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Assessing the sublethal impacts of sulfoxaflor on the physiology and behavior of Daphnia magna

Mary M. Hoffman

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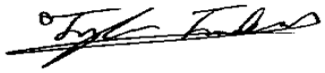

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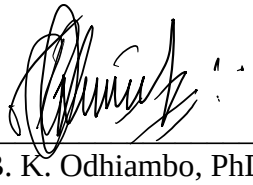

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Assessing the sublethal impacts of sulfoxaflor on the physiology and behavior of *Daphnia magna*

By

Mary Hoffman

Thesis submitted to the faculty of the University of Mary Washington
in partial fulfillment of the requirements for graduation with
Honors in Earth and Environmental Sciences
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ABSTRACT

Agricultural pest species are a growing concern due to increasing resistance to neonicotinoids. Sulfoxaflor, a sulfoximine pesticide recently approved by the USEPA, was developed to replace neonicotinoids and has shown to have high efficacy in the field. Environmental introduction is primarily caused by wet spray application or agricultural runoff. Sulfoxaflor binds to insect nicotinic acetylcholine receptors, triggering overactivation that leads to paralysis and death. Preliminary exposure studies have shown neonatal effects and development of liver tumors in rats and mice at 500 and 750 ppm, respectively. Little research into the effects on aquatic nontarget invertebrates has been conducted; as such, this research aims to identify potential physiological and behavioral impacts of sulfoxaflor on juvenile *Daphnia magna* at concentrations of 0, 0.1, 0.5, 1, 5, and 10 µg/L. HPLC analysis indicated that sulfoxaflor does not readily degrade under laboratory conditions. Despite low sample sizes, trends in increased mortality and length of apical spine were observed for 7-day exposures. Potential decreases in heart rate and mobility parameters such as average speed, acceleration, and total distance after 7-day exposures were also identified. This research aims to help elucidate the potential sublethal impacts of sulfoxaflor on non-target aquatic invertebrates at environmentally relevant concentrations.

ACKNOWLEDGEMENTS

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faced with unexpected challenges, to give his students the best and most fulfilling college adventures of any other department, and in my humble opinion, any other college.

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CHAPTER 1 – INTRODUCTION

PESTICIDES

As worldwide agriculture grows to meet increasing food demands, insect pest populations have proven to be a significant concern. Pesticides such as neonicotinoids, a recently developed class of insecticides, have been used to treat and reduce the impact of pests on agricultural yields. Due to the prevalence and continued use of these insecticides, observed resistance to these chemicals is also beginning to rise (Oliveira et al. 2014; Xu et al. 2016). In addition to pest resistance, studies have shown that these pesticides cause significant harm to bee populations, pollinators that are vital to agriculture and the environment (Blacquière et al. 2012). Imidacloprid and clothianidin are some of the most widely used neonicotinoid pesticides, with over 2 million pounds and 3.5 million pounds respectively being applied across the US in 2014 (USGS 2016). Imidacloprid has widespread use in agriculture including applications on cotton, corn, wheat, soybeans, various vegetable crops, and grape or other fruit orchards (USGS 2016). Clothianidin is primarily used on soybeans and corn, with potential for use on other crops including vegetables and fruit (USGS 2016). Both pesticides have been shown to break down into metabolites that have cyto- and genotoxic effects, suppress immune systems, and stunt growth and reproduction in non-target vertebrate organisms at environmentally relevant concentrations (Gibbons et al. 2015). Reduced sperm count in rats was observed with clothianidin at 32 mg/kg bw/d (Bal et al. 2012). Treatments between 31.2 and 36.8 mg/kg bw/d of clothianidin was also shown to decrease body weight and sexual maturation in males and increase the number of stillbirths in females (EPA 2010). In red-legged partridges (*Alectoris rufa*), imidacloprid caused a reduction in egg width and eggshell

thickness, decreased chick survival rates, and reduced egg fertility at 31.9 mg/kg/day (Lopez-Antia et al. 2013). After 30 hours of exposure to 0.1 mg/L of fipronil, zebrafish larvae demonstrated notochord degeneration, decreased body length, and decreased locomotion (Stehr et al. 2006). While not specifically a neonicotinoid, fipronil has many of the same practical applications and similarly targets insect nicotinic acetylcholine receptors. Imidacloprid has been proven in acute exposures (48 hours) to induce oxidative stress on *Daphnia magna* at concentrations as low as 13-16.5 mg/L (Qi et al. 2018).

SULFOXAFLOR

In order to address increased insect resistance to neonicotinoid pesticides, sulfoxaflor was developed by Dow AgroSciences under the name of Isoclast™ Active as the first sulfoximine pesticide (Dow 2014). It is marketed as a valuable addition in rotational use as insects currently resistant to other pesticides showed no signs of cross-resistance to sulfoxaflor in preliminary studies (Dow 2014). In May 2013, the EPA approved registration of sulfoxaflor, but the Ninth Circuit Court of Appeals responded to the complaints of pollinator advocates who argued that the pesticide was causing mass mortality in bee populations and vacated the registration in November 2015 (EPA 2019). The EPA was instructed to supply stricter policies for use, more evidence supporting approval, and increased protection of bees (EPA 2019). In October 2016, the EPA approved the final registration of sulfoxaflor with restrictions on which crops sulfoxaflor is permitted to be used and the establishment of specific time frames for application: on crops non-attractive to bees, on crops harvested before bloom, and on bee-attractive crops post-bloom only (EPA 2019).

As sulfoxaflor is required to undergo a "drying period" after being applied as a wet spray, rain events are likely the main factor that introduces the pesticide into aquatic environments. Sulfoxaflor has a half-life of 11-64 days in aquatic environments, and application rates on crops range between 12 to 150 grams of active ingredient per hectare (Dow 2014). A benefit of sulfoxaflor in crop application is that it can impart similar benefits while requiring lower usage rates (Dow 2014). Sulfoxaflor binds to insect nicotinic acetylcholine receptors (nAChR), causing overactivation of the receptors which leads to paralysis through the central nervous system and ultimately death (Babcock et al. 2011; Sparks et al. 2013).

Due to the relatively recent development of sulfoxaflor, no studies have been done to examine the presence and concentrations of sulfoxaflor in aquatic environments. Preliminary studies conducted by Dow AgroSciences for the potential toxicological effects were provided for rats and mice, showing neonatal effects in rats and the development of liver tumors in both rats and mice after prolonged dietary exposure at 500 and 750 ppm, respectively (Lebaron et al. 2014). Slight effects to the growth of fathead minnow and moderate oral toxicity in birds were also identified above 5.05 mg/L and at 5,260 mg/kg, respectively (Dow 2014). While significant testing on non-target organisms (excluding bees) has yet to be performed, preliminary research performed by Dow AgroSciences on *Daphnia magna* has shown an acute 48-hour EC_{50} of > 399 mg/L, and a chronic 21-day NOEC of 50 mg/L (Dow 2014). Little research into sub-lethal effects for aquatic invertebrates has been conducted since its approval for use by the EPA, which concluded that sulfoxaflor would have little effect on aquatic species (EPA 2019).

TEST ORGANISMS

Daphnia magna has been selected due to its role as an EPA-recommended model organism. They are ideal for toxicity testing including reproductive endpoints due to their cyclical parthenogenesis, which establishes that most offspring produced by females are genetically identical (EPA 2002). In the presence of stressors such as high density or low food, *D. magna* produce resting eggs known as ephippia, which serve as a clear marker of reproductive stress (EPA 2002). Additionally, the production of male offspring and opportunity for sexual reproduction (which is required for ephippia) occurs as a response to extreme conditions, offering an additional insight into how toxicants can induce reproductive stress on daphnids (EPA 2002). *D. magna* possess four unique life stages: egg, juvenile, adolescence, and adult, with broods consisting of 6-10 eggs (EPA 2002). The species is also ideal for assays analyzing heart rate and metabolic processes due to their transparency, making analysis noninvasive and simple (Colmorgen and Paul 1995). Ease of culture, sensitivity to various pollutants, and ease of commercial access make *Daphnia magna* an ideal model organism for toxicity testing (EPA 2002), particularly regarding sulfoxaflor, which is intended to have a higher specificity for invertebrates (Dow 2014). In-depth knowledge of the species in terms of genetics, reproductive systems, and responses to stressors (EPA 2002) allows for strong points of comparison for behavioral and reproductive alterations caused by environmental toxins.

