.2). No degradation was observed in dark controls, which remained in the chamber for the maximum period of time (96 hours) for each media (Figure 8.7).

Table 8.2. The remaining concentrations after 96 hours of light exposure and half-lives of benzobicyclon hydrolysate in four aqueous phases (n=5). One-way ANOVA was used to determine differences between media. No significant differences were observed were rice waters, but all remaining media were significantly different.

| | DI water | 2.5% seawater | Filtered Rice Water | Unfiltered Rice Water |
|---|---|---|---|---|
| Half-life (hours) | 76.7 ± 2.878 | 3.4 ± 0.1966 | 38.7 ± 1.561 | 34.1 ± 3.282 |
| Remaining Concentration | 0.41 mg/L | 0 mg/L * | 0.18 mg/L | 0.14 mg/L |
| * All benzobicyclon hydrolysate was degraded after 24 hours light exposure. | | | | |

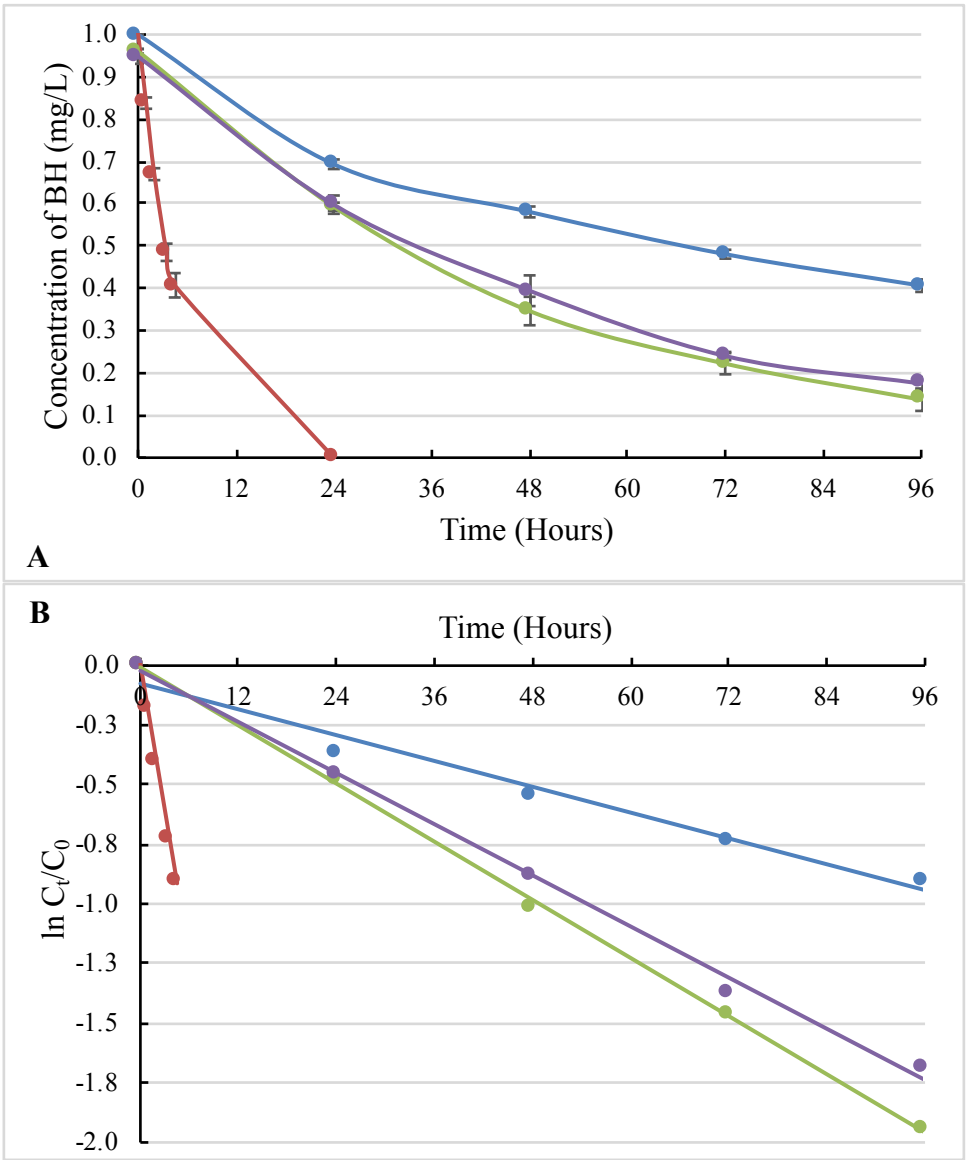


Figure 8.7. The photodegradation (A) and degradation rate (B) of BH in distilled water (blue), 2.5% seawater (red), unfiltered rice field water (green), and filtered rice field water (purple); error bars indicate standard deviation (n=5).

A one-way ANOVA ($\alpha = 0.05$) with a Tukey's multiple comparison test determined statistical differences between distilled water, unfiltered rice field water, filtered rice field water, and seawater. BH in seawater was fully degraded and not detected by HPLC after 24 hours of light exposure. P-values in each comparison were <0.0001, with the exception of filtered vs unfiltered rice water which produced a p-value of 0.0310; significant differences between media were apparent. No significant degradation was observed in dark controls; [average initial

concentration, t = 0] ± 0.02 mg/L of the average calculated concentration at time 0 hours for all media.

Williams *et al* observed similar half-lives for the photodegradation of BH in purified water and rice water. Previously reported half-lives of BH in filtered California rice was 46 hours and 170 hours in high-purity water in simulated sunlight. In filtered Louisiana rice water, the half-life was 38 hours and 77 hours in distilled water. Therefore, similar observations were observed by Williams *et al* and in this dissertation. The increased degradation rate of BH in seawater may be a result of indirect photolysis or influences of ions in the seawater.

### 8.3.2. Irradiated water-sediment: benzobicyclon and benzobicyclon hydrolysate

Water-sediment systems were dosed with 1 mg/L benzobicyclon or benzobicyclon hydrolysate for both distilled water and artificial seawater for light and dark trials (n = 3 for each treatment). The concentrations and masses of BZB in freshwater and seawater in dark and light trials (Table 8.3); table 8.3. includes the hydrolysis half-life of BZB to BH and does not include the final concentration of BZB in water, due to 0 mg/L remaining after <48 hours after dosage.

Table 8.3. The half-life, concentration, and mass of benzobicyclon remaining after a maximum of 96 hours of degradation or dissipation between distilled water and seawater in water-sediment systems (n=3).

| | Distilled water and sediment | | 2.5% Seawater and sediment | |
|---|---|---|---|---|
| | Dark[1] | Light[2] | Dark[1] | Light[2] |
| Concentration water (mg/L) | 0.0 ± 0.000 | 0.0 ± 0.000 | 0.0 ± 0.000 | 0.0 ± 0.000 |
| Concentration (BH) water (mg/L)* | 0.69 ± 0.011 | 0.30 ± 0.045 | 0.74 ± 0.035 | 0.32 ± 0.009 |
| Half-life (hours)** | 6.71 ± 0.325 | 10.3 ± 1.833 | 5.79 ± 1.779 | 10.0 ± 0.247 |
| Concentration (BZB) sediment (mg/kg) | 0.16 ± 0.009 | 0.67 ± 0.0313 | 0.32 ± 0.001 | 0.35 ± 0.020 |
| Mass in water (BZB) (µg) | 0.0 ± 0.000 | 0.0 ± 0.000 | 0.0 ± 0.000 | 0.0 ± 0.000 |
| Mass in sediment (BZB) (µg) | 3.89 ± 0.219 | 16.8 ± 0.784 | 7.88 ± 0.029 | 16.5 ± 2.632 |
| Mass in water (BH)* (µg) | 104.1 ± 1.699 | 44.5 ± 6.764 | 110.3 ± 5.221 | 47.4 ± 1.333 |
| * BZB hydrolyzes to BH, ** hydrolysis half-life (BZB), [1]96 hours, [2]72 hours | | | | |

The concentrations and masses of BH in freshwater and seawater in dark and light trials (Table 8.4) showed the differing behaviors of both chemicals under varying simulated environmental scenarios.

Table 8.4. The half-life, concentration, and mass of benzobicyclon hydrolysate remaining after [photo]degradation or dissipation between distilled water and seawater in water-sediment systems and sediment-free systems (n=3).

| | Distilled water and sediment | | 2.5% Seawater and sediment | |
|---|---|---|---|---|
| | Dark | Light | Dark | Light* |
| Concentration water (mg/L) | 1.07 ± 0.033 | 0.31 ± 0.009 | 1.17 ± 0.030 | 0.43 ± 0.032 |
| Half-life (hours) | n/a | 36.5 ± 1.463 | n/a | 25.3 ± 0.885 |
| Concentration sediment (mg/kg) | 0.19 ± 0.008 | 0.26 ± 0.025 | 0.26 ± 0.020 | 0.09 ± 0.005 |
| Mass in water (μg) | 149.7 ± 5.170 | 46.9 ± 1.301 | 156.6 ± 2.232 | 64.9 ± 4.828 |
| Mass in sediment (μg) | 9.68 ± 0.397 | 13.2 ± 1.241 | 12.8 ± 1.017 | 4.43 ± 0.260 |

Both freshwater and seawater dark trials were sampled every 24 hours for 96 hours (Figure 8.8), while freshwater and seawater light trials were sampled every 24 hours for 72 hours (Figure 8.9). Irradiated seawater jars were also sampled at 1, 2, and 5 hours to monitor the dissipation, hydrolysis, and photolysis more closely due to the previously observed rapid photodegradation of BH in seawater. Water recoveries were >90%, and sediment recoveries were 86% for benzobicyclon. Irradiated samples were not sampled at 96 hours, due to low quantified measurements of BZB in water at the 72-hour sampling interval.

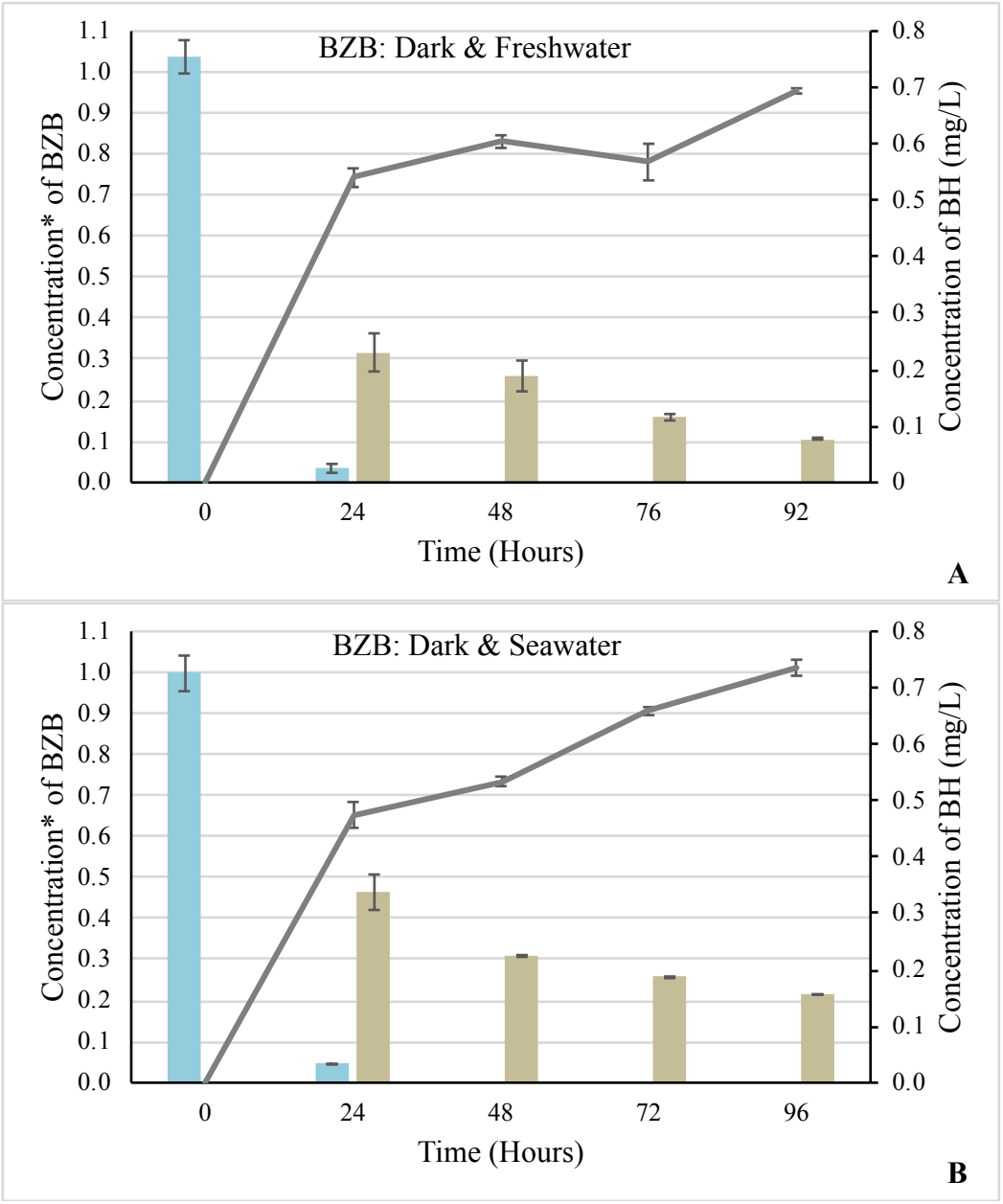


Figure 8.8. The hydrolysis of benzobicyclon in water (blue) to benzobicyclon hydrolysate (gray) and dissipation of benzobicyclon into sediment (brown) over 96 hours without sunlight in freshwater (A) and seawater (B). Concentration (*) of BZB in water was measured in mg/L and mg/kg in sediment; error bars indicate standard error (n=3).

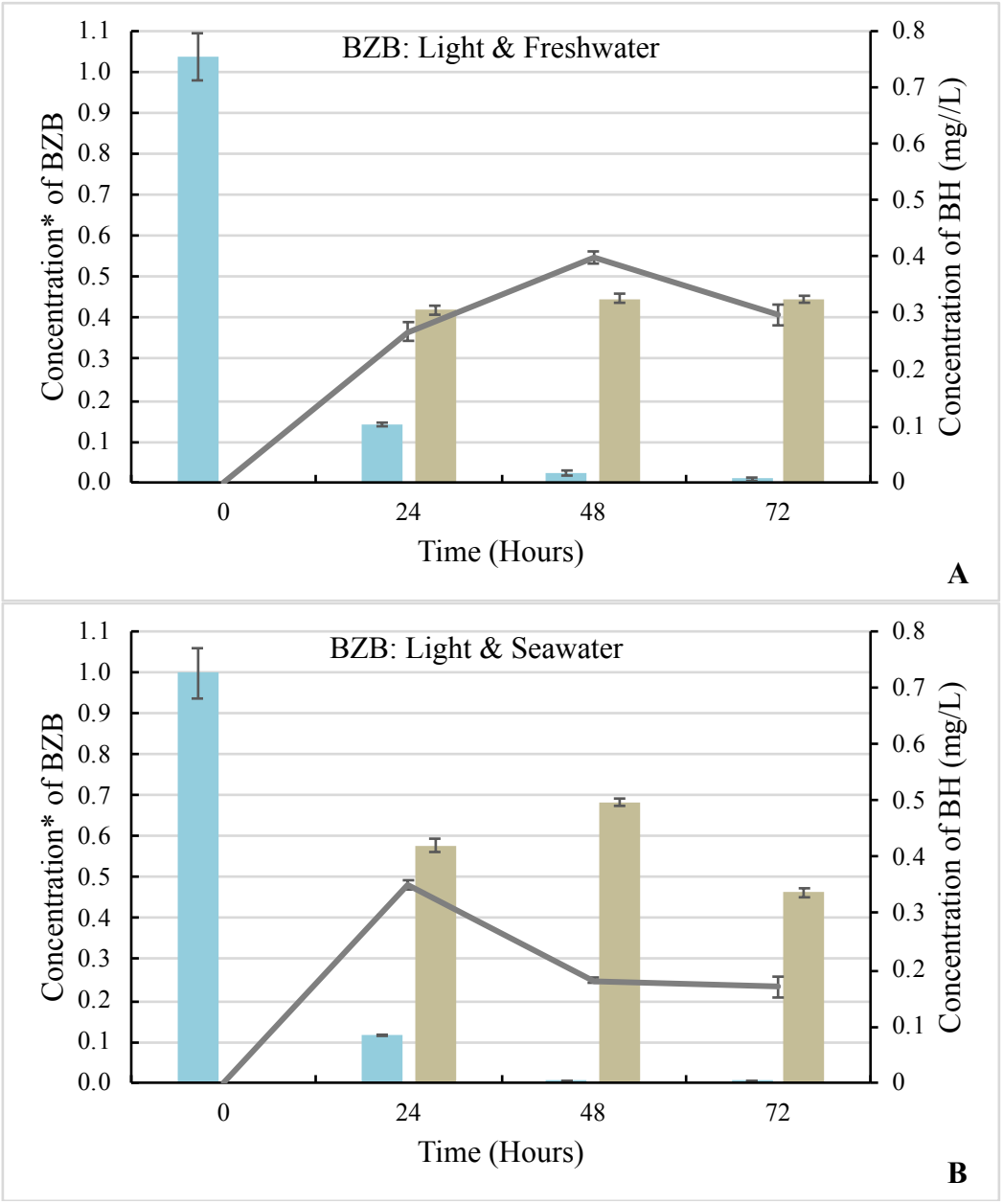


Figure 8.9. The hydrolysis of benzobicyclon in water (blue) to benzobicyclon hydrolysate (gray) and dissipation of benzobicyclon into sediment (brown) over 72 hours in artificial sunlight in freshwater (A) and seawater (B). Concentration (*) of BZB in water was measured in mg/L and mg/kg in sediment; error bars indicate standard error (n=3).

As the photodegradation of benzobicyclon hydrolysate resulted in a half-life of 3.4 hours and the estimated hydrolysis of benzobicyclon was 9.2 hours in seawater, samples were collected more frequently during the first 24 hours in the irradiated water-sediment analysis of BZB. Water and sediment samples (n = 3) were analyzed after 0, 1, 2, 5, and 24 hours of light

exposure; the dissipation of BZB into the sediment, hydrolysis of BZB to BH, and possible photodegradation of BH are shown in Figure 8.10.

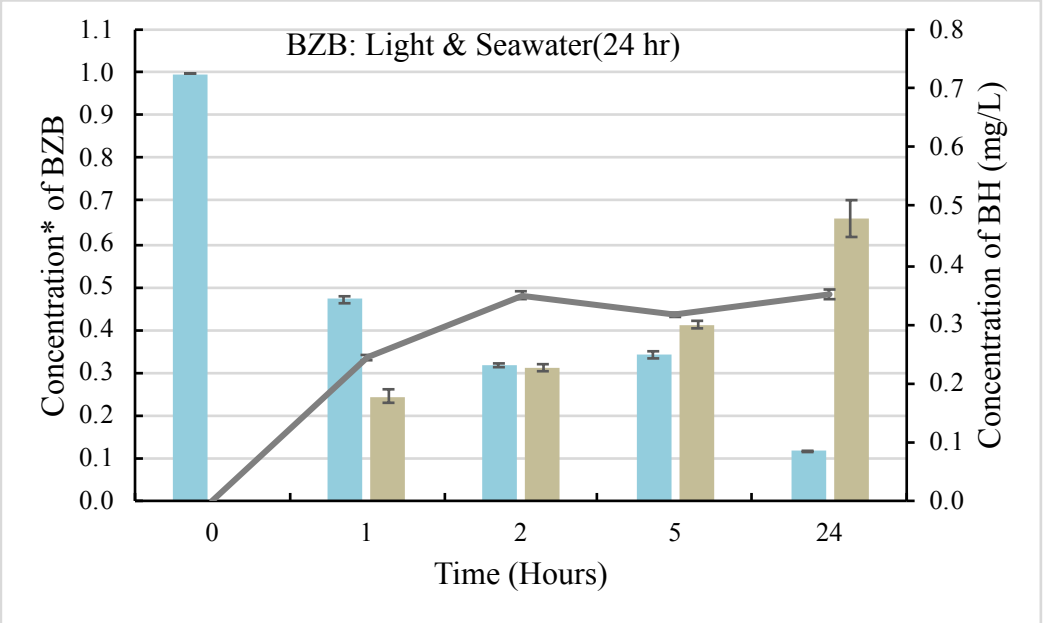


Figure 8.10. The hydrolysis and dissipation of benzobicyclon in water (blue) and sediment (brown), and the formation of benzobicyclon hydrolysate (gray) in seawater and artificial sunlight. Concentration of BZB (*) in water is measured in mg/L and in sediment mg/kg; error bars indicate standard error (n=3).