SUBLETHAL ENDPOINTS

Given the mode of action of sulfoxaflor, key endpoints useful in analyzing the sublethal impacts of sulfoxaflor include mobility parameters, heart rate, and organism growth. Mobility analyses help determine whether the mode of action of sulfoxaflor is shared

between pest organisms and non-target aquatic invertebrates, and whether run-off from fields at environmentally relevant concentrations is of concern for the health of non-target species. A study conducted on the blue-tailed damselfly (*I. elegans*) in 2019 discovered that thiacloprid levels above 1.0 ug/L led to a decrease in mobility and swimming activity of nymphs (Barmentlo et al. 2019). Heart rate analyses give valuable insight into the physiological impacts of exposure and how survival may be adversely affected in the wild, such as paralysis leading to higher rates of predation. The pesticide Lamba-cyhalothrin has been shown to decrease heart contraction frequency in *Daphnia magna* above 5 ug/L (Bownik et al. 2019). Both apical spine length and body size have been shown to increase as a morphological response to predator presence in *Daphnia magna*, with apical spine length being the most prominent defense for juvenile *D. magna* (Rabus et al. 2013). The apical spine length, body size, and the ratio of these two parameters were used to determine investment in growth, and to determine if chemical exposure induced stress similar to predatory pressure. Investment in body size and apical spine length may detract from investment in other primary functions such as reproduction, negatively impacting the overall fitness of the species as a result of exposure.

This study aims to identify the presence and degradation of sulfoxaflor in water using HPLC as well as the potential sublethal effects of sulfoxaflor on *Daphnia magna* by analyzing various endpoints including mobility, heart rate, and growth.

CHAPTER 2 – MATERIALS AND METHODS

ANIMAL CULTURE

Adult *Daphnia magna* were purchased from the Carolina Biological Supply Company and maintained in the Jepson Science Center at the University of Mary Washington. Cultures were kept at a pH between 6 - 8.5 and an optimal temperature range between 20 ± 2°C (EPA 2016). Dissolved oxygen levels were maintained at > 3 mg/L and a consistent photoperiod of 16 hours light: 8 hours dark (EPA 2016). Cultures were fed a diet of lyophilized spirulina algae meeting dietary needs of 0.2 mg of carbon a day per daphnid (EPA 2016) with feedings taking place once every two days. Cultures were maintained in synthetic water, a mixture of deionized water and necessary ions (See Table 2.1). Adult *Daphnia* with visible eggs were separated out from the main population in a one-liter beaker, and juveniles born within 24 hours from the separated population were then used for experiments.

Chemical Formula	Concentration (g/L)
$NaHCO_3$	0.192
$CaSO_4 \cdot 2H_2O$	0.120
$MgSO_4$	0.120
KCl	0.008

Table 2.1: Synthetic water composition; concentration of necessary ions added to deionized water for use in animal culture and exposure studies.

CHEMICAL CONCENTRATIONS

Due to the recent nature of its development, there is no information regarding the environmental concentration of sulfoxaflor. As such, experimental concentrations are based upon the environmental concentrations of the previous class of pesticides, neonicotinoids. Various neonicotinoid chemicals have been detected in water bodies ranging in concentration from 0.001 to 225 µg/L, though data from a combination of studies reported a geometric mean of 0.13 µg/L as an average concentration in surface water (Morrissey et al. 2015). Based on these values, treatment concentrations were selected to be 0, 0.1, 0.5, 1, 5, and 10 µg/L. Lyophilized sulfoxaflor powder (CAS:946578-00-3) was purchased from LGC Standards in 10 mg quantities and dissolved in 10 mL of 100% ethanol vehicle (CAS 64-17-5), creating a 1,000,000 µg/L stock concentration. Serial dilutions were performed with ethanol to create subsequent superstocks for concentrations of 500,000 µg/L, 100,000 µg/L, 50,000 µg/L, and 10,000 µg/L. Treatment concentrations were then created using a 1:100,000 dilution from each superstock with water to control for total amount of ethanol in each treatment. A 10mL ethanol control was also prepared for the 0 µg/ treatment. All chemical was stored in 60 mL amber bottles and wrapped with parafilm, and stored away from light exposure in a freezer at -4°C. All exposures had 0.0001% ethanol in the final stock solutions.

CHEMICAL DEGRADATION TESTING

Given the relatively recent development of sulfoxaflor, few studies have been performed examining its degradation in water, emphasizing the importance of being able to compare nominal versus actual concentrations used over the course of the study. Thus, the first assay in the study was based on determining the degradation of sulfoxaflor in

synthetic water used for exposures to determine appropriate timing for exposure solution replacement in chronic studies. Diluted concentrations used for animal exposures were too low for detection using HPLC. As such, HPLC was conducted on intermediate solutions to assess the accuracy of the treatment concentrations over the course of each study. Samples were run on an Agilent 1100 Series HPLC System with Diode Array Detector. The UV wavelength used was 210 nm, previously determined using UV spectroscopy with the express purpose of use in HPLC. The samples were run through a C18 column with a mobile phase composition of 30/70% H_2O/MeOH. Concentrations of 0, 50, 100, 500, 1000, and 1500 µg/L were analyzed over four samples of two injections each in order to determine a standard curve for the chemical in synthetic water. The standard curve was then used to determine retention time of 1000 µg/L of the chemical in synthetic water after 0, 24, and 48 hours and in a 1000 µg/L solution created from ethanol superstocks stored for 3 months, 6 months, and 12 months in order to ensure accuracy of treatment concentrations during exposure trials.

ASSAY SETUP

Assays included two different exposure trials drawn from same stock of juveniles: an acute 48-hour trial and a chronic 7-day trial. All 48-hour exposures were prepared in 50 mL beakers with 20 mL of a given test solution. Juvenile *Daphnia magna* under 24 hours of age were collected from culture and distributed between the test groups, with one individual per beaker. Test containers were covered in tinfoil with holes for air exchange and kept in ambient air temperatures within ideal range over the course of the study. All 7-day exposures followed the same procedure, with the addition of static replacement of treatment solution every 48 hours, determined to be appropriate as a result of the HPLC

degradation assay. During each static replacement, feeding was conducted using newly prepared test solution including spirulina powder equivalent to 0.4 grams of carbon (2x daily amount) (EPA 2016). Mortality was assessed daily and heart rate, mobility, and growth assays were conducted at 48 hours and 7 days, respectively. Tested individuals were used for all assays in the following order: mortality, mobility, heart rate, growth.

RANGEFINDER ASSAY

At 48 hours, test groups were checked for mortality. Signs of paralysis or death were determined by use of a well slide and compound microscope. As paralysis is a potential response to exposure, all immobile specimens were checked for viability defined as the observation of a heartbeat and eye movement. Paralyzed individuals were considered alive and included in analyses. Data was analyzed based on total mortality observed at each concentration, with the intent to define or specify the no observed effect concentration (NOEC), the lowest observed effect concentration (LOEC), the lethal concentration where 20% of individuals exhibit mortality (LC_{20}), or lethal concentration where 50% of individuals exhibit mortality (LC_{50}), as well as to determine whether the chosen concentrations were appropriate for use with subsequent assays.

MOBILITY ASSAY

Individuals were placed in a 50 mL beaker with 5 mL of synthetic water, then placed within an environment with a consistent light source. A 3-minute acclimation period was utilized to minimize disruptive effects from handling. A Logitech HD Pro Webcam C920 camera was utilized to take a top-down video of the beaker using the LogiTech Capture (v1.01.19) program from a connected computer. After taking a 3-minute recording, the

program ToxTrac (v2.84) was used to analyze the video and calculate data regarding mobility parameters over the first minute for each individual daphnid (See Table 2.2). Tracking output was used to conduct quantitative analysis (See Figure 2.1).

Mobility Parameters	Description	Units
Average speed	Average speed of individual during recording	mm/s
Average mobile speed	Average speed of individual only when in motion	mm/s
Average acceleration	Average acceleration of individual from standstill	mm/s^2
Total distance	Total distance traveled by individual during recording	mm
Total frozen events	Total number of times spent immobile longer than 0.5 seconds	# total instances

Table 2.2: List and description of mobility parameters identified by ToxTrac program.

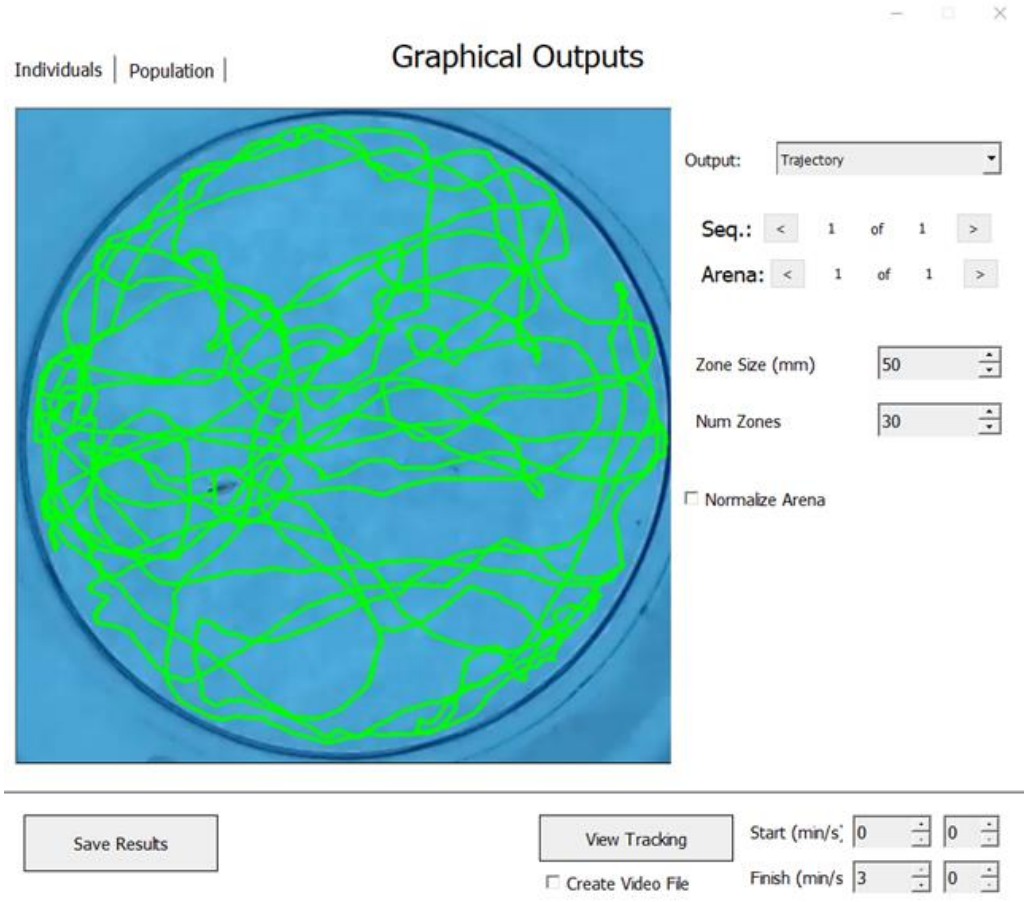

Figure 2.1: Graphical output of ToxTrac program; green line indicates tracked path of single *Daphnia magna* over the course of a 3-minute recording.

HEART RATE ASSAY

Individuals heart rates were assessed using a compound microscope, digital microscope camera, and 3 mm well slides. An OMAX 3.0 USB microscope camera was attached to the compound microscope and a computer with the ToupView (x64, v4.7) program used to obtain video footage. A digital lux meter was used to adjust the compound microscope light intensity to 200 lux. All other light sources were removed during heart rate analyses to prevent environmental bias. An individual daphnid was placed on the well slide and immobilized using wet cotton fiber. A 5-minute acclimation time was utilized to limit impact of stress from handling and a 1-minute recording of cardiac function obtained using ToupView (See Figure 2.2). Heart rates recordings were analyzed by reducing the speed of the video to ¼ speed using Windows Movie Maker 2012 and counting individual beats to determine heart rate in beats per minute.

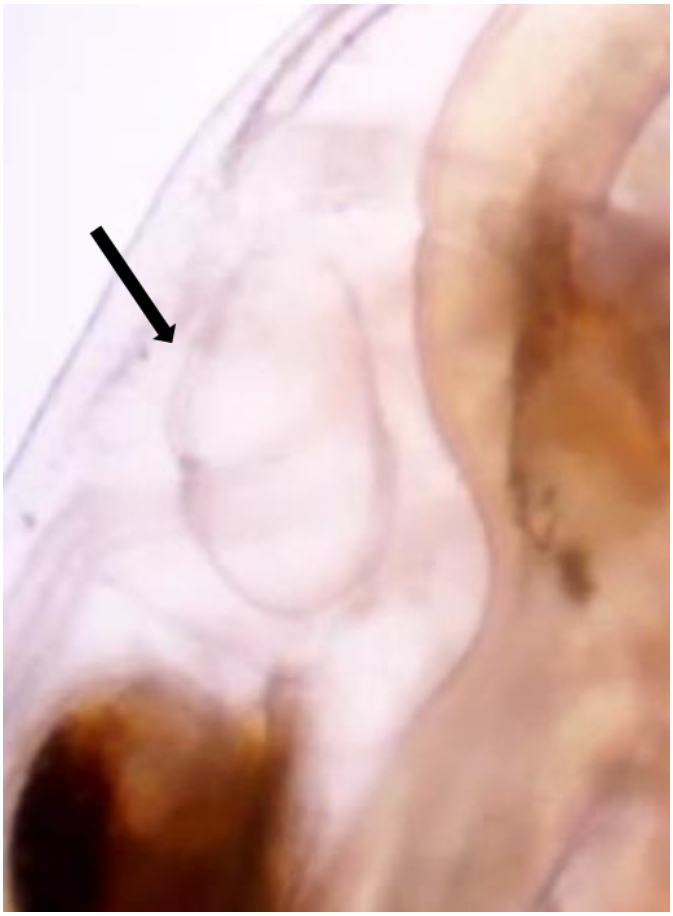

Figure 2.2: Microscope imaging of *Daphnia magna* heart visibility, indicated by arrow, using an OMAX 3.0 USB microscope camera and ToupView (x64, v4.7) program.

GROWTH ASSAY

Individuals were placed on a micrometer slide beneath a dissecting microscope. An OMAX 3.0 USB camera and ToupView (x64, v4.7) program were utilized to take a clear picture of the individual with the full length of the micrometer unobstructed. The program Fiji ImageJ (v1.8.0) was used to measure the length of the body and apical spine separately of each individual, with micrometer used to define pixel counts within the ImageJ program for accuracy of calculated lengths.

CHAPTER 3 - RESULTS

CHEMICAL DEGRADATION

A standard curve for sulfoxaflor concentration in synthetic water was successfully derived using the average value across injections, with an R^2 value of 0.998 (See Figure 3.1). The standard curve was then used to determine the actual concentrations of the various stock and treatment solutions. The actual concentration of the 1000 ug/L nominal solution created from superstock stored for 12 months ranged between 159-322 µg/L, up to an 84% decrease from the expected concentration. The actual concentration of the 1000 µg/L nominal solutions created from superstocks stored for 3 and 6 months ranged between 945-993 µg/L and 934-993 µg/L, respectively. Degradation in synthetic water was analyzed to determine appropriate timing for static replacement exposures. Initial concentrations ranged between 957-980 µg/L, with concentrations at 24 and 48 hours ranging between 1021-2092 µg/L and 1049-1090 µg/L, respectively.