The hydrolysis of BZB in freshwater and seawater was monitored in artificial sunlight and sediment (Figure 8.11), dark and sediment (Figure 8.12), and dark without sediment (Figure 8.13) for 24 hours, with samples removed at 0, 1, 2, 5, 12, and 24 hours.

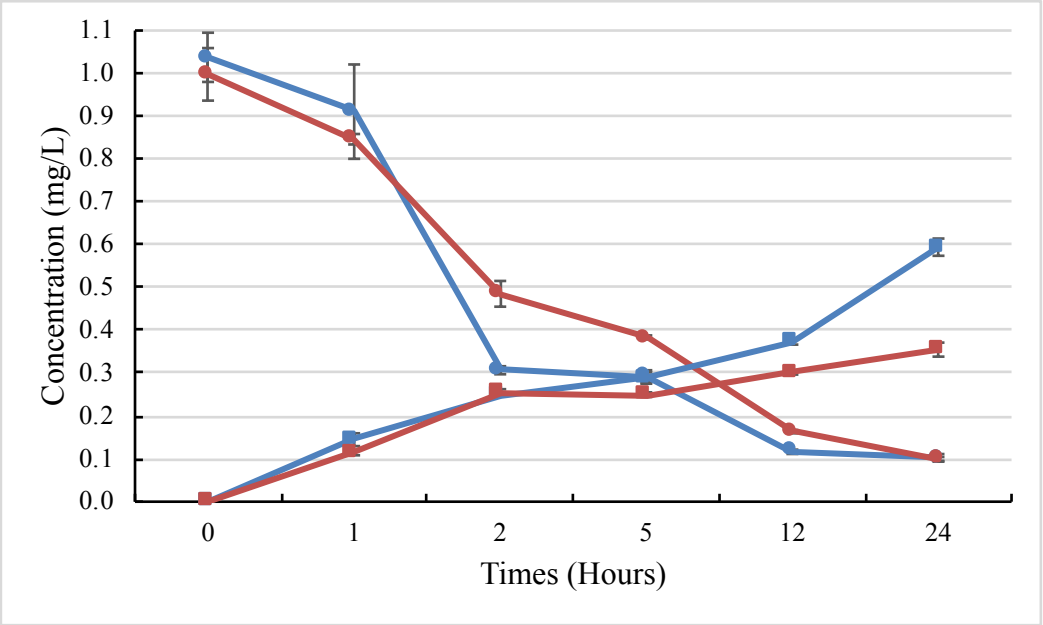


Figure 8.11. The hydrolysis of benzobicyclon (●) and formation of benzobicyclon hydrolysate (■) in freshwater (blue) and seawater (red) in the presence of artificial sunlight and sediment; error bars indicate standard error (n=3).

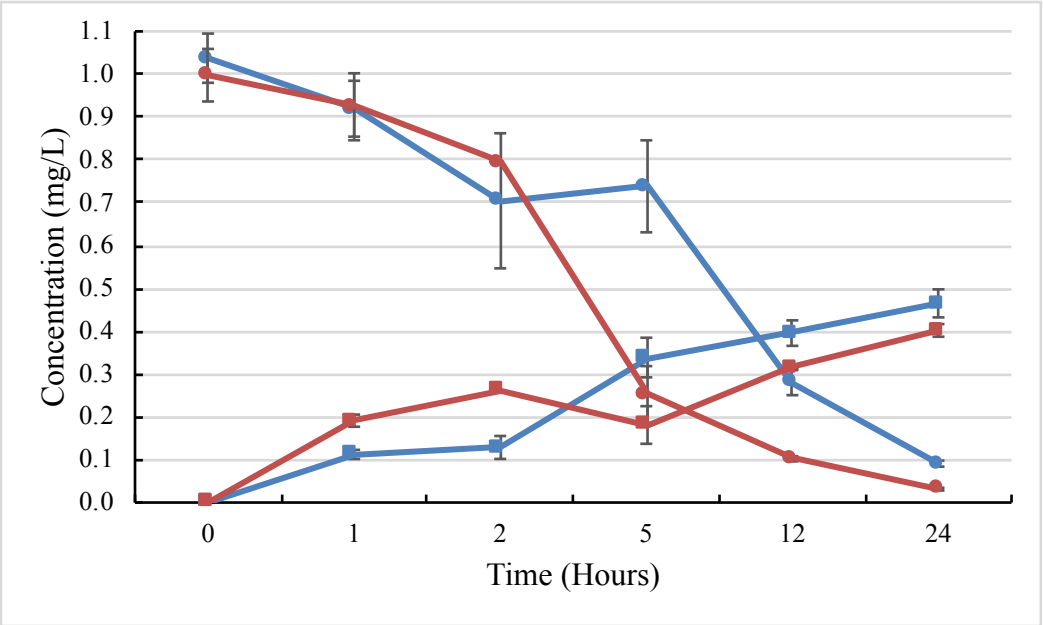


Figure 8.12. The hydrolysis of benzobicyclon (●) and formation of benzobicyclon hydrolysate (■) in freshwater (blue) and seawater (red) with sediment and without artificial sunlight; error bars indicate standard error (n=3).

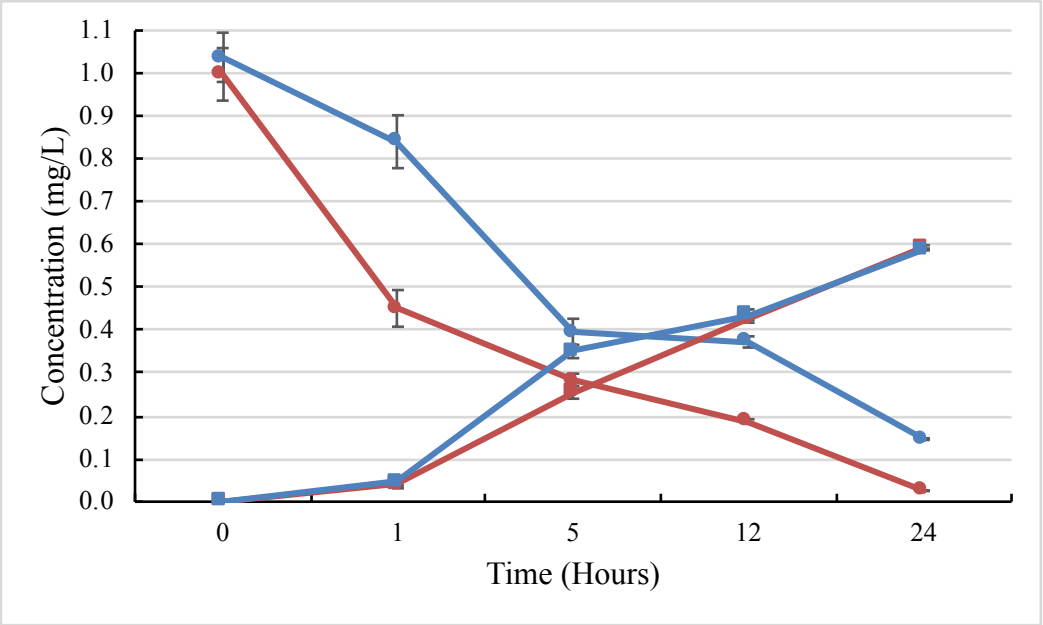


Figure 8.13. The hydrolysis of benzobicyclon (●) and formation of benzobicyclon hydrolysate (■) in freshwater (blue) and seawater (red) without artificial sunlight or sediment; error bars indicate standard error (n=3).

The photodegradation of benzobicyclon hydrolysate in both freshwater and seawater was monitored in methods mimicking those previously described for BZB in both dark (Figure 8.14), freshwater light (Figure 8.15), and seawater light (Figure 8.16). Dark trials were sampled every 24 hours for 96 hours; light trials for freshwater were sampled over 72 hours, while seawater was sampled for 24 hours due to its rapid photodegradation. Water recoveries for BH were >90%, while sediment recoveries were <20%; although, BH does not dissipate into sediment in large concentrations in comparison to BZB, BH seems to prefer the aqueous phase.

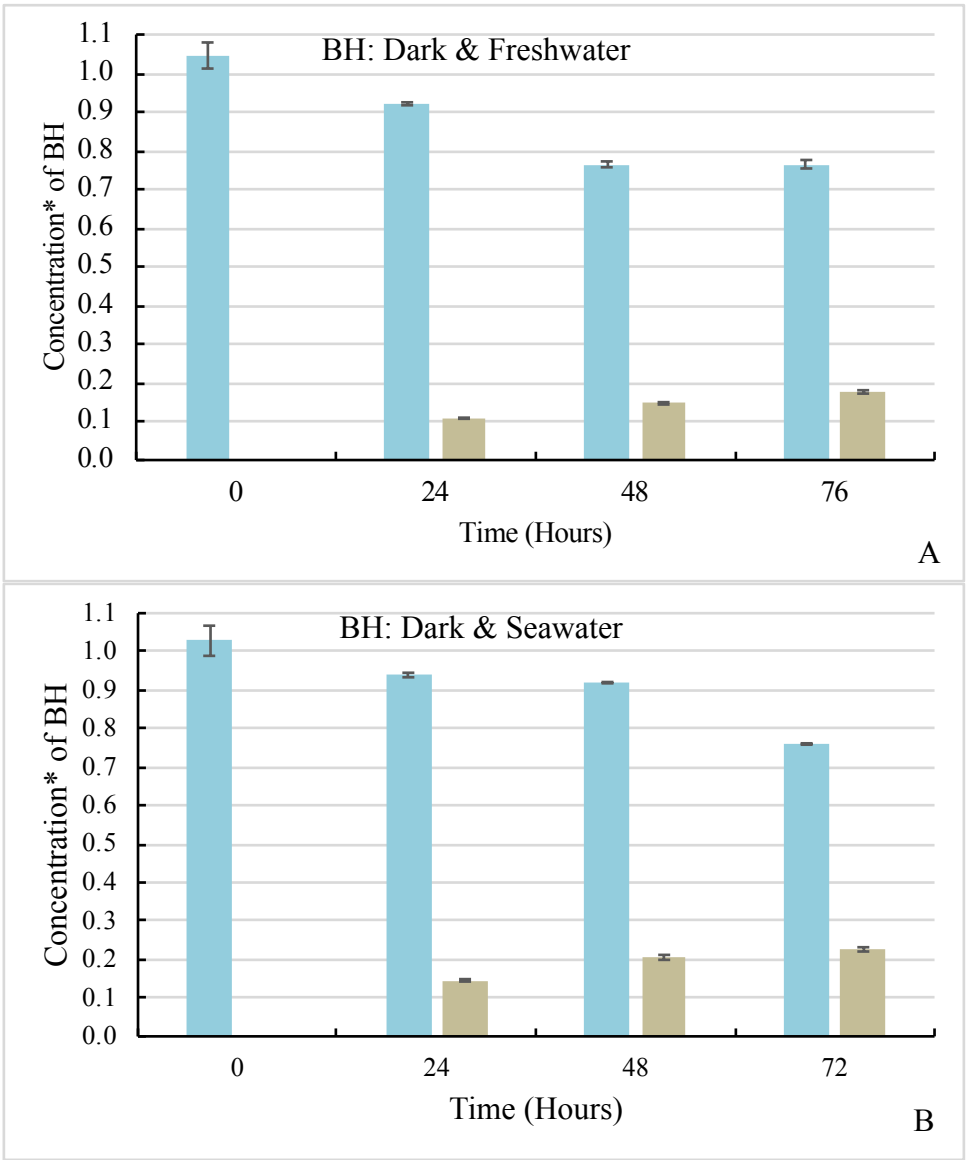


Figure 8.14. The dissipation of benzobicyclon hydrolysate into sediment (brown) from water (blue) in freshwater (A) and seawater (B) without artificial sunlight; asterisk (*) indicates water concentration measured in mg/L and sediment in mg/kg. Error bars indicate standard error (n=3).

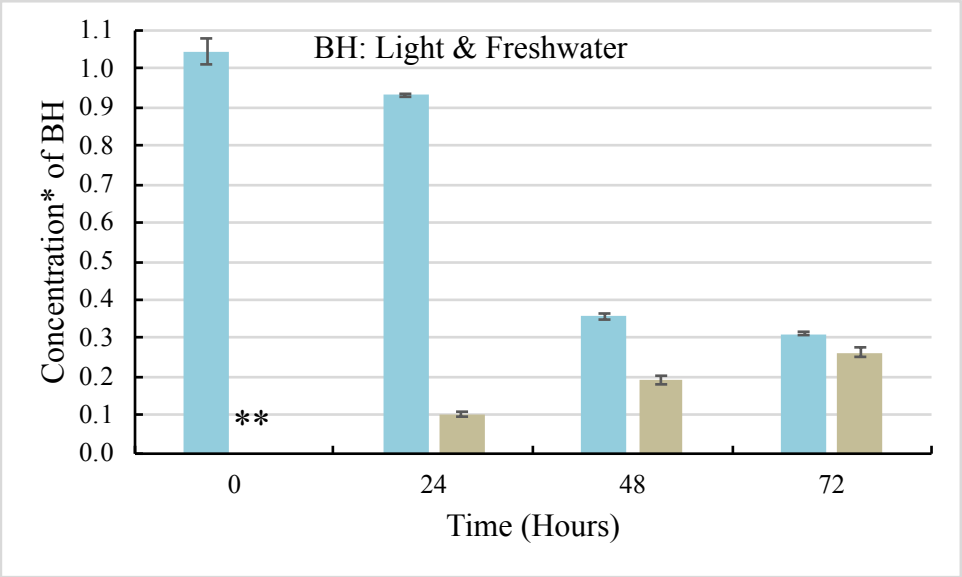


Figure 8.15. The photodegradation and dissipation of benzobicyclon hydrolysate in freshwater (blue) and sediment (brown). Samples were measured over 72-hours; (*) water was measured in mg/L and sediment in mg/kg. No BH was measured in sediments at time 0 hours (**) (n=3).

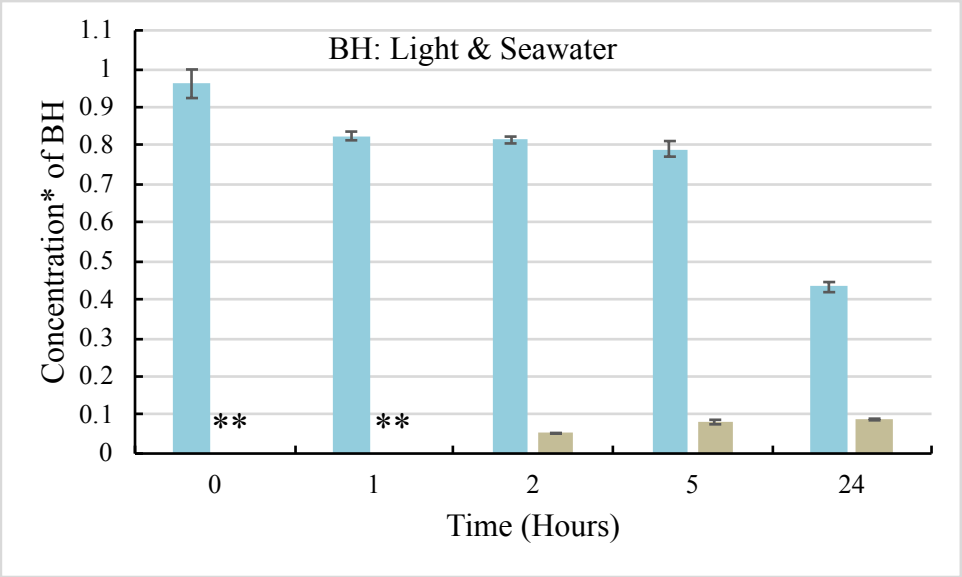


Figure 8.16. The photodegradation and dissipation of benzobicyclon hydrolysate in seawater (blue) and sediment (brown). Samples were measured over 24-hours, due to faster photodegradation of BH in seawater; (*) water was measured in mg/L and sediment in mg/kg. No BH was measured in sediments at time 0 or 1 hours (**) (n=3).

### 8.3.3. Phototoxicity of benzobicyclon to juvenile red swamp crayfish

Crayfish are an epibenthic macroinvertebrate that spend most of their life burrowed in the sediment or foraging at the sediment-water interface, which is a unique environment especially

related to chemical behaviors. The shallow-water systems used in these analyses were designed to approximate processes that can be observed at the sediment-water interface and the behavior of the chemical that may be observed in the field in this region and that may directly impact crayfish.

Juvenile red swamp crayfish were exposed to various concentrations of benzobicyclon with and without artificial sunlight for 48 hours. No mortality was observed in any treatment (n = 10, individuals) or were any obvious behavioral changes (i.e. activity, responsive to disturbances, etc.).

Benzobicyclon is applied at rates of 0.27 lb ai/ha therefore, relevant potential environmentally relevant concentrations, as well as elevated concentrations were monitored (0.05, 0.10, 0.25, 0.50, and 1.0 mg/L). No negative impacts with the endpoint as death were observed. Juvenile crayfish are a more sensitive population, as opposed to adults, to investigate chemical impacts as their carapace has not fully hardened or developed the red coloration making them more susceptible to chemical uptake as well as potential UV penetration within their bodies. Crayfish molt 7-11 times within their first year and impacts to juvenile populations can be detrimental to populations as a whole, and molting exposes softer tissues therefore the risk of exposure increases (McClain et al., 2007).

#### 8.3.3.1. Sub-lethal impacts of benzobicyclon exposure to red swamp crayfish

Surviving crayfish were preserved in TRIzol® Reagent for qPCR analysis of relative mRNA gene expressions. Seven genes (Table 8.5) related to immune function were chosen to monitor the relative gene expression for each treatment (Chen et al., 2013; Jiang et al., 2015; Li et al., 2010; Liu et al., 2017; Meng et al., 2013; Wang et al., 2011; Yi et al., 2017; Zhang et al., 2011; Zhang et al., 2013).

Table 8.5. Genes used for expression analysis.

| Function | Gene | Identity | Sequence |
|---|---|---|---|
| Reference | GAPDH | GAPDH | AGGCTGTCGGAAAGGTTATTC<br>CCAGCCTTAGCGTCAAAGAT |
| Reference | ACTB | β-Actin | TGCGACTCTGGTGATGGTGT<br>AGCGGTGGTGGTGAAGGAAT |
| Immune function | LGBP | lipopolysaccharide and beta-1,3-glucan binding protein | TCGAGAACTTCGCCTTCAAATA<br>GTGGGTCCTCTCATACAAGTTAG |
| Immune function | Lec4 | C-type Lectin | GGTGTGGAAGTGGGTCAATG<br>AGTAGTTATGGTCGCTCGTGAT |
| Immune function | SPINK1 | Kazal-type serine proteinase inhibitor 1 | CATTCGCCTTAATCGCCTTCT<br>GACATTTCCCTTCATAATCCACC |
| Immune function | Ast | Asticidin | ATGCGTCTTCTCCATCTCC<br>TTACTTGCCTGGACGGTA |
| Immune function | Fer | Ferritin | GAGTCAAGTGATGAAGAGCG<br>ATGAAGAGTACCCCATTCTTG |

GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was chosen as the reference gene for the gene expression analysis and β-actin was the secondary reference gene. The relative gene expression (mRNA) for asticidin (Figure 8.17), C-type lectin (Figure 8.18), Ferritin (Figure 8.19), and Kazal-type serine proteinase inhibitor 1 (Figure 8.20) were analyzed for statistical differences using a one-way ANOVA ($\alpha = 0.05$) with Tukey's analysis of variance.

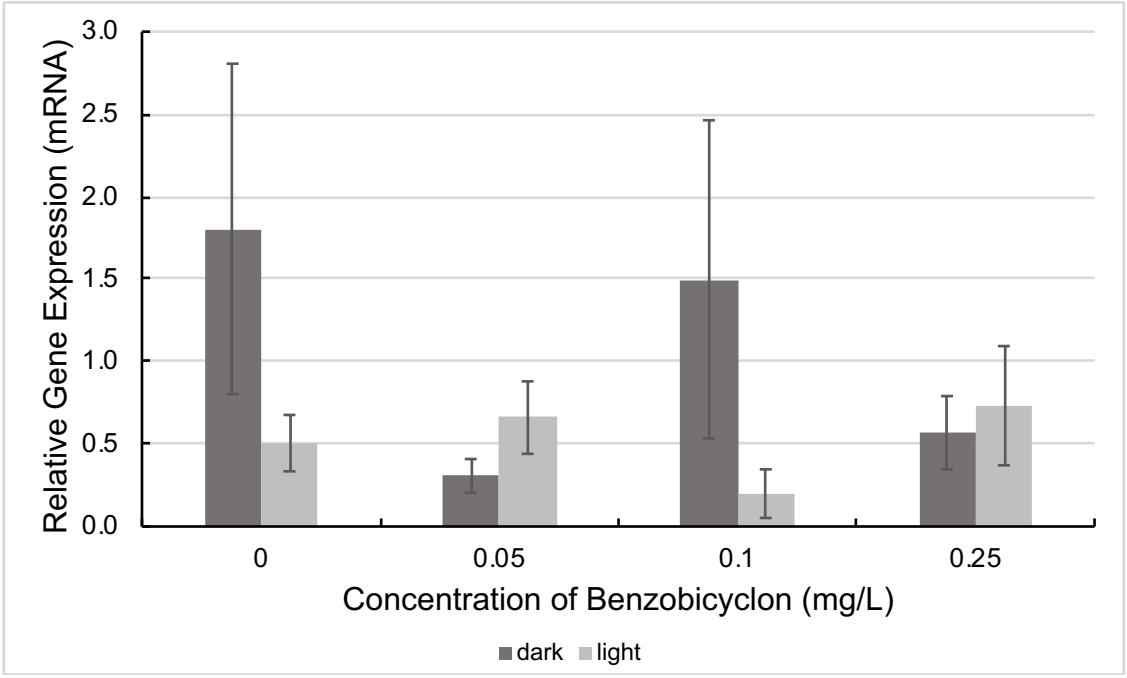

Figure 8.17. Relative gene expression of asticidin.

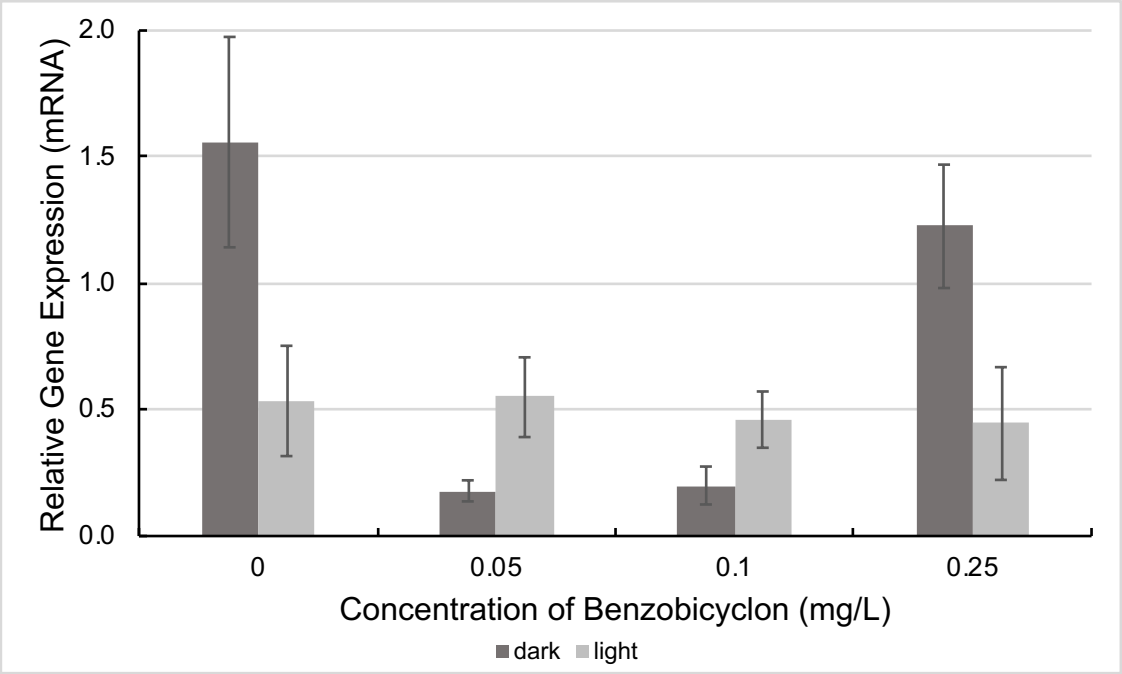

Figure 8.18. Relative gene expression of C-type lectin.

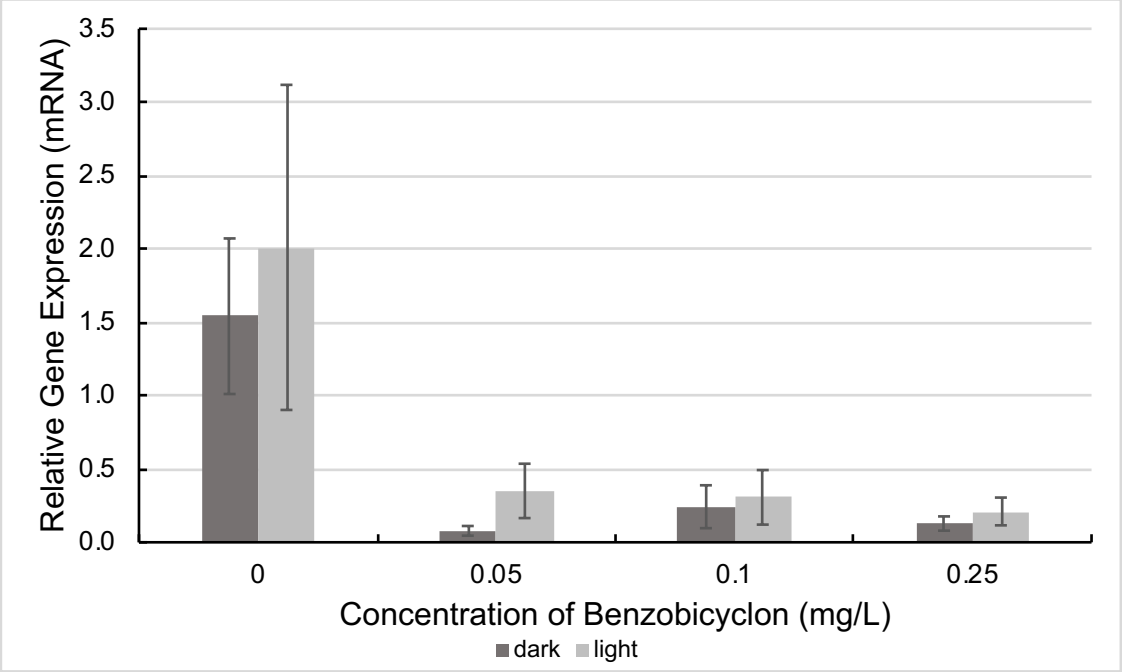

Figure 8.19. Relative gene expression of ferritin.

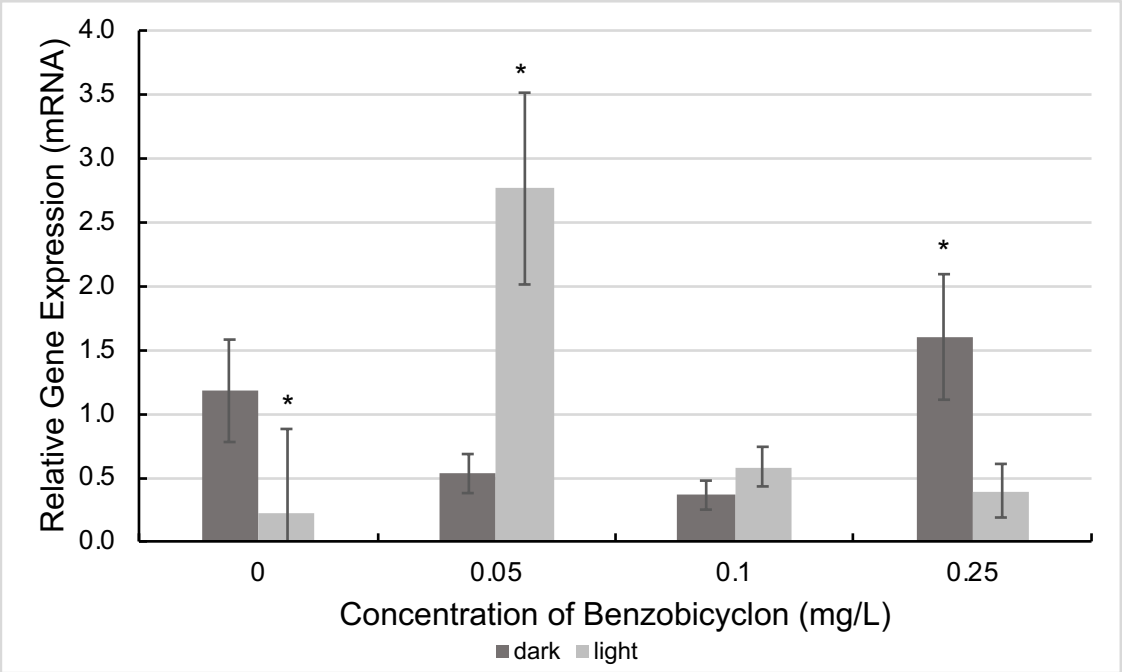

Figure 8.20. Relative gene expression of Kazal 1; asterisks (*) indicate significant differences.

Significant differences were observed for Kazal 1 for light controls, 0.05 mg/L light and 0.25 mg/L dark samples with *p*-values 0.0230, <0.0001, and 0.0126 respectively compared to dark controls. Differences were also calculated between light control and 0.05 mg/L light (*p*-value <0.0001), 0.05 mg/L dark and light (*p*-value <0.0001), 0.05 mg/L and 0.50 mg/L dark (*p*-value 0.0017), 0.05 mg/L and 0.10 mg/L light (*p*-value <0.0001), 0.05 mg/L and 0.50 mg/L light (*p*-value <0.0001), 0.10 mg/L and 0.50 mg/L dark (*p*-value 0.0018), and 0.50 mg/L dark and light (*p*-value 0.0010).

## 8.4. Implications and conclusions

The chemical behavior of benzobicyclon and benzobicyclon hydrolysate are impacted by salinity, presence of sediment, and sunlight. The risk for rice herbicides (or any rice-approved pesticide) to enter brackish waters is limited, south Louisiana is coastal, and many rice fields are located in the region. The Atchafalaya Basin is a large rice-growing region, and the Atchafalaya River drains into the Gulf of Mexico. Therefore, when flooded rice paddies are drained after the

season, remaining BZB or BH in any of the waters has the potential to be transported into coastal waters. Salinity has previously been shown to enhance toxicity, but it has also shown to increase the photodegradation rate of BH to be fully degraded in a sediment-absent system after roughly 24 hours of light exposure.

BZB favors sediment and it appears to partition into the water column or sediment porewater where it hydrolyzes to BH; as shown in this dissertation, after 24 hours, BZB began to dissipate into sediment and simultaneously, the compound that remained in the water column hydrolyzed. As time went on, the concentration of BZB in sediment decreased while BH in the water column increased. BH was non-quantifiable in BZB water-sediment systems, however when the water-sediment systems were dosed with a standard of BH, low levels of BH were detected in sediments <0.10 mg/kg with the bulk mass of the application remaining in the water column.

While crayfish are not a standard toxicity testing species, they are a good indicator species with ecological importance in regions where they have economic value. Previous studies have used crayfish in toxicological analyses. Barbee and Stout compared pesticide impacts on red swamp crayfish during rice crop rotations. Acute toxicity (96 hr) of five [neonicotinoid and pyrethroid] insecticides to juvenile red swamp crayfish were observed with calculated $LC_{50}$ values of 59 μg/L for clothianidin, 0.16 μg/L for λ-cyhalothrin, 2032 μg/L for dinotefuran, 0.29 μg/L for etofenprox, and 967 μg/L for thiamethoxam. Sublethal observations of increased aggressiveness in crayfish exposed to thiamethoxam and lethargy when exposed to λ-cyhalothrin were also reported. Therefore, impacts of insecticides used on rice fields can play a role in the potential survival of juvenile red swamp crayfish (Barbee et al., 2009).

The insecticide chlorantraniliprole was also reported to cause lethal toxicity to red swamp crayfish with an estimated $LC_{50}$ (96 hr) of 0.95 mg/L, much less toxic than the previously reported pyrethroid insecticides (Barbee et al., 2010). Exposure to BZB (and BH, by means of hydrolysis) did not result in lethal or behavioral effects to red swamp crayfish, unlike other pesticides that are used on Louisiana rice fields. As an emerging pro-herbicide with anticipated registration in Louisiana, the lack of lethal-toxicity to crayfish is an added bonus to rice farmers that see potential in the success of this new herbicide, especially in management of weed species resistant to herbicides with other modes of action. Agronomic research suggests the potential success for the use of benzobicyclon in other rice-growing states. However, the need for chronic exposure investigations exists as previous organisms have shown decreased egg counts and stunted growth as a result of chronic exposure to BZB and/or BH (USEPA, 2017). Juvenile crayfish appear gray or brown, and slightly translucent, and therefore there is potential risk for light penetration through their carapace to activate accumulated chemicals in their blood or hemolymph however, unlike dicloran, specific phototoxic effects were not observed in this investigation.

Herbicide exposure, especially to the more-sensitive juvenile populations, can potentially be chronically detrimental to crayfish populations; adults exposed to it may have decreased egg counts impacting the populations as a whole.

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# CHAPTER 9: SUMMARY OF CONCLUSIONS

Sunlight is well established as an important force driving chemical degradation and has also been shown in some cases to drive toxic responses in organisms exposed to contaminants in aquatic ecosystems. However, gaps in knowledge for how a pesticide may interact with an organism or within an ecosystem exist due to registration studies for pesticides not accounting for environmental parameters that may be important to the behavior of a particular chemical in a particular environment. All pesticides undergo field and laboratory testing prior to registration, however the behavior of the pesticide may vary dependent upon local environmental characteristics. In addition to local variability, current regulations do not consider important variables that may be important to a particular chemical in a particular location. For example, chemicals can exhibit dramatically different behavior in aqueous systems as a function of salinity, however these are not considered in current studies. Additionally, while the potential of a chemical to degrade photochemically is a required study, the potential for it to be phototoxic to aquatic organisms is not assessed in any current registration requirement. The overarching purpose of this dissertation was to account for differences in organismal responses during chemical exposure to environmental factors including sunlight, salinity, and sediment. Select compounds were chosen for this purpose based upon results in previously published works.

## 9.1. Effects of salinity, sunlight, and sediment on chemical behavior

Sunlight and salinity appear to play a major role in the behavior, specifically the degradation, of all chemicals analyzed in this dissertation. Dicloran's photodegradation rate and half-life (at 60 $W/m^2$) were previously shown to not be impacted by salinity, though a decrease in light intensity has proven in this work to affect the photodegradation rate as well as product distribution. At 40 $W/m^2$ light intensity, dicloran in distilled water has an estimated half-life of

12.9 hours and in 2.5% ASW it has an estimated half-life of 14.8 hours, which are statistically different by means of a one-sided t-test ($\alpha = 0.05$) opposed to the previously published data of 7.6 hours in distilled water and 7.4 hours in 3.2% ASW (60 W/m$^2$).

### 9.2. Effects of salinity and sunlight on toxicological responses

Dicloran exposure to inland silversides at three salinities resulted in differences in response at each salinity. The highest $LC_{50}$ was observed at the highest salinity, 0.18 mg/L at 2.5% salinity. While the lowest $LC_{50}$ was observed at 1.2% salinity, 0.08 mg/L in light; mortality in the dark was also observed at 1.2% salinity and approximately 50% of the fishes had a lethal response to 0.75 mg/L dicloran in exposures in the dark.

Sub-lethal responses varied among salinities as well. Severe gill damage was seen at chemical exposures across all salinities, but the severity varied; at 0.5 and 1.2% salinities, necrosis was be observed in the gills of fish 0.17 and 0.25 mg/L, respectively, in irradiated dicloran exposures.

### 9.3. Dicloran as a model pesticide for chemical and toxicological implications

Dicloran was phototoxic to all organisms tested in this dissertation. It has previously been shown to cause phototoxicity in cardiomyocyte cultures of the eastern oyster (*Crassostrea virginica*). Overall, dicloran photodegradation and phototoxicity did not appear to be predictive of the phototoxic potential of 4-hydroxychlorothalonil and benzobicyclon. Chemically, all three pesticides undergo photolysis and salinity [slightly] impacts their half-life with the exception of benzobicyclon, which is significantly impacted by salinity. Additionally, dicloran and 4 – hydroxychlorothalonil have similar photodegradation pathways. However toxicologically no correlation could be made for these chemicals as the organisms exposed to BZB and 4-OH-CHT did not exhibit any lethal response.

## 9.4. Objectives revisited

In Chapter 1, specific objectives were established alongside general hypotheses and the results from this work addressing them are summarized below.

Objective 1: Determine the distribution of dicloran in a simulated shallow water (water-sediment) system under the influence of sunlight and salinity.

In sediment-water systems, dicloran dissipated into the sediment where it persisted over a 24-hour period in the absence of sunlight. In the presence of light dicloran dissipated faster in distilled water compared to artificial seawater (ASW) systems with and without sediment present and dicloran partitioned into sediments at higher concentrations in distilled water compared ASW.

Objective 1: Determine the distribution of dicloran in a simulated shallow water (water-sediment) system under the influence of sunlight and salinity.

In sediment-water systems, dicloran dissipated into the sediment where it remained at higher concentration without sunlight. Dicloran photodegraded faster in distilled water compared to artificial seawater (ASW) with and without sediment present and dicloran partitioned into sediments at higher concentrations in distilled water compared ASW.

Objective 2: Determine the potential for dicloran to be phototoxic to a regulatory freshwater fish species.