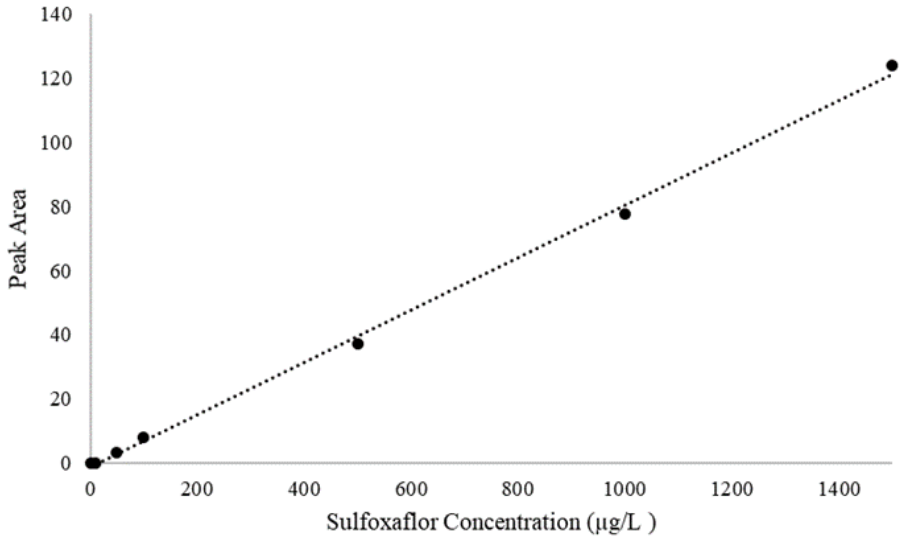

Figure 3.1: HPLC Standard Curve of sulfoxaflor at 210 nm between 0 and 1500 µg/L; $y=0.0815x -1.1197$, $R^2=0.998$.

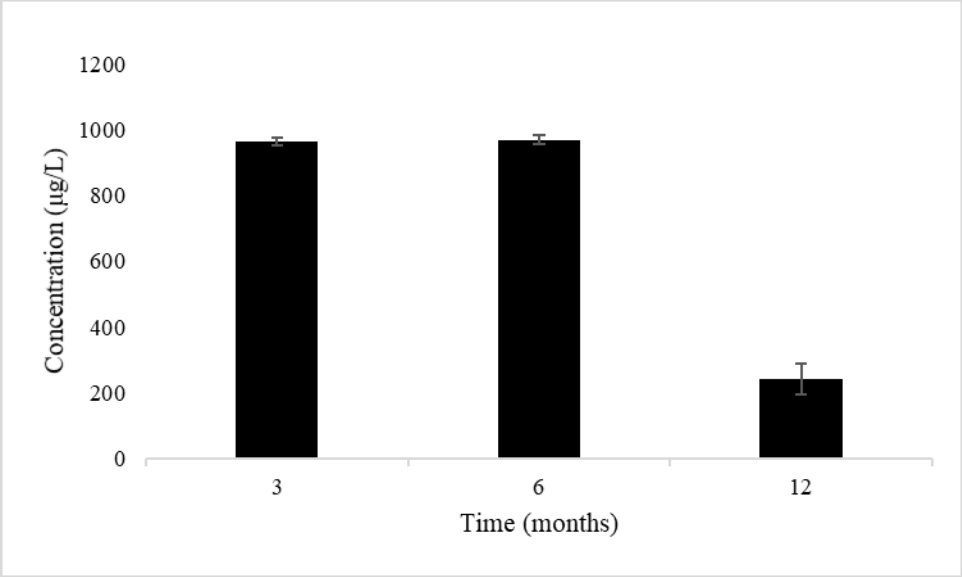

Figure 3.2: Actual concentration ± SEM through HPLC analysis of 1000 ug/L solutions created in synthetic water from stocks that had been in storage for 3, 6, and 12 months post-creation.

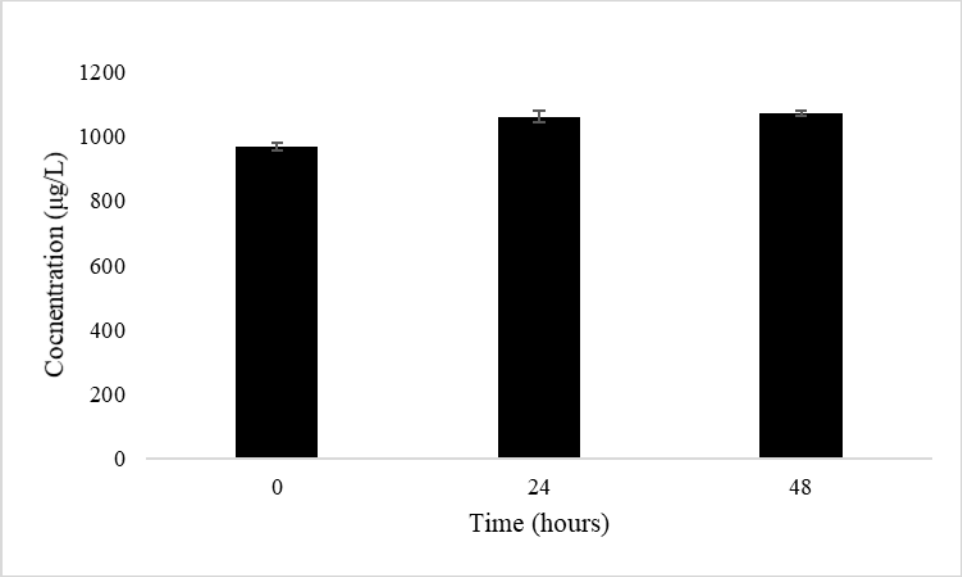

Figure 3.3: Actual concentration ± SEM through HPLC analysis of 1000 ug/L solutions created in synthetic water after 0, 24, and 48 hours of creation; error bars are SEM.

RANGEFINDER ASSAY

Mortality was assessed at 48 hours for 48-hour trials and 7 days trials. While no consistent trend in mortality was observed at 48-hours, there was a trend in mortality observed at 7 days (See Table 3.1). An LC_{50} (lethal concentration where 50% of individuals experience mortality) plot analyzed using probit analysis returned an LC_{50} value of 5.85 µg/L (See Figure 3.4).

	Mortality	
Concentration (µg/L)	48 Hours (% / # individuals)	7 Days (% / # individuals)
0	0 / 0	25 / 1
0.1	25 / 2	25 / 1
0.5	0 / 0	25 / 1
1	12 / 1	50 / 2
5	0 / 0	50 / 2
10	12 / 1	75 / 3

Table 3.1: Mortality of juvenile *Daphnia magna* after exposure to various concentrations of sulfoxaflor at 48-hours (n=8) and 7 days (n=4); percentage of total sample size experiencing mortality at each respective time point.

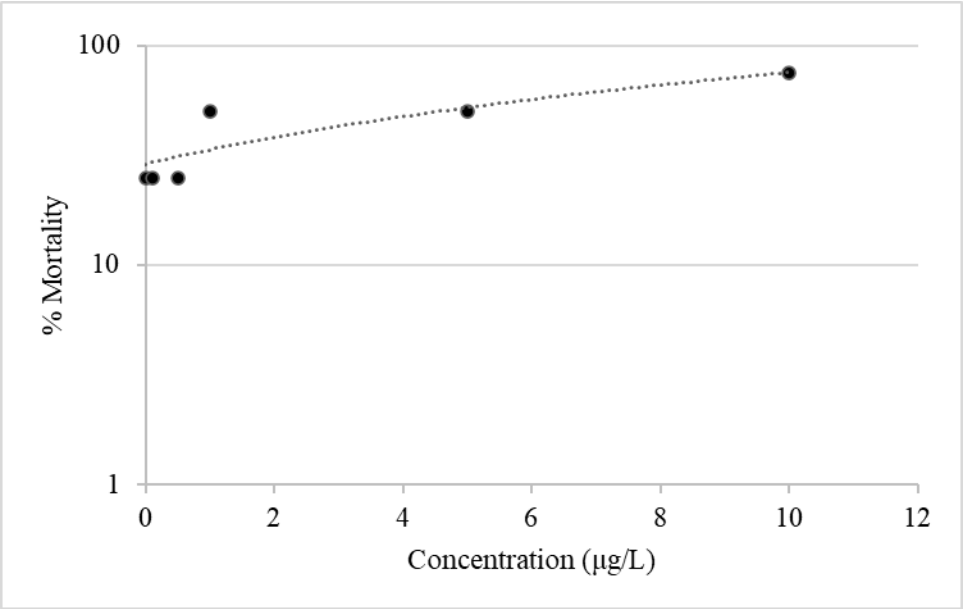

Figure 3.4: LC_{50} plot through probit analysis of *D. magna* mortality to increasing concentrations of sulfoxaflor; y = 4.6473x + 28.809 and R^2=0.8328.