Exposure to dicloran and artificial sunlight for 48 hours resulted in a phototoxic response from juvenile fathead minnows, resulting in an $LC_{50}$ of 0.19 mg/L. Without light, no mortality was observed in fathead minnows exposed to dicloran, 1,4-benzoquinone, or 2-chloro-1,4-benzoquinone. Surviving fishes in both dark and light trials exhibited sublethal responses to dicloran including cell proliferation and apoptosis within their gill filaments. The severity of

responses increased with increasing concentrations in the presence of sunlight. Similar comparisons were observed for the gene expressions of fathead minnows. All light treatments resulted in statistically significant responses compared to the dark controls; cyp1a, ESR1, and MMP9 showed an upregulation in relative expressions in correlation to increasing concentrations of irradiated dicloran. Cpy1a is associated with metabolism, ESR1 is hormone function, and MMP9 is immune function and increased expressions could be indicative of potential long-term impacts to fishes exposed to them. Further research following fish through an entire life-cycle or through multi-generational studies would be necessary to determine if these increases in expressions result in long-term impairment of the organism.

Objective 3: Determine the potential for dicloran to be phototoxic to an estuarine fish species.

The photodegradation of dicloran and well as the phototoxic response of a euryhaline fish was measured at three salinities. While no statistical difference was observed between salinities for the photodegradation of dicloran, the half-life appeared to slightly increase as salinity increased (12.6 hr in 0.5% and 14.9 hr in 2.5%). Inland silversides also had different responses when exposed to solutions of dicloran in both dark and light treatments at different salinities. At 2.5% salinity, the highest $LC_{50}$ was 0.18 mg/L (light), at 1.2% salinity the $LC_{50}$ was 0.08 mg/L (light), and at 0.5% salinity the $LC_{50}$ was 0.11 mg/L (light). Mortality was only observed in dark exposures at 1.2% salinity and 2.5% salinity to BQ and CBQ, possibly due to their rapid degradation in light solutions.

Sublethal impacts again varied with salinity. Histological analysis of gill filaments showed they were severely impacted at all salinities, with the most severe at 0.5% salinity; apoptosis and cell proliferation were observed for fishes exposed to dicloran in both dark and light treatments as well as the potential for aneurisms and necrosis at higher concentrations of

irradiated dicloran. There were few statistical differences observed in gene expression possibly resulting from low sample sizes, although obvious trends in variation are apparent.

Objective 4: Determine the potential for a compound with a similar photodegradation pathway as dicloran, 4-hydroxychlorothalonil (4-OH-CHT), to be phototoxic to an estuarine fish species.

While 4-OH-CHT and dicloran appear to have similar proposed photodegradation pathways and both are slightly impacted by salinity (longer half-lives at higher salinities), the same trend does not exist toxicologically. Inland silversides exposed to 4-OH-CHT in dark and light treatments did not result in lethal impacts. Gene expression was relatively low but significant differences were observed for MMP9 (immune function), suggesting possible impairment through long-term exposure. Additional research would need to be conducted to support these suggestions.

Objective 5: Determine the potential for dicloran to be phototoxic to a benthic aquatic invertebrate (crayfish).

Previous reports have determined red swamp crayfish to be more resilient to pesticide exposure compared to freshwater fishes and that appears to be the case in this investigation as well. Crayfish were exposed to dicloran in both dark and light treatments, resulting in both lethal and sublethal responses. At exposures of 0.75 mg/L dicloran and light, about 50% mortality was observed while 100% mortality was observed at 1.5 mg/L. Less than 10% mortality was observed for dark exposures. A downregulation in gene expression from 0.50 mg/L to 1.0 mg/L was observed for crayfish in dark exposures for asticidin and c-type lectin, while the highest expression for crayfish exposed to irradiated dicloran was at 0.75 mg/L for all three gene analyzed.

Objective 6: Apply this system to assess the potential of a new pesticides, benzobicyclon [hydrolysate] (BZB/BH), to be phototoxic to crayfish.

Benzobicyclon hydrolyzes to benzobicyclon hydrolysate (BH) within < 1 day and BH was degraded by sunlight with a media dependant half-life. Dicloran has shown to photodegrade and cause phototoxic impacts to inland silversides, fathead minnows, and red swamp crayfish; 4-OH-CHT was shown to photodegrade but not be phototoxic to inland silversides. BZB/BH appeared to behave similar to 4-OH-CHT, undergoing photolysis but not resulting in lethal phototoxic responses from aquatic organisms. No crayfish exhibited lethal responses to benzobicyclon exposure in dark or light trials, but sublethal responses in gene expressions were observed. Kazal 1 exhibited the highest gene expression at 0.05 mg/L BZB and light, while a downregulation in expression level was observed in asticidin, c-type lectin, and ferritin, in comparison to the dark control. These genes code for immune function and may be indicative of potential sub-lethal impacts, however further research would need to be conducted following juvenile organisms through an entire life-cycle and/or multiple generations to assess this possibility.

Overall, phototoxicity appears to be chemical dependent and while a chemical may photodegrade this does not mean it will cause phototoxic impacts to non-target aquatic organisms. Dicloran is a model fungicide for the photochemical processes of pesticides, but it is not a (photo)toxicological model for pesticide response by non-target organisms. chemical degradation alone can also be media (specifically salinity) dependent. Currently, from a regulatory perspective, there are no protocols for assessing chemical phototoxicity to aquatic organisms, or the impacts of light in mixed-media (water-sediment) pesticide degradation investigations. In addition, the impacts of salinity are not addressed in protocols assessing

chemical environmental fate. Such protocols may be needed as higher-tiered studies for chemicals based upon the results of lower tiered studies as well as the physical and chemical properties of the chemical of interest. Dicloran may be a potential candidate as a "positive control" to if there was a desire or need to assess if a chemical has the potential for phototoxicity.

## APPENDIX A. GENERAL SUPPLEMENTARY INFORMATION

Table A.1. HPLC parameters for each pesticide analyzed: dicloran, 2-chloro-1,4-benzoquinone, 1,4-benzoquinone, 4-hydroxychlorothalonil, benzobicyclon, and benzobicyclon hydrolysate.

| Chemical | Mobile phase | | | | Retention time (min) | Wavelength (nm) | Injection volume (µL) |
|---|---|---|---|---|---|---|---|
| | Water | ACN | 0.1% formic acid in water | 0.1% formic acid in ACN | | | |
| Dicloran | X | X | | | 12.1 | 380 | 40 |
| CBQ | X | X | | | 7.5 | 254 | 40 |
| BQ | X | X | | | 5.5 | 254 | 40 |
| 4-OH-CHT | X | X | X | | 11.8 | 280 | 20 |
| BZB | X | | X | X | 14.5 | 280 | 20 |
| BH | X | | X | X | 11.9 | 340 | 20 |

## APPENDIX B. SUPPLEMENTARY INFORMATION FOR CHAPTER 3

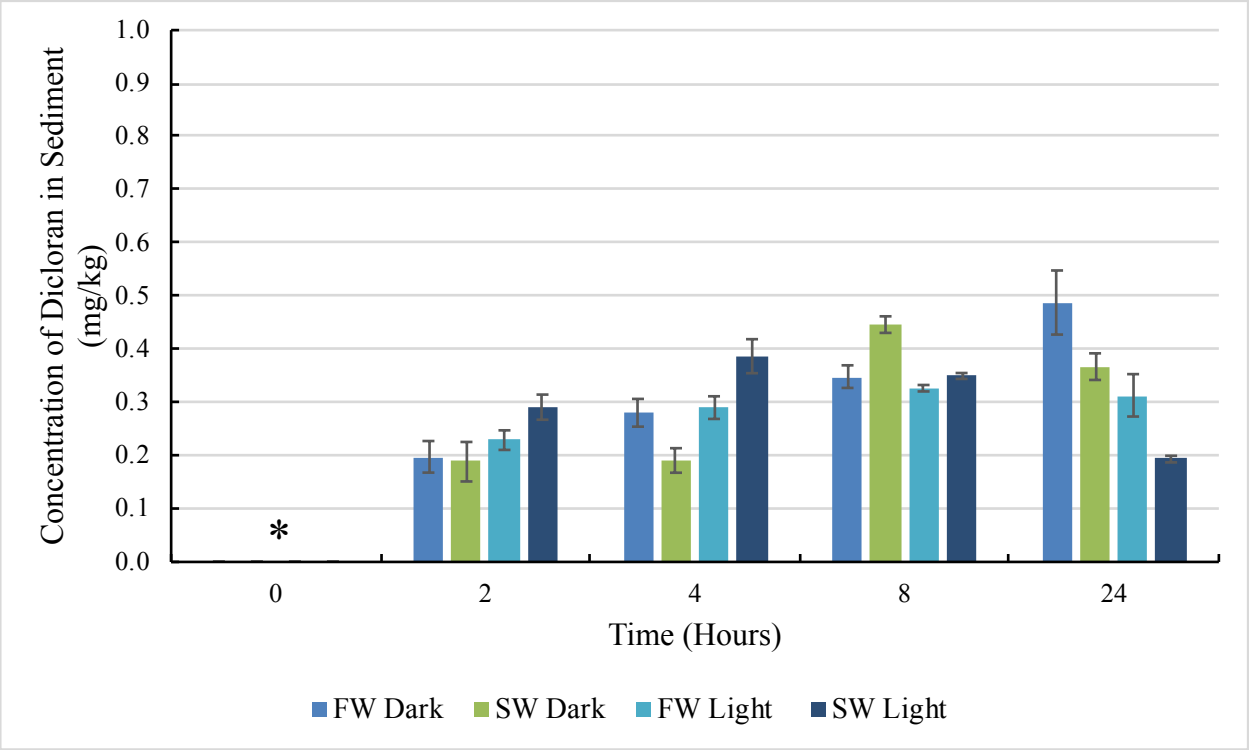


Figure B.1. The dissipation and degradation of dicloran in sediment. Legend identifies freshwater dark, seawater dark, freshwater light, and seawater light sediment samples. Asterisk (*) indicates 0 mg/kg dicloran at time 0 hours; error bars indicate standard error.

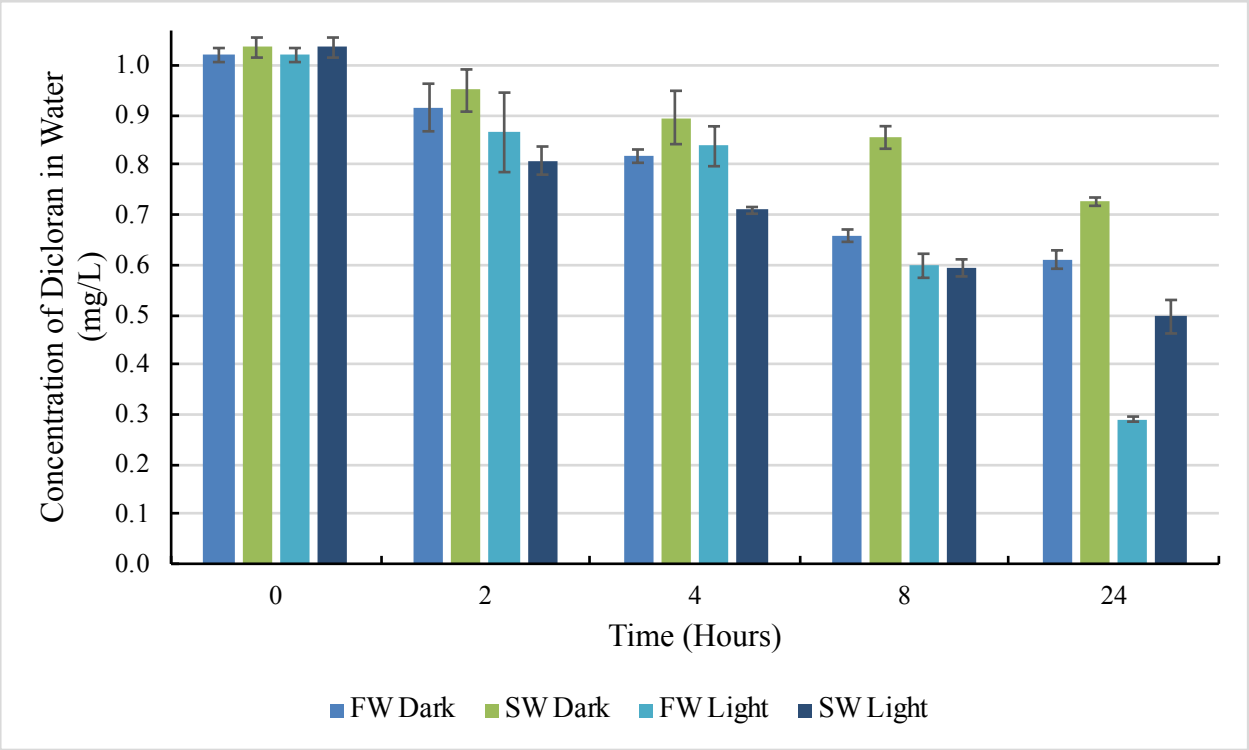


Figure B.2. The photodegradation of dicloran in water and dissipation of dicloran into sediment, measured in the water. Legend identifies freshwater dark, seawater dark, freshwater light, and seawater light water samples. Error bars indicate standard error.

## APPENDIX C. SUPPLEMENTARY INFORMATION FOR CHAPTER 5

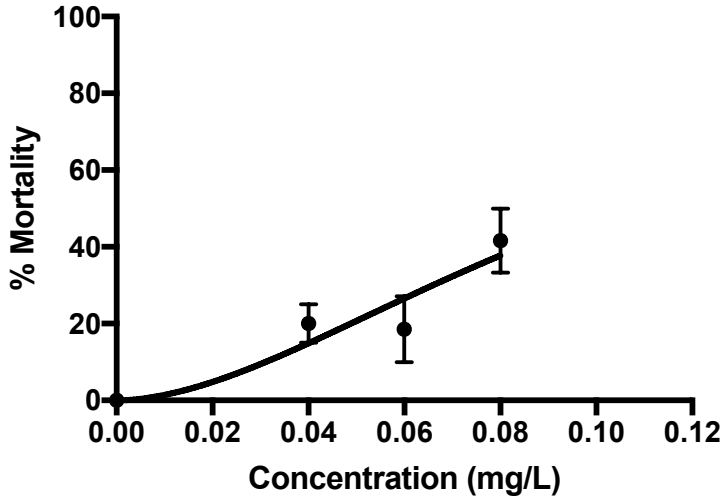


Figure C.1. The dose-response curve for inland silversides (*M. beryllina*) exposed to 1,4-benzoquinone for 48 hours in 2.5% seawater; error bars indicate standard error.

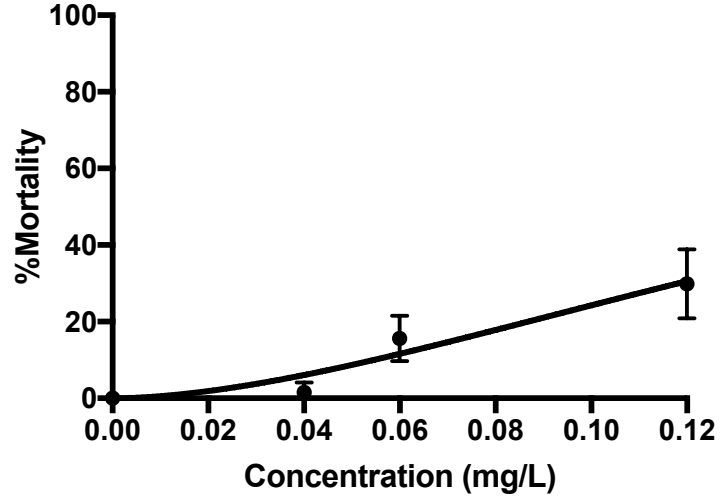


Figure C.2. The dose-response curve for inland silversides (*M. beryllina*) exposed to 2-chloro-1,4-benzoquinone for 48 hours in 2.5% seawater; error bars indicate standard error.

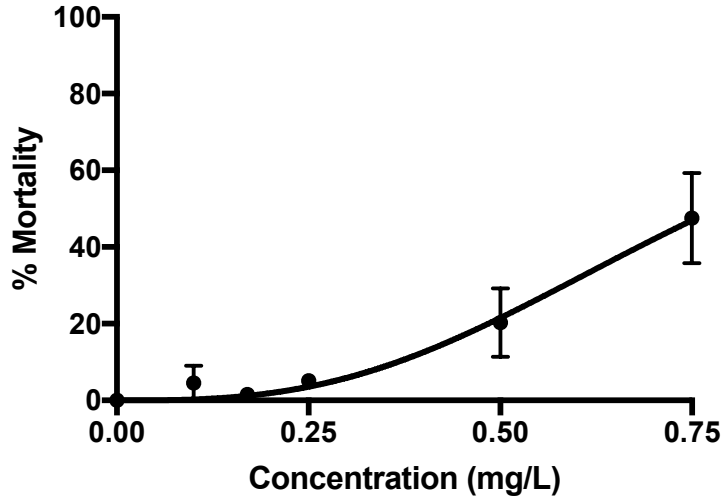


Figure C.3. The dose-response curve for inland silversides (*M. beryllina*) exposed to dicloran for 48 hours without sunlight in 1.2% seawater; error bars indicate standard error.

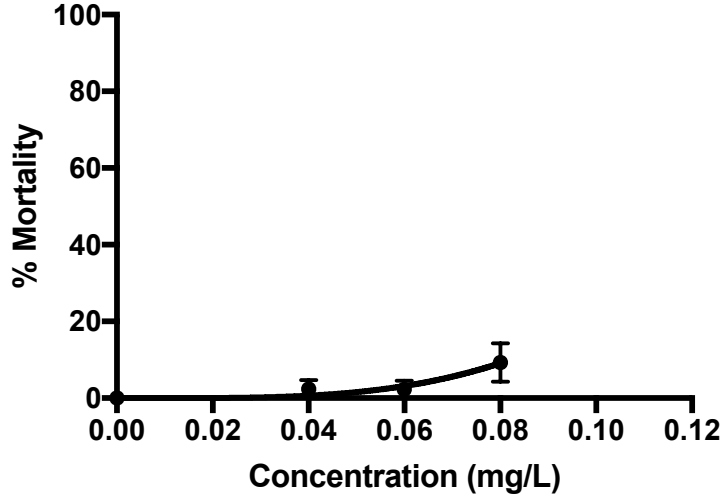


Figure C.4. The dose-response curve for inland silversides (*M. beryllina*) exposed to 1,4-benzoquinone for 48 hours in 1.2% seawater; error bars indicate standard error.

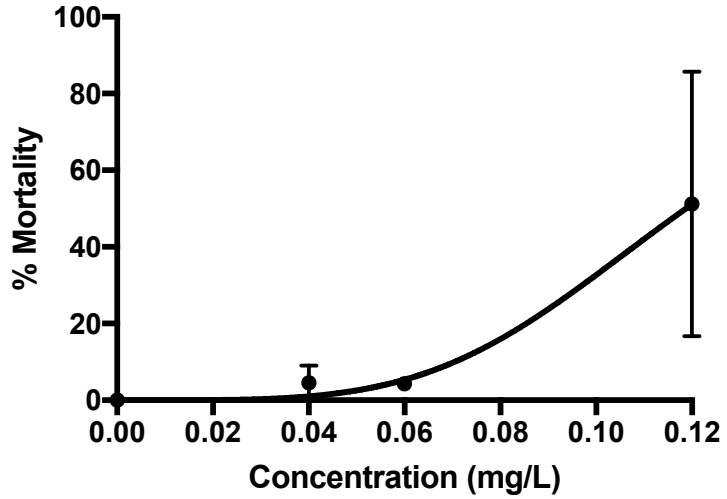


Figure C.5. The dose-response curve for inland silversides (*M. beryllina*) exposed to 2-chloro-1,4-benzoquinone for 48 hours in 1.2% seawater; error bars indicate standard error.

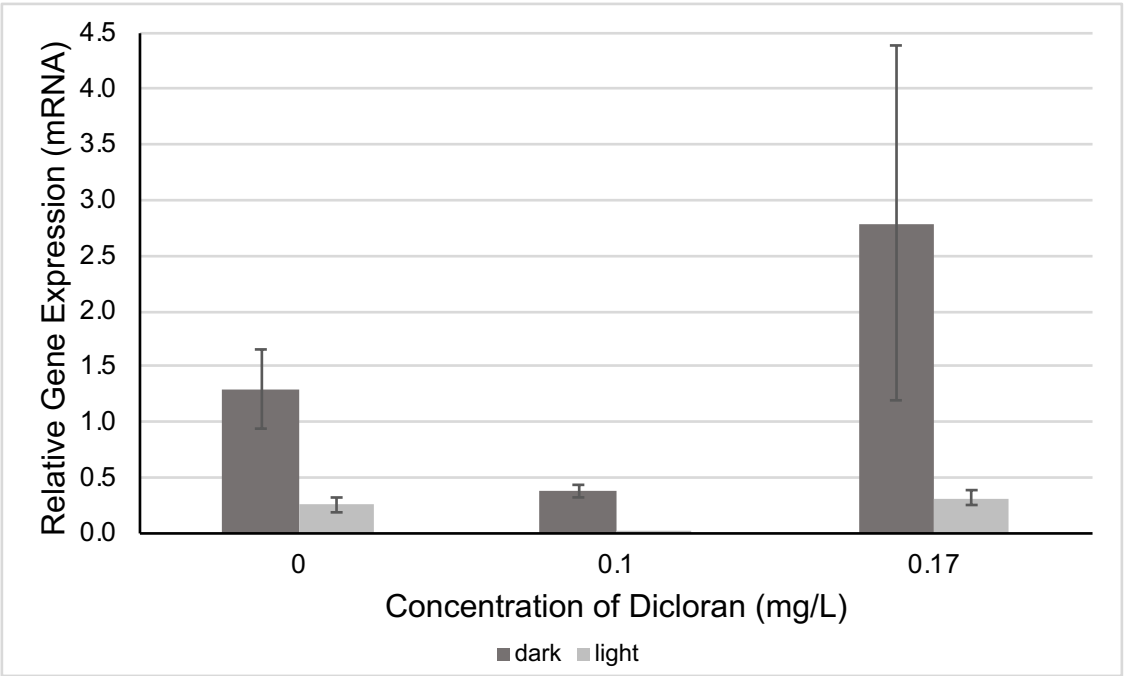


Figure C.6. The relative gene expression of ATP1A1b of inland silversides (0.5%); EF-1α was used as the reference gene.

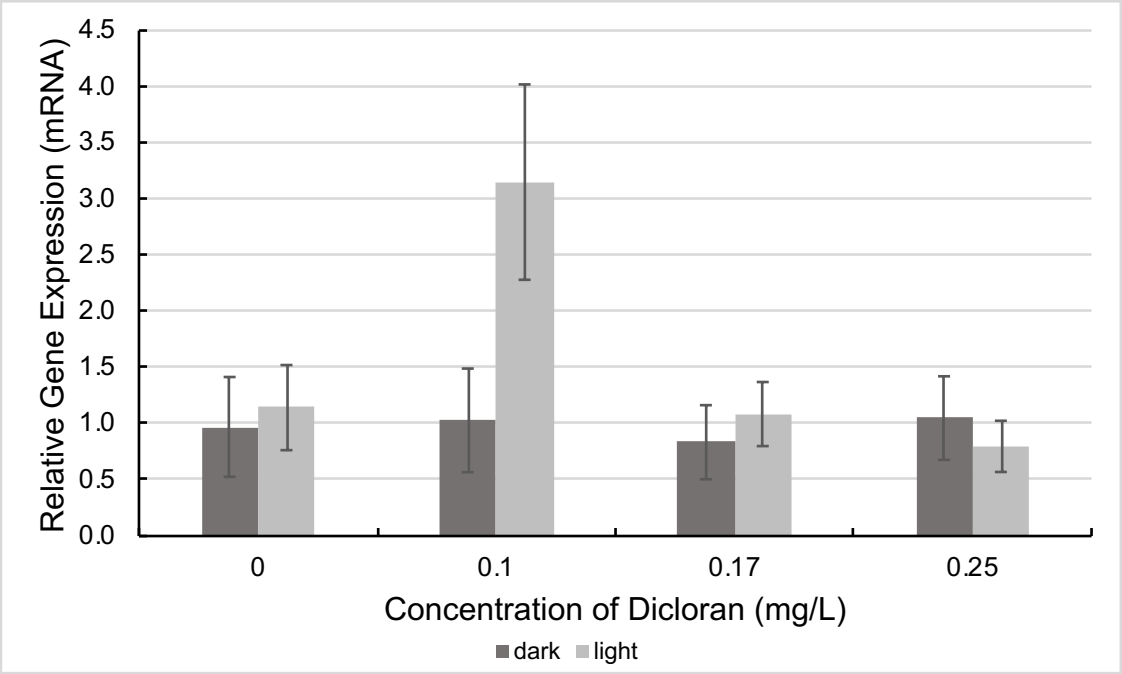


Figure C.7. The relative gene expression of ATP1A1b of inland silversides (1.2%); EF-1α was used as the reference gene.

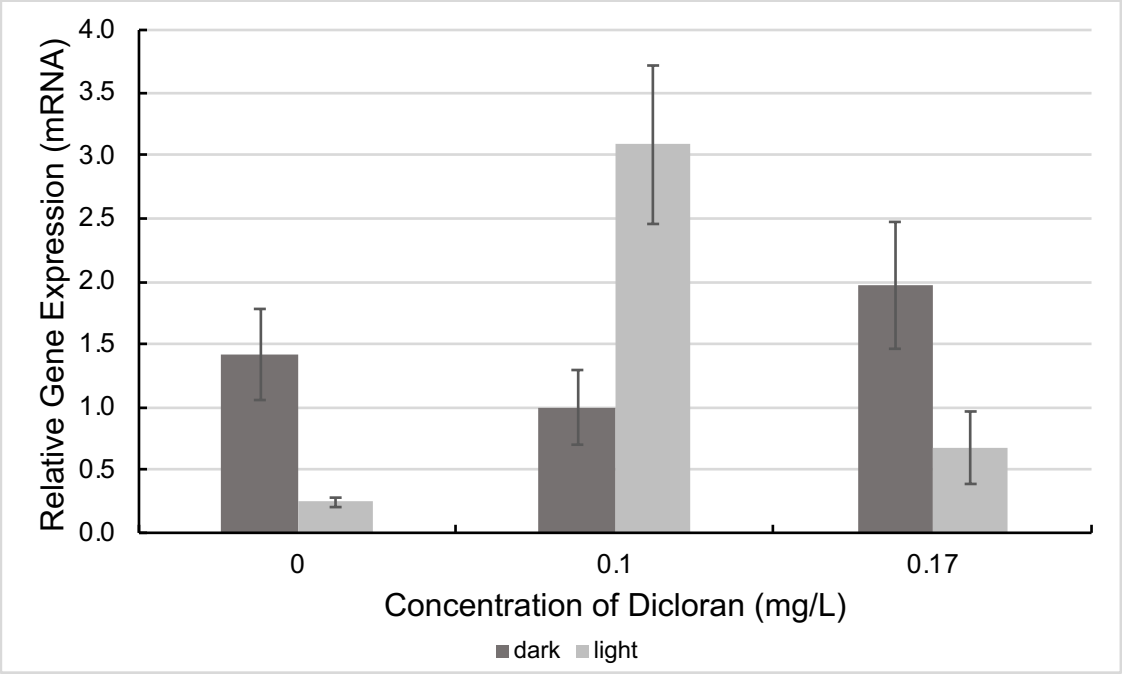


Figure C.8. The relative gene expression of Trα of inland silversides (0.5%); EF-1α was used as the reference gene.

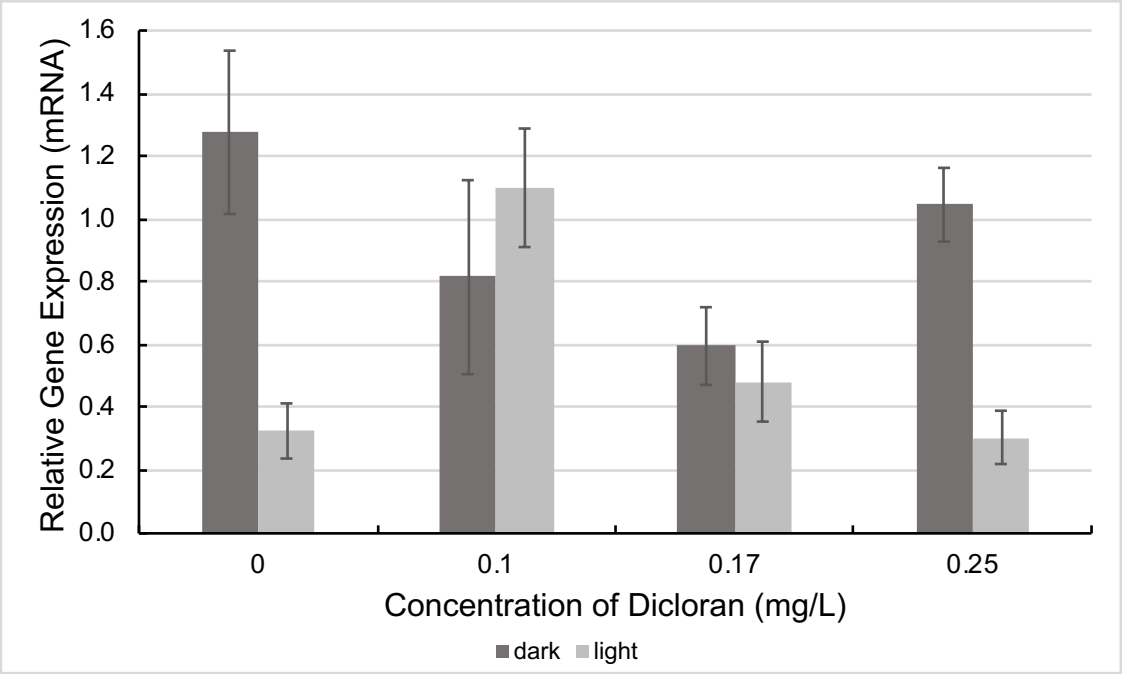


Figure C.9. The relative gene expression of Trα of inland silversides (1.2%); EF-1α was used as the reference gene.

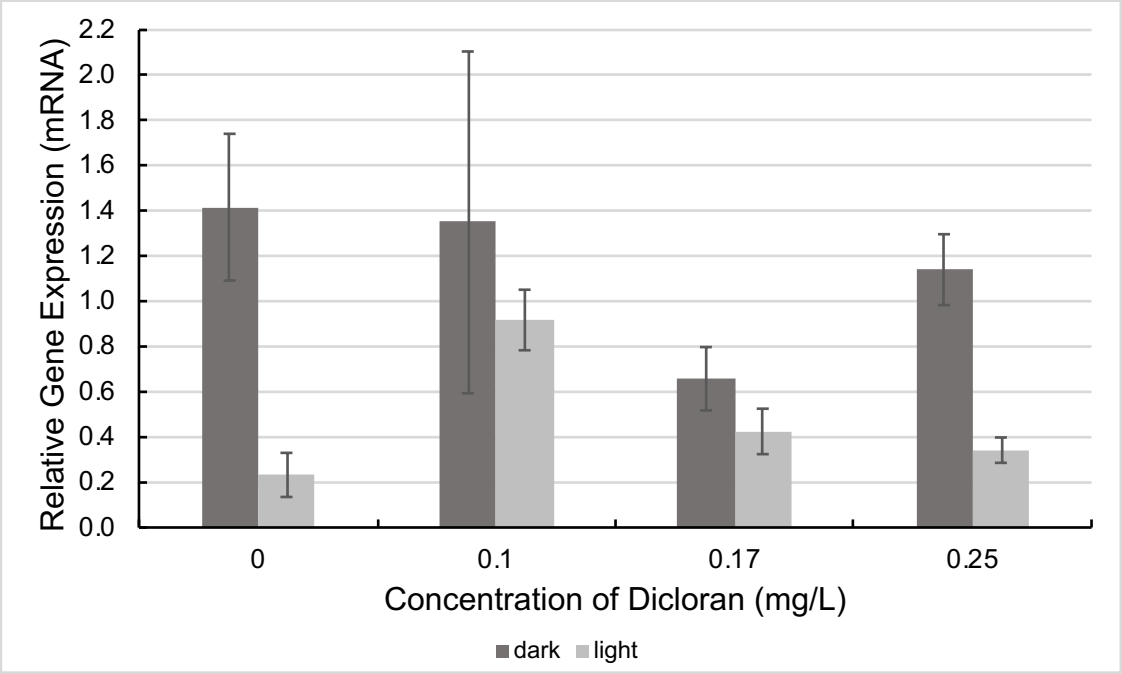


Figure C.10. The relative gene expression of Trα of inland silversides (1.2%); GAPDH was used as the reference gene.

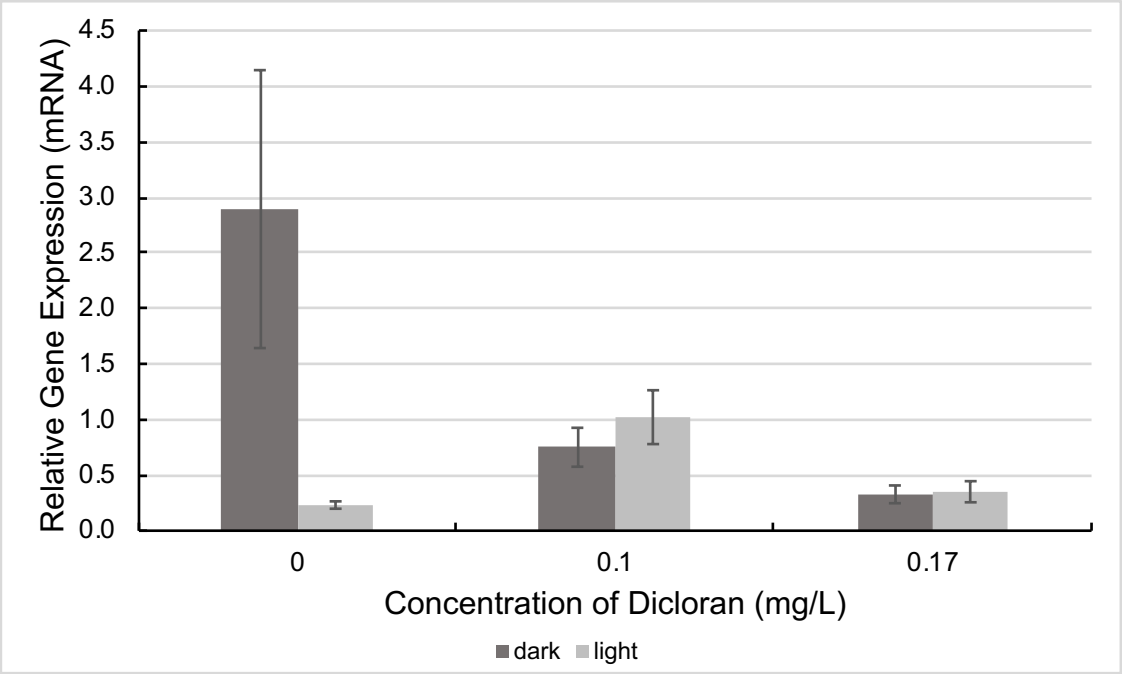


Figure C.11. The relative gene expression of MMP9 of inland silversides (0.5%); EF-1α was used as the reference gene.

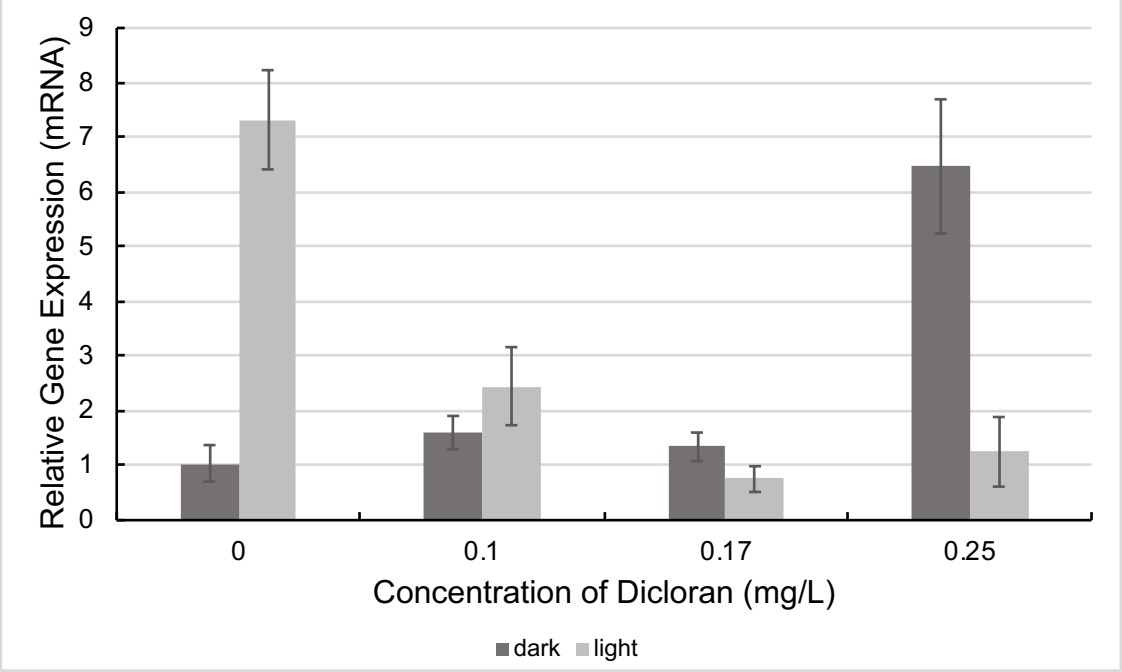


Figure C.12. The relative gene expression of MMP9 of inland silversides (1.2%); EF-1α was used as the reference gene.

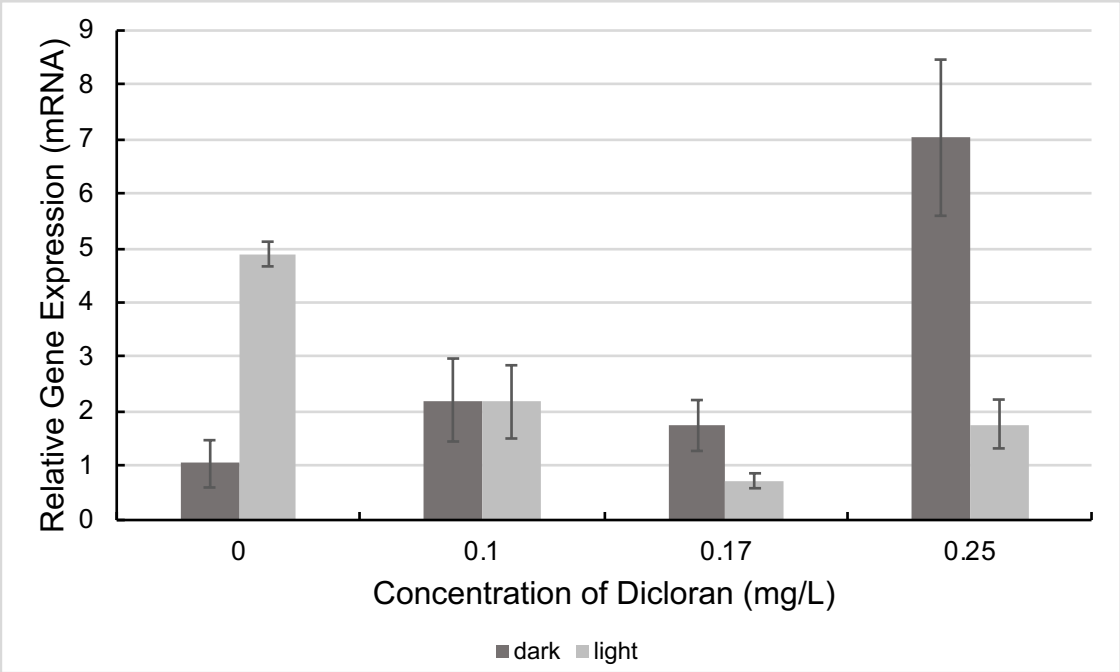

Figure C.13. The relative gene expression of MMP9 of inland silversides (1.2%); GAPDH was used as the reference gene.