MOBILITY ASSAY

Mobility was assessed after 7 days, with trends indicating decreased speed, mobility, and acceleration at higher concentration levels (See Figure 3.5). Observed trends in increased number of frozen events were observed at 1 and 5 µg/L. Results had low statistical significance due to low samples sizes of treatments at the 1, 5, and 10 µg/L treatments (n=2, 2, and 1 respectively). Due to time constraints induced by global pandemic, analyses on mobility were not able to be completed on 48-hour exposures at the time of thesis finalization.

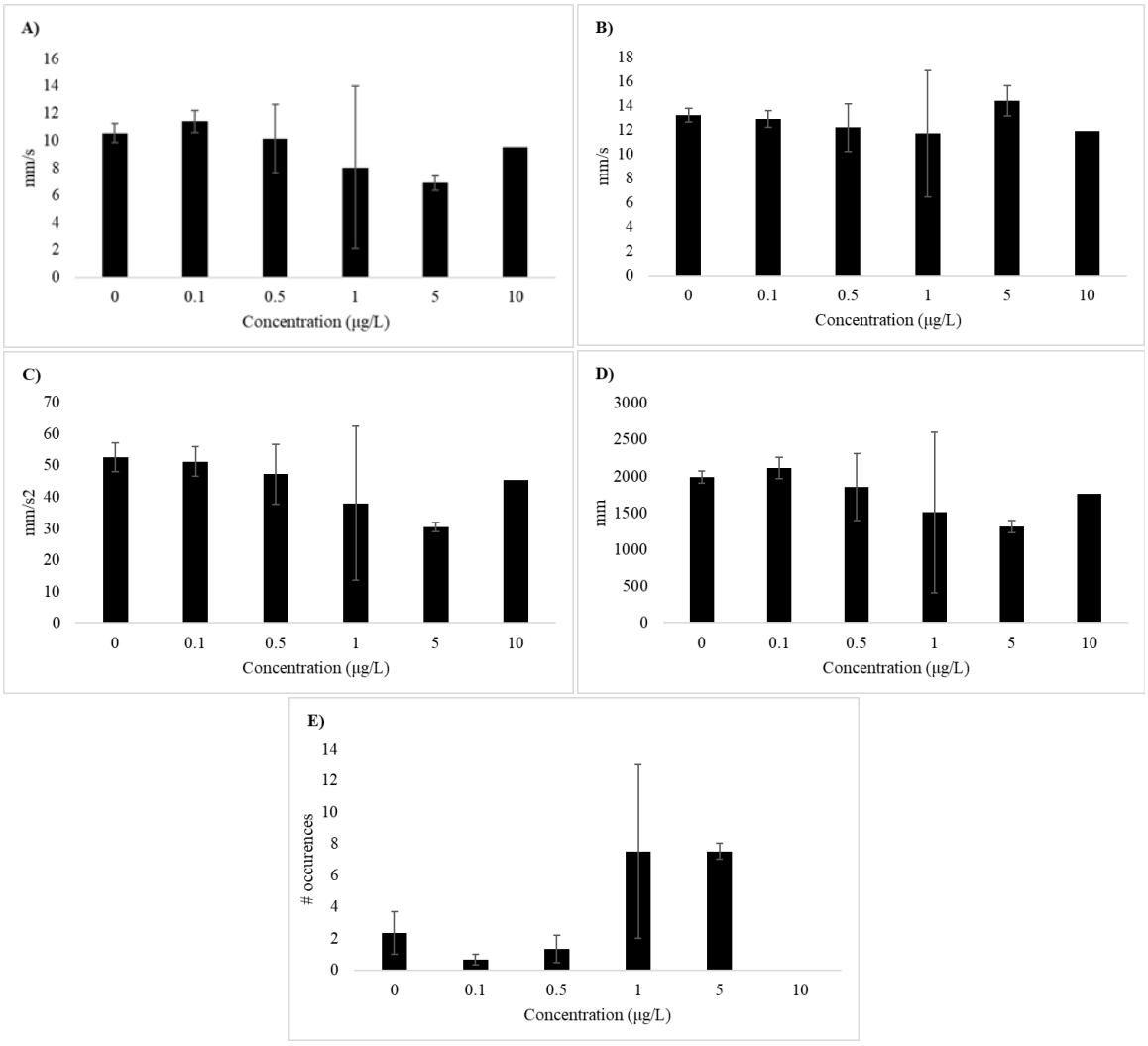

Figure 3.5: Effects of various concentrations of sulfoxaflor exposure to juvenile *D. magna* after 7 days on different mobility parameters, including: average speed ± SEM (**A**), average mobile speed ± SEM (**B**), average acceleration ± SEM (**C**), total distance traveled ± SEM (**D**), and total number of frozen events ± SEM (**E**) over the course of a 3 minute exposure period (n=3,3,3,2,2,1).

HEART RATE ASSAY

Analysis at 48 hours did not indicate any clear trend in impact on heart rate in juvenile *D. magna*, however, potential differences at higher concentrations of the chemical during 7-day exposures were observed (See Figure 3.6). Low sample sizes at 1, 5, and 10 µg/L (n=2, 2, and 1 respectively) led to incomplete statistical analyses of heart rate data for 7-day exposures.

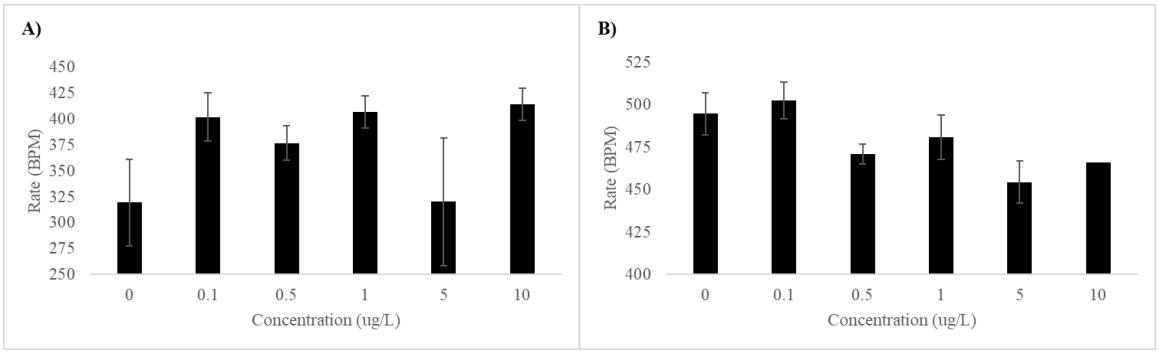

Figure 3.6: Average heart rate in beats per minute (BPM) ± SEM of juvenile *Daphnia magna* after 48 hours (**A**) and 7 days (**B**) of exposure to concentrations of sulfoxaflor.

GROWTH ASSAY

Juvenile *D. magna* growth did not differ between treatments at 48 hours (See Figure 3.7), however, apical spine length and body length: apical spine length ratio appeared to show an increase in higher treatments after 7 days of exposure (See Figure 3.8).

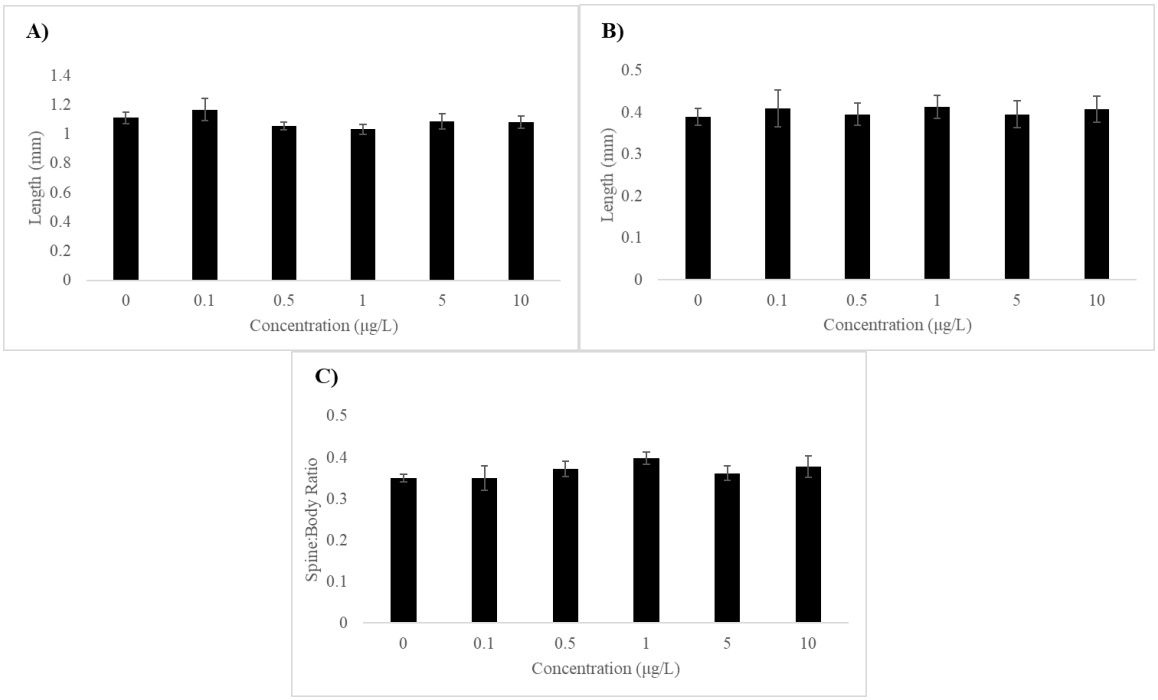

Figure 3.7: Average growth values ± SEM of body length (**A**), apical spine length (**B**), and the ratio of these apical spine length to body length (**C**) in juvenile *D. magna* after 48 hours of exposure to various concentrations of sulfoxaflor.

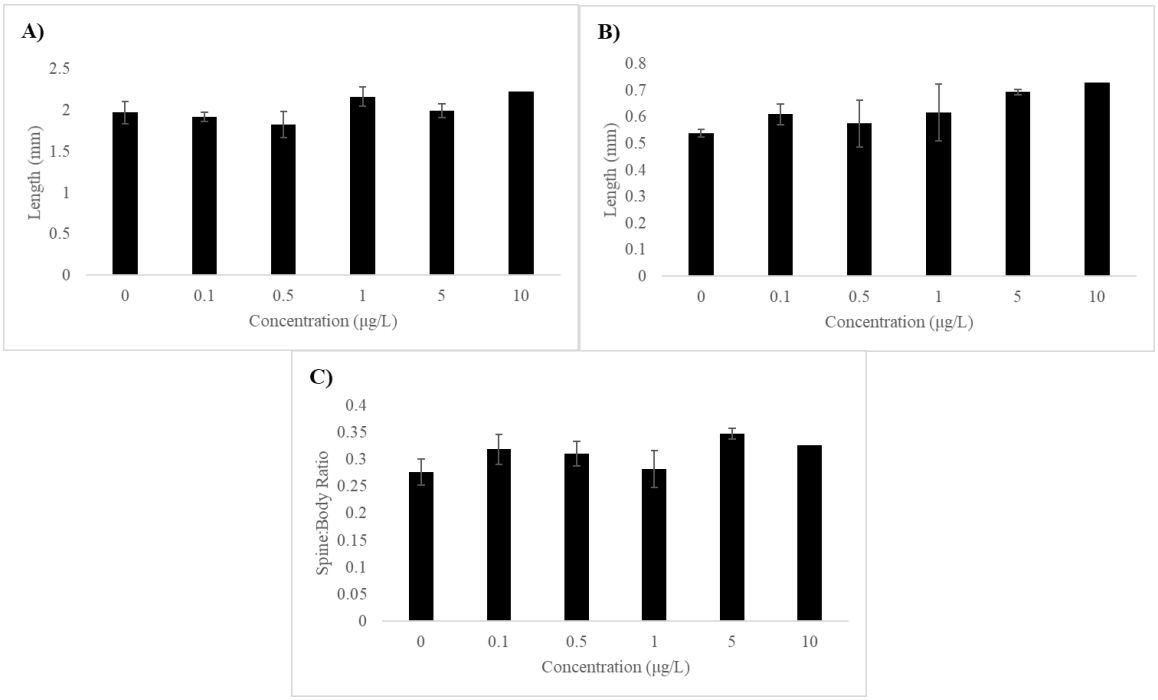

Figure 3.8: Average growth values ± SEM of body length (**A**), apical spine length (**B**), and the ratio of these apical spine length to body length (**C**) in juvenile *D. magna* after 7 days of exposure to various concentrations of sulfoxaflor.

CHAPTER 4 - DISCUSSION

CHEMICAL DEGRADATION

Analysis of chemical degradation in ethanol superstocks allows insight into persistence of the sulfoxaflor under storage conditions and supports the conclusion that the expected concentrations of the superstocks that had been in storage for less than 6 months were accurate (See Figure 3.2). Additionally, analysis on degradation under exposure conditions indicates that sulfoxaflor does not degrade under test conditions over a 48-hour period, with slight increases in concentration attributable to evaporation, indicating a 48-hour solution replacement was appropriate for chronic studies (See Figure 3.3). A recent publication successfully isolated the bacterial strain *Aminobacter* sp. CGMCC 1.17253 and determined the half-life of sulfoxaflor exposed to these bacteria in soil was 6.97 days (Yang et al. 2020). The same study also found that in the absence of *Aminobacter* sp. CGMCC 1.17253, the chemical had a half-life of 27.68 days in soil (Yang et al. 2020). Values provided by Dow Agrosciences indicated average half-lives of 4 days in soil, 11-64 days in water, and 37-88 days in sediment/water conditions (Dow 2014). The comparison of these studies indicates that the presence of bacteria in both soil and water is key for chemical degradation in the environment, and that degradation of the chemical is slowed in water compared to soil systems. This has important implications for lab exposures attempting to investigate the environmental effects of the chemical. Sulfoxaflor may have varied half-lives depending on the presence of sulfoxaflor-degrading bacteria in local environments, altering the potential toxicity of the chemical and potential for interaction with non-target organisms within application range.

Sulfoxaflor is shown to degrade into various metabolite compounds in animal, plant, and soil systems. There are eight different metabolites that result from metabolic breakdown of sulfoxaflor, primary among these being X11719474 (CAS 1186104-89-1) which is predicted to be the metabolite most present in soil and aquatic environments with a field-based half-life of around 76 days (Terry et al. 2015). Metabolite X11719474 was found to be significantly less toxic than the parent compound, either producing no effect or only incurring an effect at a higher dose than sulfoxaflor in rats, mice, and dogs (Terry et al. 2015). Relative toxicity was maintained through impacts on the liver, but metabolite X11719474 did not exhibit similar impacts on neonatal survival and development in rats as the parent compound (Terry et al. 2015). Other soil-based metabolites include X11579457 and X11519540. While X11579457 has been found to be nontoxic during rat *in vitro* studies, metabolite X11519540 was found to be more toxic than the parent compound, ranging between 3.3-16 and 10-15.1 times more toxic to male and female rats, respectively, than sulfoxaflor in short term exposures (Terry et al. 2015). The data presented by Terry et al. indicate that metabolic breakdown of sulfoxaflor has the potential to either increase or decrease toxicity of the parent compound, depending on which metabolite is generated in soil and water. Presence of these metabolites under different conditions will alter the potential toxicity and risk presented by sulfoxaflor application to various environments, indicating that further research into the existence of these metabolites in the field are necessary to understand the impact of the chemical on natural environments. While current testing shows that only the less-toxic metabolite X11719474 is present in water sediment systems (Terry et al. 2015), more testing is necessary to

determine environmental presence of the sulfoxaflor and subsequent metabolites in various waterways and environmental conditions.