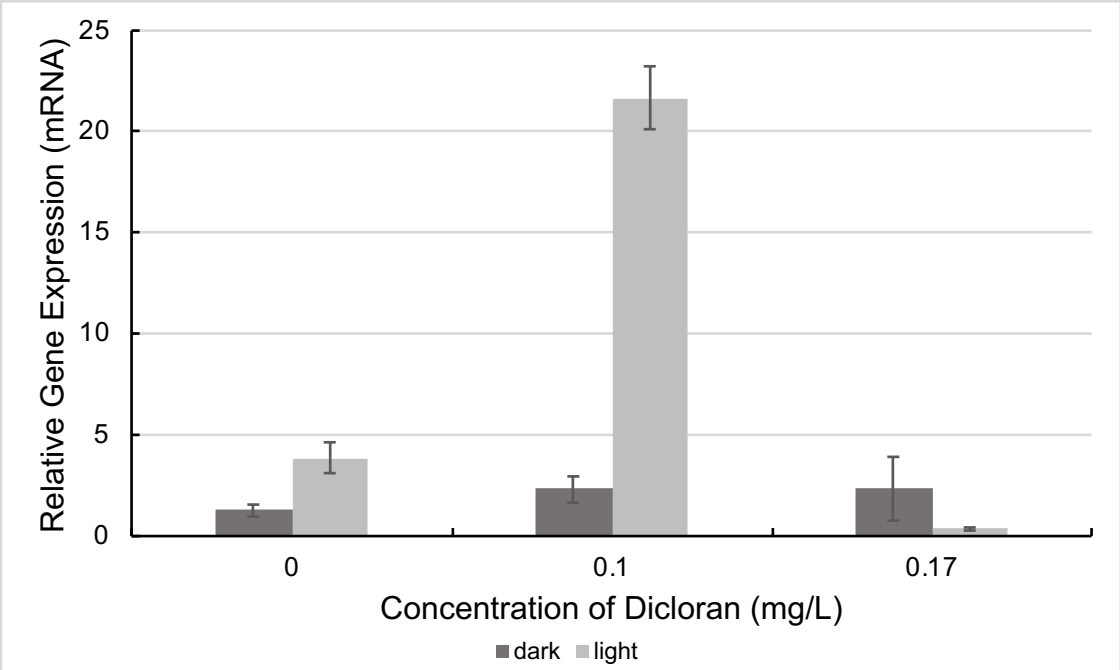

Figure C.14. The relative gene expression of ALOX5AP of inland silversides (0.5%); EF-1α was used as the reference gene.

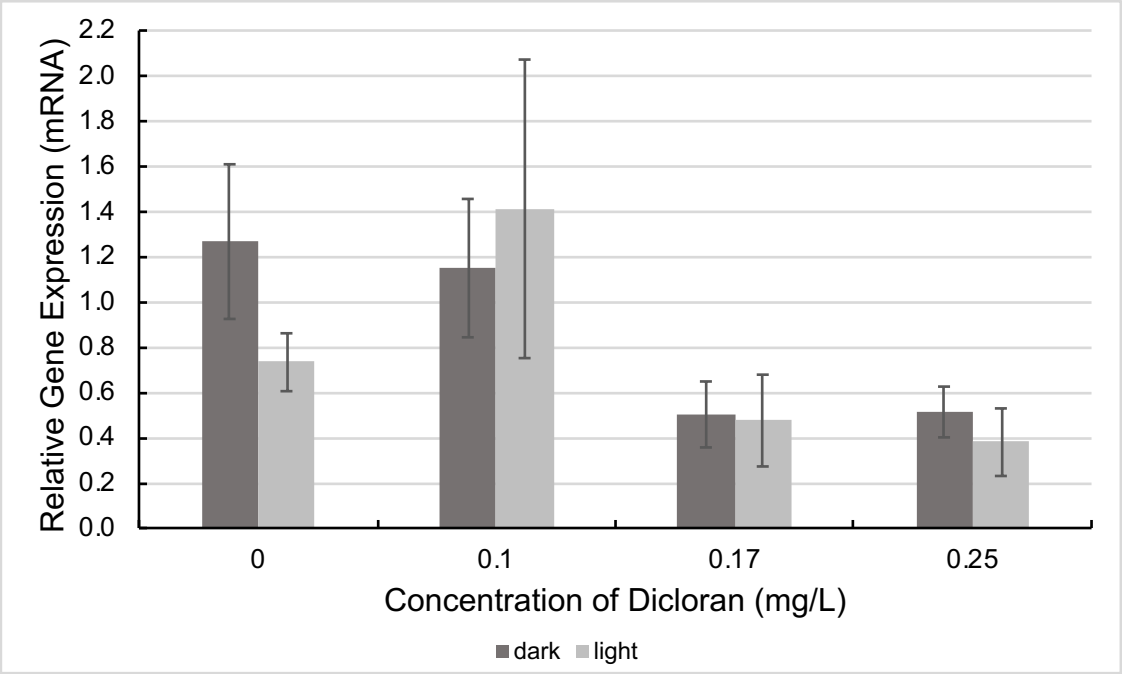


Figure C.15. The relative gene expression of ALOX5AP of inland silversides (1.2%); EF-1α was used as the reference gene.

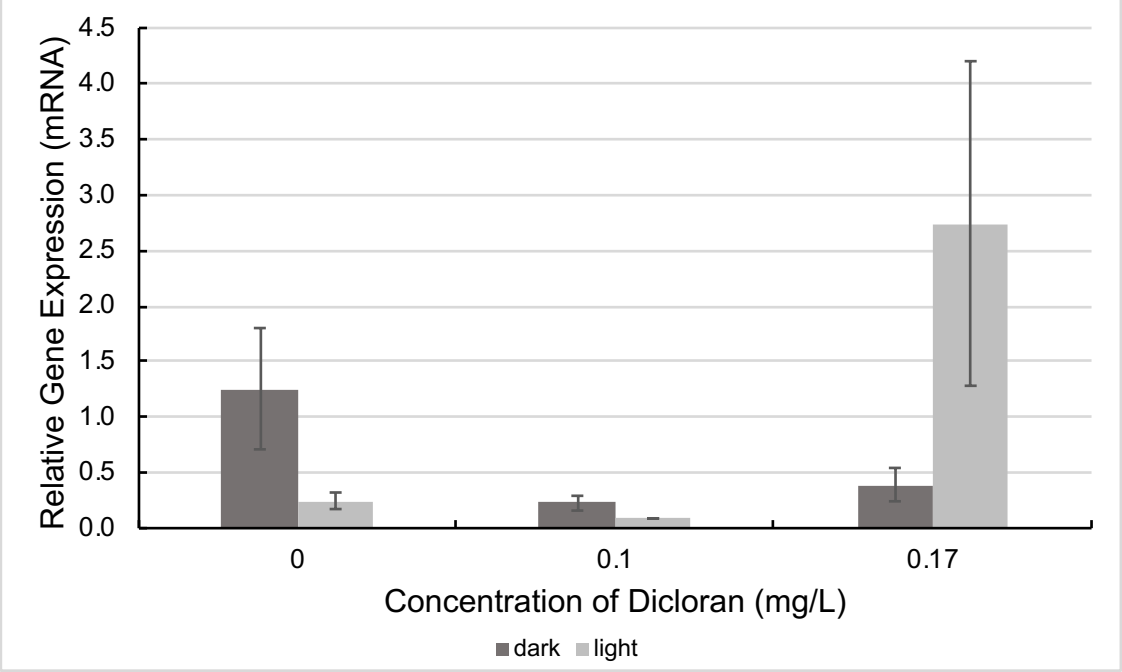


Figure C.16. The relative gene expression of ALOX5AP of inland silversides (0.5%); GAPDH was used as the reference gene.

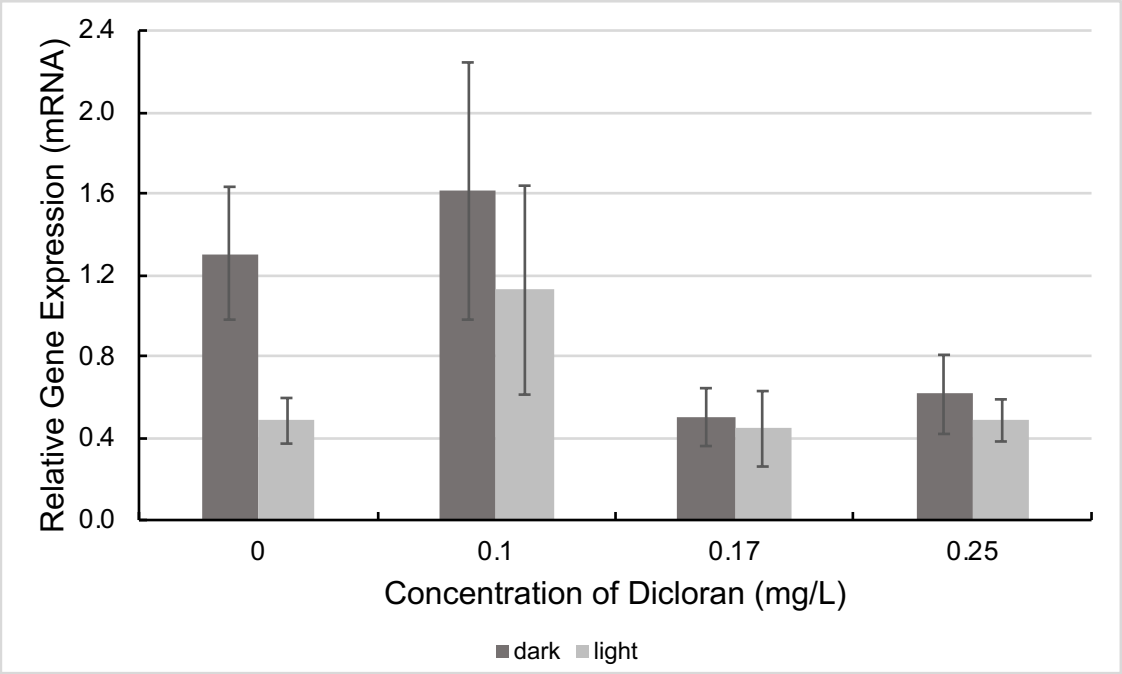

Figure C.17. The relative gene expression of ALOX5AP of inland silversides (1.2%); GAPDH was used as the reference gene.

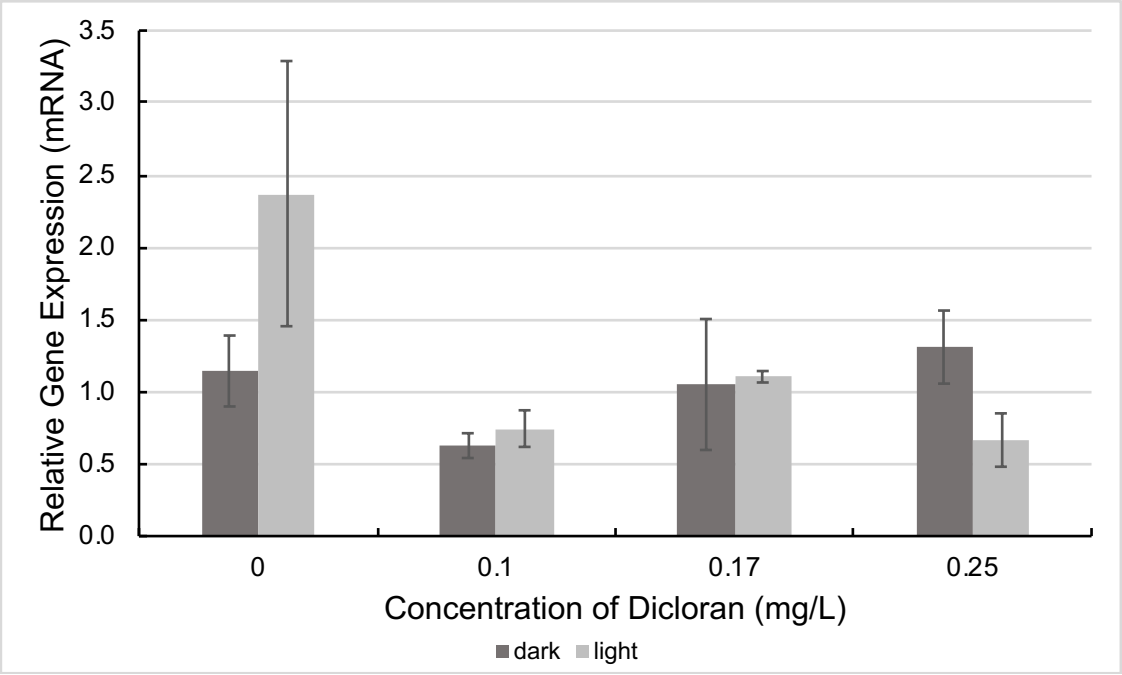

Figure C.18. The relative gene expression of GPR30 for inland silversides at 1.2% salinity, quantified with EF-1α as the reference gene.

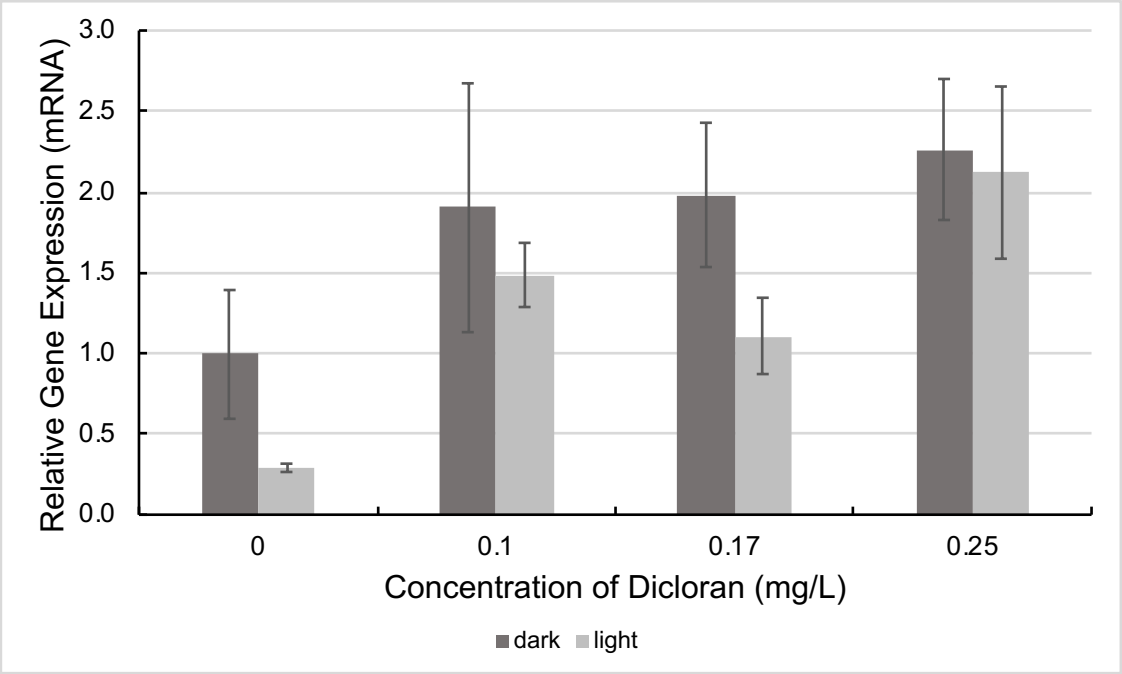


Figure C.19. The relative gene expression of GSR for inland silversides (1.2%) exposed to dicloran in dark and light treatments using GAPDH as the reference gene.

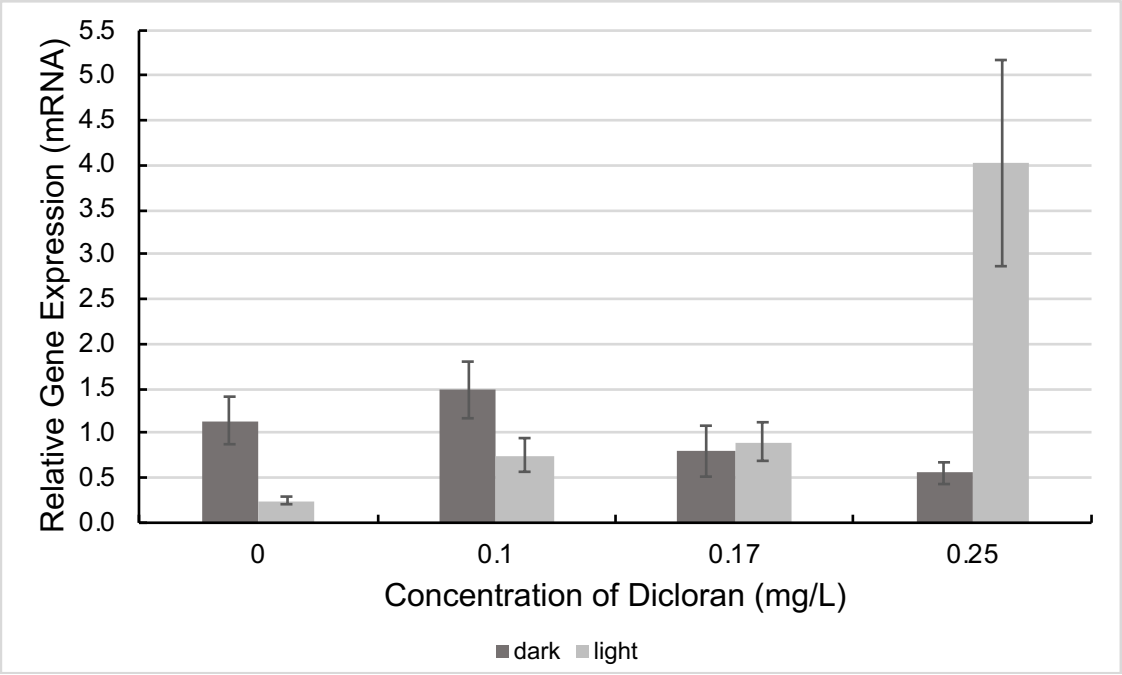


Figure C.20. The relative gene expression of GSR for inland silversides (2.5%) exposed to dicloran in dark and light treatments using EF-1α as the reference gene.