As of now, little research into the environmental presence of sulfoxaflor has been conducted in locations where the chemical is in use. Application of sulfoxaflor, based on the guidelines for use of the market product Transform®, typically does not exceed 2-4 applications per crop (Dow 2016). Based on this information as well as the chemical half-life, it is unlikely that repeat use of sulfoxaflor will lead to accumulation of the chemical in soil environments but may lead to accumulation in waterways. Additionally, persistence of metabolites in both soil and water systems may lead to potential increases in concentrations over time. These data collectively indicate the need for further investigation into environmental presence of the chemical and changes in concentrations of both parent material and metabolites throughout agricultural application.

RANGEFINDER ASSAY

Concentrations used in this study reflect the expected environmental range of *D. magna* concentrations of sulfoxaflor in the environment. No significant mortality to sulfoxaflor was observed at 48 hours between concentration levels (See Table 3.1), indicating that acute exposure to the chemical is unlikely to influence survival of juvenile *D. magna*. Given a half-life of 11-64 days in water, however, acute 48-hour analyses do not accurately reflect expected mortality in affected environments. Though not statically significant, preliminary analysis at 7 days suggests an increase in mortality occurring at 1 µg/L with 50% mortality, increasing to 75% mortality at 10 µg/L with a LC_{50} of 5.85 µg/L (See Figure 3.4). These data show a considerable increase in mortality with prolonged exposure to the pesticide, indicating that introduction into an aquatic environment may

have significant impacts on *D. magna* survival, though further analysis may better elucidate these correlations. Previous analyses on various neonicotinoid pesticides report EC_{50} values of 16.5 mg/L for imidacloprid, 14.7 mg/L for cycloxaprid, and 13.0 mg/L for guadipyr for juvenile *D. magna* after 48 hours of exposure (Qi et al. 2018). Data reported by Dow Agrosciences demonstrate a 48-hour EC_{50} (concentration where 50% of individuals exhibit an effect) greater than 399 mg/L, and a 21-day NOEC of 50 mg/L (Dow 2014), falling significantly higher than reported findings for neonicotinoid pesticides. Furthermore, this study indicates potential effects at concentrations as low as 1 µg/L after 7 days, warranting further analysis into whether sulfoxaflor is less toxic to juvenile *D. magna* than neonicotinoids. Additionally, the studies conducted by Dow fail to identify whether juvenile or adult individuals are used in exposures, raising questions as to how the chemical might impact the species at different time points in their life cycle. *D. magna* have been shown to respond differently to exposure to the pesticide pyriproxyfen as juveniles and mature adults (Ginjupalli and Baldwin 2013). While the pyriproxyfen exposure led to increased output of male offspring by mature *D. magna*, it had a much stronger impact on juveniles, leading to slower recovery from chemical exposure and delayed reproductive maturity (Ginjupalli and Baldwin 2013). This lends support for the idea that analysis of *D. magna* at a single time point in toxicity studies may not provide enough insight into the effects of a chemical on wild populations, and that further investigation into the effects of sulfoxaflor at different ages of *D. magna* are needed to fully comprehend its impacts in aquatic environments.

Given expected environmental conditions, 48-hour exposures do not accurately portray potential mortality based on the persistence of sulfoxaflor in aquatic environments.

Longer-term analyses, including 7-day exposures, are more indicative of environmental conditions based on sulfoxaflor half-life (11-64 days) under aquatic conditions. While wild *D. magna* populations would be expected to decline, the species serves as key organisms in freshwater ecosystem interactions (Miner et al. 2012) whose presence as a primary consumer impacts many of the other species around them. Loss of *D. magna* populations could lead to decreased consumption of phytoplankton, leading to the potential for overgrowth of the phytoplankton and potential eutrophication of affected waterways. Additionally, loss of the species could result in decreased food availability for higher trophic level predators, including planktivorous fish and predatory invertebrates (Miner et al. 2012), whereby bottom-up control would lead to an overall decrease in these populations in response. The key trophic position of *D. magna* makes mortality data particularly important for analysis of the effects of sulfoxaflor on aquatic environments and indicates that wildlife populations unaffected by the pesticide itself may still be impacted by chemical introduction into the environment through eutrophication and/or decreased food availability.

MOBILITY ASSAY

Mobility as a behavioral endpoint has been used consistently when examining the impacts of toxicants on *D. magna* (Bownik 2017). Various programs and systems have been used for analysis, including BehavioQuant and DaphTox ® (Bownik 2017), but these systems are often expensive and not readily accessible. The mobility data in this study was analyzed using ToxTrac (v. 2.90), an open-source and freely available program that specifically tracks organisms and reports quantitative data for toxicological purposes (See Figure 2.1). Studies have shown that ToxTrac and related software can be used to collect

comprehensive mobility data on other aquatic invertebrate species such as *Planorbella duryi* (Frankel et al. 2020). This study found that at higher concentrations of sulfoxaflor, juvenile *D. magna* exhibited decreased speed, acceleration, and total travel distance after 7 days of exposure (See Figure 3.5). Additionally, increases in the number of frozen events were identified at 1 and 5 µg/L. While sample sizes at 1, 5, and 10 µg/L were small (n= 2, 2, and 1 respectively), these data present a potential trend in decreased mobility after prolonged exposure to sulfoxaflor, suggesting that the chemical may impair juvenile *D. magna* mobility under expected environmental conditions. Reduced mobility can have ecological consequences for wild populations, as inhibition of swimming behaviors can lead to higher rates of predation due to a decreased ability to escape (Bownik et al. 2019). Higher rates of predation could subsequently lead to decreased population numbers in the environment, increasing the likelihood of eutrophication or decreasing food availability for secondary consumers that utilize *D. magna* as a food source (Miner et al. 2012).

HEART RATE ASSAY

Sulfoxaflor acts as an insect nicotinic acetylcholine receptor (nAChR) agonist, leading to excitatory responses in the central nervous system (CNS) of arthropods (Sparks et al. 2013). This activation typically leads to tremors, leg extension and curling, partial or complete paralysis, and death in affected insects (Sparks at al. 2013). Many studies have been conducted analyzing the impacts of various chemicals on the heart rate of *D. magna*. This study aimed to determine if sulfoxaflor interacted with non-target invertebrate nAChR receptors in the same manner, and whether agonistic impacts affected heart rate in *D. magna*. After 48 hours, no clear correlation between heart rate and chemical exposure can be determined (See Figure 3.6). Acute exposures do not seem to induce any effect on heart

rate in exposed juveniles. While a slight decrease in heart rate is observed after 7 days of exposure at higher treatment levels (See Figure 3.6), larger sample sizes are necessary to delineate the magnitude of this effect. Impacts on heart rate can lead to decreased mobility in the water column as well as paralysis. Based on the data presented in this study, sulfoxaflor does not appear to cause any effect to juvenile *D. magna* heartrate at expected environmental concentrations, though further analysis into chronic exposures based on chemical half-life are necessary to determine the impacts of the chemical under environmental conditions.

Alterations in heart rate can lead to impacts on ability of the heart to transport oxygen through the body, leading to a reduction of oxygen delivered to vital organs and potential for increased susceptibility to disease (Bownik et al. 2019). Decreased ability to maintain cellular homeostasis and increased energy consumption are also potential detrimental impacts of impaired circulatory function (Lari et al. 2017). Alterations in heart rate can be compensated by *D. magna* through the use of thoracic limb movement, which can often support the heart in supplying oxygen to the body (Lari et al. 2017), indicating that even under conditions that impair heart function, overall survival may not be significantly altered. It is important to connect this with impacts on mobility, however, as contaminants such as sulfoxaflor that target the central nervous system in control of muscular contractions may subsequently limit movement of the thoracic limbs. Future studies into impacts of sulfoxaflor on *D. magna* might focus on thoracic limb movement to identify whether impaired heart rate carries significant complications for survival in natural environments.