## APPENDIX D. SUPPLEMENTARY INFORMATION FOR CHAPTER 8

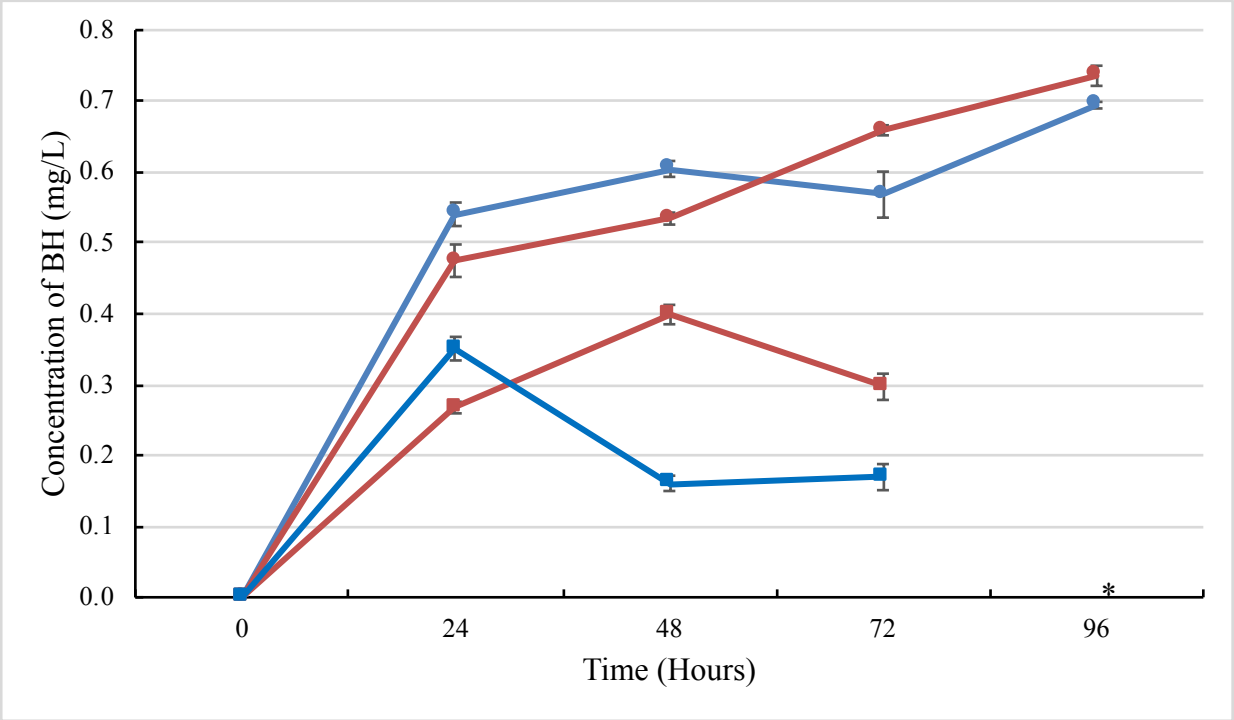


Figure D.1. The hydrolysis and photolysis of benzobicyclon to benzobicyclon hydrolysate in dark (●) light (■) in freshwater (blue) and seawater (red); error bars indicate standard error and asterisks (*) represent unsampled time points at 96 hours for light trials.

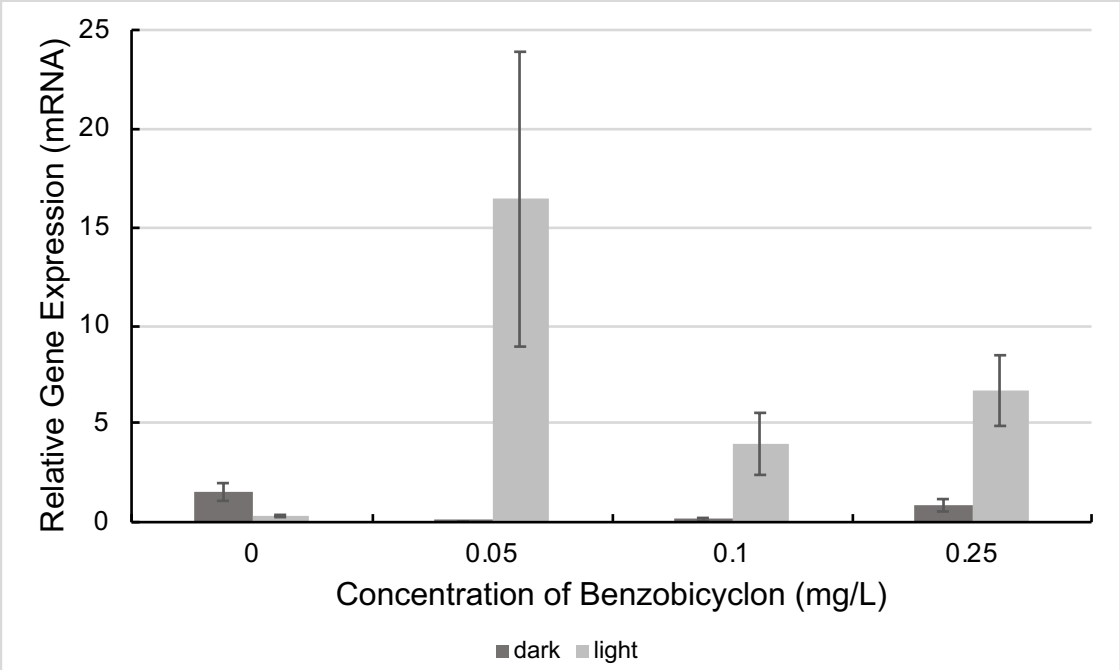


Figure D.2. Relative gene expression for LGBP in response to crayfish exposure to benzobicyclon.

# APPENDIX E. IACUC PROTOCOL

Revised March 2014

**INSTRUCTIONS FOR OBTAINING APPROVAL FOR THE USE OF ANIMALS**
**In Research/Teaching/Extension**
**WITHIN THE LSU AGRICULTURAL CENTER (AgCenter)**

The **objective** of the review process:

All projects involving animals must be reviewed by the AgCenter's IACUC including noninvasive projects with agricultural animals that utilize accepted or routine management practices. Via the protocol form, the scientist supplies the committee with enough information to ensure humane animal treatment and compliance with the Animal Welfare Act. By federal law, approvals for ALL projects cannot exceed three years. All animals must be on a protocol whether it is experimental or health maintenance.

Getting the correct form:

Protocols for animals to be housed in the SVM or Division of Laboratory Animal Medicine (DLAM) facilities should be submitted to the DLAM IACUC office (Room 1502 SVM). The main campus form is available at http://www1.vetmed.lsu.edu//ORAS/index.html

Researchers using animals to be housed at AgCenter facilities should use the AgCenter form which is available from the AgCenter committee chair or downloaded from the AgCenter/LAES Intranet Project Admin website. http://intranet.lsuagcenter.net/unit/LAES/Pages/TheExperimentStationProject.aspx

Where and how to submit:

AgCenter IACUC forms (protocols for animals to be housed on AgCenter properties – including off campus locations) should be submitted to Dr. Philip H. Elzer, LSU AgCenter, 102 J.N. Efferson Hall. The original, typed protocol and 14 copies should be submitted.

Please pay close attention to the following:

Complete **ALL** Sections of the protocol form.
Submit 14 copies plus the original (**Fifteen** TOTAL). Form must be typed.
Signature of animal housing representative on the original form.
AgCenter: Joe Navarre, Randy Wright, Mike Canal, or Tony Bridges
Station Animal coordinator: individual in charge of animal husbandry
If applicable attach any wildlife permits
Signature of PI, CoPI, and Surgeon (as applicable) on the original form
Hazardous Material Information section filled out properly.
Include approval from IBRDSC if using biological or recombinant DNA.
If using hazardous chemicals, include approval from Chemical Safety Committee
Type of project must be checked regarding pain levels.
Answer all questions and use NA if applicable.
DO NOT attach inserts from your grant application. This protocol form serves as a "stand alone" document. Standard operating procedures (SOPs) referenced in protocol should be provided.

0

**PROTOCOL NUMBER: _____**
**APPROVAL DATE: _____**

# LSU AgCenter PROTOCOL FOR ANIMAL CARE AND USE

## SECTION 1: Principal Investigator

| | |
|---|---|
| Name: Wei Xu | Department: Aquaculture Research Station |
| Office Phone: 225-765-0107<br>Home Phone: 516-784-6675 | E-mail Address:wxu@agcenter.lsu.edu |

## SECTION 2:

**A. Project Title (Enter the name of your project/course number below.)**

| |
|---|
| **Photo-Inducted Toxicity in Freshwater and Seawater of Various Compounds including Pesticides, Pharmaceuticals, and Petroleum Compounds on Zebrafish, Fathead Minnow, and Inland Silverside** |

**B. Anticipated Project Start Date (animals may not be used until approved!)**

| |
|---|
| |

## SECTION 3:

**A. Animal Species**

| | |
|---|---|
| Species (common name): *Danio rerio* (zebrafish), *Pimephales promelas* (fathead minnow), and *Menidia berylline* (inland silverside) | Strain: |

| | | |
|---|---|---|
| Number of animals needed:<br>Year 1: 360 *D. rerio*; 360 *P. promelas*; 360 *M. berylline*<br>Year 2: 360 *D. rerio*; 360 *P. promelas*; 360 *M. berylline*<br>Year 3: 360 *D. rerio*; 360 *P. promelas*; 360 *M. berylline*<br>*TOTAL over 3 years 1,080 per species (3,240 for all species)<br>(*animals carried over are not counted twice) | Maximum number needed at one time: 180 for each species | Number of animals to be placed in each group: 30 |

| | | |
|---|---|---|
| Yes: | No: X | Are you using wild, invasive, or non-native species for which permits are necessary? (ATTACH COPY OF PERMIT)<br>Note: a copy of the permit(s) must be received before animal work begins. |

**B. Source of Animals**

| | |
|---|---|
| | Order through DLAM or AgCenter unit (list which one) |
| X | Other (list source): CK Associates (Baton Rouge, LA) |
| | Transfer from Approved Protocol (list protocol number): |

**C. Location of Animal Housing (check or fill in appropriate space)**

| | | | |
|---|---|---|---|
| | DLAM Vivarium | | Life Sciences Vivarium |
| | SVM Barns (list site): | | SVM Fish Building |
| | SVM Research Herd | | |
| | LAES Reproductive Biology Unit | | LAES Infectious Disease Isolation Facility |
| | LAES Aquaculture Unit | | |
| X | LAES Research Station (list site) Aquaculture Research Station | | |
| | Other (list site): | | |
| | Field Study **(Do not complete D and E)** | | |

Animal housing and veterinary care have been coordinated with DLAM office or LSU AgCenter Unit.
Yes: __ X _____
No: _____
*Name of Animal Housing Representative Contacted (typed): **Robert Reigh**

Signature (required): _____
*(person responsible for assuring space and animal care personnel are available)

**C. Special Husbandry Requirements**

**Do your animals have special needs to be addressed by DLAM or research unit?**

| | |
|---|---|
| | Housing under the direct care of DLAM is not required. (e.g. SVM fish building) |
| X | NO. Animals will be cared for according to standard operating procedures of DLAM or unit |
| | YES (complete table below) |

| | |
|---|---|
| TEMPERATURE RANGE | (F) Humidity: (%) |
| LIGHT CYCLE | Hours light: Hours dark: |
| CAGING | Type: Size: ABSL2: ABSL3: |
| BEDDING/LITTER | Type: Autoclaved: Changes/week: |
| WATER | Sterile: De-ionized: Acidified: Tap: Other: |
| DIET | List Special Feeding Requirements: |
| OTHER SPECIAL NEEDS | List: |

D. **Animal Management**

**Individual (or groups of) animals are identified by:**

| | |
|---|---|
| | Tag |
| | Tattoo |
| X | Cage, Tank, or Stall Card |
| | Other. List type of identification: |

**Check all applicable below:**

| CARE OF SICK ANIMALS | | DISPOSAL OF DEAD ANIMALS | | PEST CONTROL | |
|---|---|---|---|---|---|
| X | Call Investigator | X | Call Investigator | X | Call Investigator |
| | Clinician to Treat | | Necropsy | | Pesticides OK |
| | Euthanasia | | Disposal. List any special requirements: incineration | | No Pesticides |

### E. Disposition of Animals

**What will be done with any animals at the conclusion of the project? Mark all that apply.**

| | |
|---|---|
| X | Animals will be euthanized. |
| | DLAM/LAES has permission to REASSIGN animals to another IACUC-approved protocol. |
| | TRANSFER animals to the following IACUC-approved protocol(s).<br>List Protocol Number(s): |
| | Catch and release (applies to field studies). |
| | Return to owner/supplier. |
| | Other (please state): |

### SECTION 4: Layman's Summary of Research/Teaching

Provide a brief (**100 word** maximum), non-scientific (i.e., no jargon, not your grant abstract) explanation of the purpose, materials, and methods in the block below for the benefit of reviewers and animal handlers who need to understand the research project. Why is project being performed? If this is a holding protocol, please state that no experimental design is involved.

**This proposed study will utilize three fish species to determine changes of chemical toxicities under exposure of light. Toxicities of ocean contaminants, such as pesticides, pharmaceuticals, and petroleum compounds, can be modified by a number of environmental factors. Changes of the contaminant toxicities need to be evaluated to provide supports for environment protection and ecosystem restoration. Fish have been widely used in toxicological studies. The two freshwater fish species (zebrafish and fathead minnow) and one saltwater species (inland silverside) will be used to help us understand the changes of contaminant toxicities under exposure of sun light along the Gulf coast.**

**SECTION 5: Investigator's Statement. Assurances for the Humane Care and Use of Vertebrate Animals.**

By signing this form, we agree to abide by the Policy for the Care and Use of Animals of Louisiana State University AgCenter. This project will be in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*, the FASS *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (except as explained in the accompanying protocol), and the Louisiana State University AgCenter Animal Welfare Assurance on file with the U.S. Public Health Service.

We further assure the Committee that: 1) We will abide by all federal, state, and local laws and regulations governing the use of animals in teaching and research; 2) the investigators and technicians are adequately trained to perform the research techniques required in these studies; 3) the fewest number of animals required to produce valid results are being used in this study; 4) the PI will notify the attending veterinarian or Committee of unexpected animal injury, illness, or disease; and 5) emergency veterinary care will be available if needed.

(Add additional rows as needed)

| | | | |
|---|---|---|---|
| Principal Investigator Signature: | Principal Investigator Name (Typed): Wei Xu | Title/Rank: Assistant Professor | Date: 3/24/2017 |
| Co-Investigator Signature: | Co-Investigator Name (Typed): Kevin Armbrust | Title/Rank: Professor | Date: 3/28/2017 |
| Surgeon Signature: | Surgeon Name (Typed): | Title/Rank: | Date: |

## SECTION 6: Hazardous Materials

Will zoonotic or recombinant, radioactive, or hazardous chemical agents be **PRESENT IN THE ANIMAL ROOM?**

If zoonotic (infectious to humans) or recombinant organisms are to be used, this protocol request must be submitted to the IBRDS Committee for approval **PRIOR TO CONSIDERATION** by the IACUC. Final approval will not be granted until IBRDSC approval is received by the IACUC. Similarly, if hazardous chemicals are to be used in the animal room, submit the proposal to the Chemical Safety Committee for prior approval. **P.I. MUST PROVIDE health and safety measures for animal technicians and facility maintenance personnel. In Standard Operating Procedure (SOP) form, describe any precautions, procedures, or personal protection required in handling animals or waste containing listed agents or compounds, or in working in or around the animal room (including air handling system), and attach a copy of your SOP(s) to this protocol proposal.**

Will Zoonotic Agents be used? __ YES _X_ NO

List agents:__._____

Has request for use of agents been submitted to the Institutional Biological Recombinant DNA Safety (IBRDS) Committee? ___ YES ___ NO

If not, please contact either Dr. Greg Hayes, Biological Safety Manager, at (225) 578-4658 / ghayes@lsu.edu in the Office of Occupational and Environmental Safety; or Dr. Gregg Pettis, Chair of the IBRDS, at (225) 578-2798 / gpettis@lsu.edu in the Department of Biological Sciences.

Will Recombinant DNA and/or Virus Vectors be used? __ YES _X_ NO
List:_____

Has request for use been submitted to the IBRDS Committee? __ YES _X_ NO (Pending)

If not, please contact either Dr. Greg Hayes, Biological Safety Manager at (225) 578-4658 / ghayes@lsu.edu in the Office of Occupational and Environmental Safety; or Dr. Gregg Pettis, Chair of the IBRDS, at (225) 578-2798 / gpettis@lus.edu in the Department of Biological Sciences.

Will radioisotopes be used? ___ YES _X_ NO

List isotope(s):_____

Are you certified by the Radiation Safety Committee? ___ YES ___ NO

Will hazardous chemicals be used? _X_ YES __ NO

List compound(s): 2,6-dichloro-4-nitroaniline; 1,4-benzoquinone; 2-chloro-1,4-benzoquinone; 4-hydroxychlorothalonil; propiconazole; flutolanil_____

Please note that approval from the Mr. Jerry Steward, Chemical Safety Manager, is required when using hazardous chemicals in the animal facilities. You can contact him at (225) 578-5640 / jsteward@lsu.edu regarding a list of hazardous chemicals, and approval of these chemicals.

**SECTION 7: Type of Project and Narrative Statement**

| | |
|---|---|
| | **TYPE B –** – Animals being bred, conditioned, or held for use in teaching or research but not yet used for such purposes – a herd of cattle/horses/goats on a station on which no data is collected; non research protocol for animals that may be transferred to an experimental protocol; a field study that involves observation only. |
| | **TYPE C -** Animals for teaching, research, experiments involving NO pain, distress, or use of pain relieving drugs. Pain or distress will not be induced; animals will only be used for routine animal husbandry, non-irritating injections, standard blood collections, or procedures causing nothing more than minor discomfort. If tissues are to be collected, animal is first humanely euthanized. |
| | **TYPE D -** Animals for teaching, research, experiments involving pain or distress which is relieved by appropriate therapy, e.g. tranquilizers, analgesics, anesthetics, or euthanasia. |
| X | **TYPE E -** Animals for teaching, research, experiments involving pain or distress and where drug intervention would interfere with the test protocol **(If this block is checked, specific justification MUST be provided here.)** |

**All projects must complete number 1 below**: Federal regulations mandate that you provide **written, narrative statements** for all projects and state that this is not a duplication of previous work.

1. You must state that **"the proposed activities do not unnecessarily duplicate previous experiments"**. In this statement, include sources used to make such a determination (e.g., Databases, workshops, expertise in the field, etc.) If an electronic database was used, include database, years and words searched, and date of search.

Non-duplication statement: The proposed activities do not unnecessarily duplicate previous experiments.

Database used:_____PUBMED and Current Research Information System (CRIS)
Years searched:_____2017_____
Words searched:_____Phototoxicity; Fish; __
Date of search:_____3/24/2017_____

Note: Address the following items only if you indicated project **Type D or E**.

2a. You must indicate that you have considered alternatives to procedures producing more than momentary or slight pain or distress. Describe any alternatives available and why they are not appropriate.

**We have considered other approaches for this project, but the fish model is not replaceable for marine toxicology studies. Since it is a toxicological study, any kind of anesthetic or analgesic treatment will interfere with the experiments. There are no alternative methods other than direct contact of chemicals in water.**

2b. If this is a Type E project, describe the anticipated effects of pain or analgesia on the research model. That is, how might pain or analgesics alter and possibly invalidate the research model?

**The toxicants applied to fish may cause disorders in nervous, endocrine, circulating, or other systems of fish. In most cases, toxicants are lethal to fish subjects but some may cause irritation of fish individuals. The behavioral changes of fish with treatment of toxicant are also important parameters to evaluate the toxicities of the compounds in this project. Any pain relieving analgesic chemicals will invalidate the results of experiments.**

3. Describe the methods you used to determine that alternatives to such procedures were not available (Databases, years and words searched, date of search etc.). Put your statements in the block below.