GROWTH

Daphnia magna have been demonstrated to morphologically change in response to increased predatory pressures, including lengthening of the body and apical spine (Rabus et al. 2013). Additionally, *D. magna* have been shown under toxicant-induced stress to increase energy consumption and decrease energy uptake, leaving less energy to allocate to other important parameters such as growth and reproduction (Villarroel et al. 2009). Decreased growth can often have direct impacts on survival of individuals, while decreased reproductive output can lead to a decline in overall population levels in the environment (Villarroel et al. 2009). Both investment in growth or lower energy allocation for growth as result of exposure can have significant impacts on individual survival in the environment. The data from this study indicate that there is no obvious effect on growth of juvenile *D. magna* between treatment levels after 48 hours (See Figure 3.7), however, an increase in apical spine length at higher treatments was identified after 7 days (See Figure 3.8). Heightened stress induced by chemical exposure may encourage growth as a morphological response, with chemical stress inducing defensive allocations rather than heightened energy consumption. Investment in growth can reduce rates of predation in the wild (Rabus et al. 2013), but in environments where predation rates are low, this energy allocation or increased energy consumption caused by stress can come at the cost of reproduction during the exposure period. Many *D. magna* populations in northern geographic ranges experience population declines during the winter, and reproductive investment in sexual reproduction and development of ephippia is vital for population regeneration in the spring (EPA 2002). If sulfoxaflor is sprayed on fall crops during this crucial period, and sufficient environmental contamination takes place, the decrease in

reproductive investment could significantly harm the ability of *D. magna* populations to recover in the spring. The EPA does currently allow usage of the pesticide on various fall crops, including different types of grains and cucurbits (EPA 2012). Impacts on growth and ability to invest in reproduction surrounding application sites could reduce overall populations in surrounding agricultural areas. *D. magna* are vital to freshwater ecosystems, as consumers of phytoplankton and as a food source for secondary consumers such as planktivorous fish and predatory invertebrates (Miner et al. 2012). Declines in *D. magna* populations could lead to overgrowth of phytoplankton and drastic decline in food availability for secondary consumers, leading to instability within the trophic web and potential declines in higher trophic level populations.

CHAPTER 5 – FUTURE STUDIES

AGE-BASED ANALYSIS

Differences in the impacts of pesticides on *D. magna* based on the age of individuals has been demonstrated in previous studies (Ginjupalli and Baldwin 2013). Due to the limited availability of studies analyzing the effects of sulfoxaflor exposure on *D. magna*, as well as uncertainty centered around current data provided by Dow Agrosciences, future studies should prioritize differentiating the potential changes in response to sulfoxaflor exposure at different ages in order to identify potential windows of sensitivity. Acute and chronic exposures are both necessary to capture impacts of the chemical in the environment based on environmental degradation times.

REPRODUCTIVE ASSAYS

Presently, no studies have been conducted analyzing toxicological impacts of sulfoxaflor on *D. magna* reproduction; however, current studies demonstrate that *D. magna* exhibit stress responses to chemical exposure. A prominent stress response of the species includes high rates of energy consumption that may lead to decreased or limited investment in reproduction (Villarroel et al. 2009). Due to this, exposure studies determining the potential for delayed or altered reproduction is an important avenue of investigation into the impacts of sulfoxaflor on wild *D. magna* populations. This additionally pairs with age-based investigations, as pesticides have been shown to impact the reproductive output of juvenile and adult *D. magna* differently (Ginjupalli and Baldwin 2013).

METABOLITE EXPOSURES TO AQUATIC INVERTEBRATES

While metabolite exposures have been conducted on various mammalian species including rats, mice, and dogs (Terry et al. 2015) to help elucidate the potential effects on humans, no research has been conducted on the potential toxicological impact of sulfoxaflor metabolites on aquatic invertebrates including *D. magna*. Given current evidence that suggest metabolites tend to persist longer in the environment than the parent chemical and may induce toxic effects (Terry et al. 2015), analysis of relevant metabolite interaction with non-target aquatic invertebrates is of vital importance for determining environmental toxicity of the chemical.

ENVIRONMENTAL PRESENCE

Due to sulfoxaflor's recent development, very few studies have looked at degradation of the chemical under various environmental conditions, and no studies have quantified the environmental presence of the chemical, nor analyzed its potential accumulation at different time points in the agricultural season. As such, understanding of the chemical's environmental relevance, as well as persistence and toxicological interactions of metabolites, are necessary to develop a basis for ecotoxicological studies attempting to analyze impacts on non-target species at environmentally relevant concentrations. Furthermore, studies investigating presence in natural environments may help direct future policy surrounding agricultural use, as potential accumulation of the chemical and metabolites following agricultural guidelines are currently not well understood.

CHAPTER 6 – FINAL CONCLUSIONS

The main objective of this study was to analyze the potential behavioral and physiological sublethal impacts of sulfoxaflor on juvenile *Daphnia magna* at environmentally relevant concentrations. Based on these findings, sulfoxaflor has been shown at environmentally relevant concentrations to produce sub-lethal impacts on juvenile *D. magna* after 7 days of exposure. Though much of the current 7-day data is not statistically significant due to low sample sizes, preliminary analysis presents trends not visible in 48-hour exposures, which exhibited no observable impact on juvenile *D. magna*. Analysis of longer exposure periods is supported by the broad estimated half-life of sulfoxaflor falling between 11-64 days in water sediment systems. HPLC analysis indicates that sulfoxaflor does not readily degrade in water under laboratory conditions, supporting findings that suggest sulfoxaflor has an increased half-life in water as opposed to soil systems.

Increased mortality was observed at higher concentrations, though 50% mortality was observed at concentrations as low as 1 µg/L, contradicting current data presented by Dow Agrosciences that states a NOEC of 50 mg/L after 21 days. This contradiction emphasizes a need for further analysis into the impacts of sulfoxaflor on non-target aquatic invertebrates, as well as identifying potential windows of sensitivity between juvenile and mature *D. magna*. Additionally, higher concentrations of sulfoxaflor identified trends of increased length of apical spine and decreased heart rate and mobility parameters such as average speed, acceleration, and total distance after 7 days of exposure. While current data on sulfoxaflor and *D. magna* discuss mortality only, many of these parameters have direct implications for *D. magna* survival in the wild, emphasizing the importance of analysis

into sublethal endpoints of chemical exposure. Increased energy consumption, decreased oxygen flow to the body, reduced or delayed reproduction, and higher predation rates due to decreased mobility are all potential impacts to *D. magna* that are not addressed or investigated in standard rangefinder assays. Mortality data can be useful in identifying major concerns with chemical or pesticide use, but clearly do not offer a full picture of impacts on environmental systems or non-target species. For species such as *D. magna*, whose presence in freshwater environments is key for both control of phytoplankton populations and as a food source of secondary level consumers, drastic changes in population numbers as a result of toxicant exposure can have significant impacts on overall ecosystem health. As such, it is important that future toxicological research include analyses that investigate the various sublethal behavioral and physiological endpoints for all investigated species that, if negatively impacted, may have severe survival implications in the wild.

Lastly, it is apparent that sulfoxaflor's interactions in the environment are not currently well understood. Toxicological impacts of the chemical have not been thoroughly investigated in any non-target species beyond rats and mice, and even fewer studies have been conducted on aquatic species. No studies on environmental presence of sulfoxaflor or its metabolites have been conducted at this time, and research into the toxicity of its metabolites is sparse. It is evident that much more research into the environmental presence and interactions of sulfoxaflor is needed before its toxicological impacts can be understood well enough to protect non-target organisms and natural ecosystems surrounding its agricultural use.

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