Database used: PubMed
Years Searched: 2007-2017
Words Searched: Phototoxicity; Fish; Models
Date of Search: 3/24/2017

**\SECTION 8: Animal Treatment Checklist**
Check "Yes" or "No" to each of the following questions. Provide an explanation in **Section 9 for any "yes" answers.**

| Q# | Yes | No | | Individual (s) responsible: |
|---|---|---|---|---|
| **1** | | X | Will animals be restrained? *(Restraint refers to immobilization or other restrictions to normal movement beyond momentary holding for injections, etc.)* | Not applicable |
| **2** | | X | Will animals be fasted? | Not applicable |
| **3** | X | | Are any ANESTHETICS, ANALGESICS, or TRANQUILIZERS to be used? Include drug, dose, route and frequency, and how animals will be monitored in Section 9.4. | **Who will administer?**<br>**Dr. Wei Xu and Emily Vebrosky**<br>**_____** |
| **4** | | X | Are neuromuscular blocking agents to be used? Include drug, dose, route and frequency, and how animals will be monitored in Section 9.4. | **Who will administer?**<br>**_____** |
| **5** | | X | Will surgical procedures be employed? Check all that apply! Are they:<br>Survival_____<br>Multiple-Major Survival _____<br>Multiple-Minor Survival _____<br>*Major survival surgery= Any procedure which penetrates **and** exposes a body cavity or alters function.<br><br>Terminal_____<br><br>In addition to describing surgical procedures in Sec. 9, you must indicate the time frame between multiple procedures.<br>**Note**: *Survival mammalian surgeries must be conducted aseptically, and major surgical procedures performed on non-rodent species must be conducted in a dedicated surgical facility.* | **Who** will perform surgery?<br>**_____**<br>If survival:<br>**1) Who** will be responsible for recovery of the animals?<br>_____<br>**2) Who** will maintain post-operative records?<br>_____<br>**3) Where** will records be maintained?<br>_____<br>**4) Who** will provide post-operative analgesics?<br>_____ |
| **6** | X | | Do you anticipate any adverse effects of the experimental procedures on the animals (e.g., pain, discomfort, reduced growth, fever, anemia, etc)? Explain in Sec. 9.5 | Not applicable. |

| | | | | |
|---|---|---|---|---|
| **7** | X | | Is death an endpoint in your experimental procedure?<br><br>**Note**: *Death as an endpoint refers to acute toxicity testing, assessment of virulence of pathogens, neutralization tests for toxins, and other studies in which animals are not euthanized, but die as a direct result of the experimental manipulation.* | Not applicable. |
| **8** | | X | Are there emergency treatments by the DLAM veterinary staff that would not be allowed? | Not applicable. |
| **9** | X | | Will animals be euthanized during or at the close of the study? | **Who will perform euthanasia?**<br><br>**Dr. Wei Xu and Emily Vebrosky** |
| **10** | | X | Will animals be used for antibody production? | Not applicable. |
| **11** | | X | Will Complete Freund's Adjuvant be used? **Must be scientifically justified in Section 9.** | Not applicable. |
| **12** | | X | Will other adjuvants be used? | **If yes, please specify here:** |
| **13** | | X | Will blood be collected?<br>**Note**: *Blood equal to 1.5% of the animal's body weight per 2 weeks represents the upper approvable limit, unless scientific justification is provided.* | **How often?**<br>**Volume?**<br>**Who will collect blood?** |
| **14** | | X | Will live animals be taken from approved housing facilities for procedures followed by their return later that day?<br><br>**Note:** *Animals may not be housed outside of the Vivarium (e.g. in a laboratory) overnight.* | **If yes, please specify to which building and room/rooms the animals will be taken:**<br><br>**Note:** *This room(s) must be approved for use before the animals can be brought there. Contact IACUC coordinator for list of approved rooms.* |
| **15** | | X | Will live animals be brought onto campus for demonstration, teaching, euthanasia, etc. for which no housing is required? | **If yes, please specify to which building and room/rooms the animals will be taken:**<br><br>**Note:** *This room(s) must be approved for use before the animals can be brought there. Contact IACUC coordinator for list of approved rooms.* |

**SECTION 9: Summary of Procedures**

Your response in this section should provide the reader with a complete description of how every animal to be used in this project is to be treated during every phase of the study. Your target audience is a faculty member from a scientific discipline unrelated to yours. If not hypothesis-driven, please explain reason for project. Do not use jargon. **Please answer each statement in its own expanding box.**

1 a: What is the rationale for using animals?
**i. Fish are the most common vertebrate species in aquatic toxicological investigations.**
**ii. The embryonic development of teleost fish follows the common procedure which is also employed by mammals including humans.**
**iii. Many physiological pathways employed by fish can be also seen in mammalian systems, which will be used to evaluate the toxicant toxicities.**
1 b: Why should this study be done?
**With the assistance of this model, we will be able to establish a laboratory testing system to evaluate the short and long term impacts of the ocean contaminants on organisms, which will contribute to the science of ecosystem recovery of a number of toxicants. We will also explore the physiological changes and genomic responses of the teleost embryos and juveniles to the environmental chemical toxicities. These results will help to address the questions in how the pollutants impact public health and other population health considerations and issues.**
1 c: What hypothesis will be tested?
**(1)Toxicities of ocean contaminants can be elevated with UV irradiation.**
**(2) Photoxicities of ocean contaminants will result in the delay of fish embryonic development.**
**(3) Photoxicities of ocean contaminants can lead to physiological and neurological changes of juvenile fish.**

2. Explain how and/or why the particular animal species was selected?
**Zebrafish is a classic fish model that has been widely used in biomedical and environmental toxicological studies. Fathead minnows are recommended by the U.S. Environmental Protection Agency (EPA) to be used for environmentally related research. Procedures for embryo production in zebrafish and fathead minnow have been well documented, which allow us to conveniently obtain embryos for the study. Moreover, the released genome information for these two types of fish gives us an opportunity to study the genomic responses of the fish embryo to phototoxic stresses. The inland silverside is also an EPA-approved marine vertebrate which is commonly used in acute and chronic toxicity tests. Compared to the freshwater fish species, the inland silverside can tolerate a wide range of salinities (5-30‰) which can well represent the estuarine environment in the Gulf of Mexico.**

3. Explain how you arrived at the number of animals to be used (e.g., power analysis in comparison studies, permitted animal limits in field studies, etc).
**We expect to test two chemical components each year for each fish. The number of the animals was estimated with power analysis using R. With an expectation of 90% power for an ANOVA with three groups (non-treated, chemical treated only, and chemical with UV**

**irradiation), and a large effect size (0.4), we will need a total number of 28 individuals per treatment group to reach the usual 0.05 criterion of statistical significance. Plus 10% of accidental loss of fish during maintenance and experiment, we propose a total number of 30 individuals for each treatment group of each chemical compound. Therefore, two compounds with 3 treatment groups in each compound will come with 180 individuals. The total number of fish of each species in each year is 360 to cover the tests for two compounds using each fish species. Since we are going to perform tests with three species of fish, a total number of 1,080 fish individuals will be requested each year.**

4. Provide a complete description of the proposed use of the animals. Describe the experimental design of the study. Include a list of any physical, chemical or biological agents (name, dose, volume, route, frequency) that may be administered. If animals are being transported between facilities, describe conditions of transport. If multiple surgical procedures are planned, you must include the time frame between those procedures. If food or fluid restriction and/or restraint are used, you must include the duration of each. Use tables and outlines to indicate group assignments and study progression.

**Both fish embryos and juvenile fish will be used as experimental subjects. The embryos or juvenile fish will be maintained in 10 gallon aquarium tanks in ARS. The tanks will be enclosed in a chamber to avoid the interference of natural lights. The light treatments will be applied using a light bulb with emission wavelengths between 300 and 800 nm to accurately simulate the distribution of sunlight. Reflective materials will be used inside the chamber to evenly distribute the light strength to each tank. The light will be temporarily turned off during sample monitoring and collections to protect the investigators.**

**Embryos will be produced with adult zebrafish and fathead minnows. The fertilized eggs will be collected and treated with chemical compounds. Development of the embryos will be monitored and used as a parameter for toxicity evaluation. The embryos in each treatment group will be checked under a microscope at 0, 4, 8, 16, and 24 hr post the start of treatment. Thereafter, the embryos will be checked every 24 hr until hatched. For each observed embryo, pictures will be taken and its development stage will be determined following the protocol for fish embryo staging. All the fish individuals will be harvested within 48 hr post fertilization. The mortality rate of the embryos in each group will also be recorded at each time point. Phototoxicity of each chemical will be determined by the dose that causes cause 50% death of embryos. Meanwhile the percentage of successfully hatched embryos in each group will be calculated. Any signs for embryo maldevelopment in each treatment group, such as pericardial accumulation, edema, or tissue lysis, will be recorded. Values for mortality rates, hatching rates, and maldevelopment rates will be used to evaluate the phototoxicities of the tested chemicals.**

**The following chemical compounds that are commonly found as ocean pollutants will be tested: 2,6-dichloro-4-nitroaniline; 1,4-benzoquinone; 2-chloro-1,4-benzoquinone; 4-hydroxychlorothalonil; propiconazole; flutolanil.**

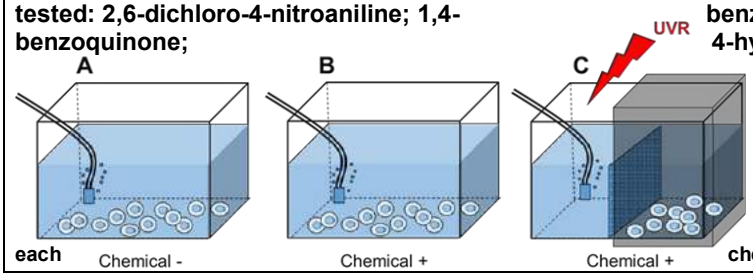


**Fig. 1. Phototoxicity studies on fish embryos. 50-60 embryos will be put in each tank for the test of each chemical compound. Samples will**

**be harvest immediately post hatching.**

**Juvenile zebrafish, fathead minnows, and inland silverside will be also used for chemical phototoxicity studies. The juveniles will be exposed to chemical compounds with or without UV irritation. The mortalities of each group of fish will be monitored daily. Phototoxicity of each tested chemical will be determined based on the dose of modified chemical applied to the juveniles. The half-life of UV activated chemical is usually very short, therefore the lethal effect of the modified chemical is expected to be observed within several hours post treatment. Juveniles will also be treated with a sublethal dose of each chemical to observe the changes in behaviors and physiology of juveniles. Fish will be checked at 0, 4, 8, and 16 hr post treatment at day 1, and every 24 hrs thereafter. At each time point, the mortality rates of the juveniles will be recorded. The movement of the survivors will be visualized under a stereomicroscope and recorded by an attached high-speed camera. The movement of each fish within one minute will be tracked and analyzed using TrackMate in ImageJ. The map of movement for each fish will be used to evaluate the impact of the toxic components to the fish nervous system. Juveniles will be all harvested if If a significant difference is seen between treatment and control groups, fish will be all harvested. Otherwise juveniles will be harvested at day 7 post treatment. Juvenile fish will be euthanized by overdosing of MS-222 (200-300 mg/L) prior to sample collection. Doses of modified or non-modified chemicals expected to cause 50% death of juveniles will be used to evaluate the toxicities.**

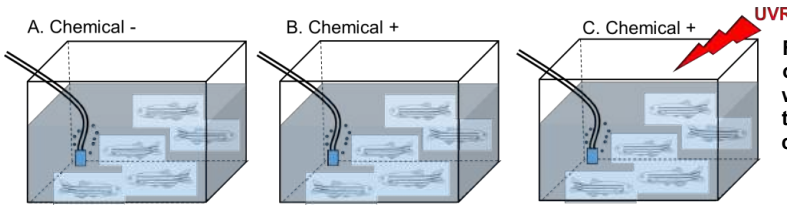


**Fig. 2. Phototoxicity studies on juvenile fish. 30 individuals will be kept in each tank for the study of each chemical compound.**

**Zebrafish will be kept in Room 142A at the Aquaculture Research Lab. The fathead minnows and inland silverside will be maintained in Room 118A which is located in the north wing of the ARS building. The aquaria can be used to keep freshwater or saltwater fish species with the temperature regulated through heat radiator and air-conditioning. The feed for fish is kept at the dry lab connected to this wet lab. The power of the wet lab is backed up with generator to maintain the continuous electricity supply during a power outage. The fish and water quality is monitored by the PI and students in the lab every day.**

**Fish are monitored daily and water quality parameters assessed on a regular basis to maintain adequate parameters for pH, ammonia, salinity, temperature, nitrite, alkalinity, and hardness. The water quality will be tested once every day during the weekday. There is a protocol in place for testing water quality. Re-circulating, closed tank systems are used. Each tank has its own drain. Water is collected from each tank to a central sump tank, and water is pumped into a biological filter to balance ammonia and nitrite levels. Water is then distributed from the biological filter to each tank through a faucet over the tanks. Room temperature is maintained at 26 °C, and the light cycle is controlled with timer.**

**Chemicals are from Sigma Aldrich and are in solid form (powder), they are kept in the dry chemical cabinet in Dr. Armbrust's lab at the Environment and Coastal Engineering. Stock solutions are kept in the lab fridge in borosilicate glass containers and prepared by authorized people only using the dry chemical and dissolved in acetonitrile or water**

**(dependent on the chemical). Chemicals will be all prepared in a hood in the lab with proper protections (gloves, goggles, lab coat).**

**The chemical containing water will be collected with biohazard buckets and disposed by the Department of Environmental Health and Safety through standard procedures.**

5 a: Describe expected adverse effects.
**The toxicities of the chemical compounds may result in the death or irritation of juvenile fish.**
5 b: What is the likelihood of these effects (high, low, unknown)?
**High.**

6. Describe procedures designed to assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research. For anesthesia and survival surgeries, include a description of post-procedural care and monitoring. Indicate how analgesic, anesthetic, and tranquilizing agents will be used where appropriate, to minimize discomfort and pain to the animals. Include any conditions where veterinary treatment would not be allowed. Specify which treatments would not be allowed, and include a scientific justification. It is advisable that you obtain input from LSU's or the AgCenter's Attending Veterinarians (Drs. David Baker or Diana Coulon) or from another veterinarian familiar with the species to be used.

**The toxicant treatment to juvenile fish is likely to cause irritation. However, the behavior changes will only be recorded in a short period of time (less than 10 min) for the study of neuron toxicity with video recording. Thereafter, the juvenile fish used for recording will be immediately sacrificed. If the movement of treated and non-treated fish showed a clear difference based on the ImageJ analyses, all the rest of the fish will be harvested. Otherwise, observation will be continued daily until day 7 post treatment. Fish embryos and juveniles will all be harvested at the end of the experiment.**

7. Describe any euthanasia method to be used; even if euthanasia is not planned, please provide a "what if" solution in the event of unforeseen circumstances. Justify any deviation from AVMA Guidelines on Euthanasia, 2013.

Text, viewable at https://www.**avma**.org/KB/Policies/Documents/**euthanasia**.pdf

**For embryos ≥ 15 days post fertilization (dpf), immobilization of fish will be performed by submersion in ice water (5 parts ice/1 part water, 0-4º C) for at least 20 minutes to ensure death by hypoxia. Alternatively, overdose of buffered tricaine methane sulfonate (MS222, 200-300 mg/l, buffered with sodium bicarbonate to pH 7.0-7.5.) can be used for at least 10 minutes following cessation of opercular movement.**
**For embryos < 15 dpf, addition of bleach solution (sodium hypochlorite 6.15%) to the culture system water at 1 part bleach to 5 parts water will be used for at least five minutes following 20 minute ice water immobilization to ensure death.**

**SECTION 10: Investigator Training**

In accordance with IACUC policy, all personnel conducting animal-based research must participate in an online Rules and Regulations Course and verify their training, experience and skills in the care and use of the animals and techniques for which they are responsible.
List all persons involved in the experimental animal care and use for this study below. Add additional lines as needed.

| Name | Rules & Regulations Course* (Indicate Yes or No) | Date taken: | Training or Experience?** (Indicate Yes or No) | Brief explanation of training/experience |
|---|---|---|---|---|
| Wei Xu | Yes | 10/9/2015 | Yes | Clinic and diagnosis of fish diseases; fish handling and dissection. |
| Kevin Armbrust | Yes | 4/20/2017 | Yes | Years of experience in environmental toxicology and petroleum chemistry |
| Emily Vebrosky | Yes | 4/20/2017 | Yes | Several years of graduate student training for toxicology and environmental sciences |
| Laura Basirico | Yes | 4/20/2017 | Yes | Several years of graduate student training for toxicology and environmental sciences |

* Personnel participating in the project must complete the Rules and Regulations course once every three years. Those who have not taken the Rules and Regulations course will have **six (6) weeks** from the approval date of the project to complete it. A copy of the AALAS Learning Library certificate or the AgIACUC quiz must be sent to the AgIACUC secretary for verification of compliance.

Rules and Regulations Courses are ONLINE either through the LSU IACUC for AAALAC/OLAW approved units (www.aalas.learninglibrary.org - contact Ms. Best-Desjardins at 578-9643 or dbest@vetmed.lsu.edu for directions to sign up) or for other projects, please use the AgCenter LAES intranet web link.

****The person named has training/experience in assigned procedures for this protocol. It is the PI's duty to verify that all personnel involved in the project have received adequate training for their responsibilities prior to participation in animal procedures.**

Who will train individuals for participation in protocol procedures? Answer in the block below.

| |
|---|
| Wei Xu |

Revised March 2014

**SECTION 11: Occupational Health and Safety**

It is the responsibility of the principal investigator to conduct a hazard analysis and risk assessment to determine if personnel involved in the proposed study should participate in the Occupational Health and Safety Program administered through DLAM or the RBC and the Student Health Center. Currently, there is no direct cost for participation in the program**. All persons listed in Section 10 must read the following and indicate level of participation with their signature. Add additional rows in the table as needed.**

The Division of Laboratory Animal Medicine operates an Occupation Heath Program (OHP). Participation is voluntary and is open to all personnel with direct or indirect contact with animals used in teaching and research, their bodily products, or materials to which they may be exposed, as described in this protocol. Eligible persons include facility services personnel, animal caretakers, principal investigators, technical staff, graduate and other student workers, and post-doctoral and visiting scientists. All medical information is kept confidential and is retained by the Student Health Center. You have the right to refuse any and all procedures recommended.

To determine the extent of your participation in the OHP, discuss with the principal investigator named on this protocol, and/or your health professional, any potential physical, chemical, or infectious hazards to which you may be exposed while working on the project. Whether or not you participate, questions related to health risks should be directed to Dr. Tim Honigman, Campus Physician, at the Student Health Center.

If you are at increased risk of illness or injury due to drug-related immune suppression, HIV infection, pregnancy, concurrent illness, musculoskeletal problems, etc., you are advised to discuss your risks with Dr. Honigman, your physician, or another health professional.

To participate in the OHP, contact Ms. Dawn Best-Desjardins at 578-9643 or dbest@vetmed.lsu.edu for information or for RBC protocols, please contact Ms. Sonyja Thomas at 225-642-5474 or SRThomas@agcenter.lsu.edu.

| | | |
|---|---|---|
| Printed Name:<br>Wei Xu | Signature: | ____I choose to participate<br>__x__I choose NOT to participate |
| Printed Name:<br>Kevin Armbrust | Signature: | ____I choose to participate<br>__x__I choose NOT to participate |
| Printed Name:<br>Emily Vebrosky | Signature: | ____I choose to participate<br>__x__I choose NOT to participate |
| Printed Name:<br>Laura Basirico | Signature: | ___I choose to participate<br>_x__I choose NOT to participate |

## VITA

Emily Noelle Vebrosky, of Frackville, PA, received her Bachelor of Science from Lycoming College in Williamsport, PA in Chemistry with a minor in Environmental Science in 2014. She received her Master of Science from Louisiana State University in Baton Rouge, LA in Environmental Sciences in 2016. She continued at LSU with her major professor, Kevin Armbrust, to pursue a Doctor of Philosophy in Environmental Sciences with a minor in Wetland Science and Management. Emily's love and interest for the environment started at a young age while trout fishing with her dad and continued to grow and flourish through high school and college. At Lycoming College, she had the opportunity to work with Dr. Mel Zimmerman and the Clean Water Institute on trout population studies (among other projects) throughout the state of Pennsylvania. This work solidified her decision to continue her schooling within the studies of environmental science and carry her love of fish and aquatic systems throughout that work. Emily has had many great educational and research opportunities throughout her studies at both Lycoming College and Louisiana State University and was able to work with a large group of very knowledgeable professionals who helped to teach her various aspects of their expertise within her education.