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An Evaluation Of Endocrine Disrupting Effects Of Emerging Contaminants Using Daphnia Pulex And Danio Rerio

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AN EVALUATION OF ENDOCRINE DISRUPTING EFFECTS OF EMERGING CONTAMINANTS USING *DAPHNIA PULEX* AND *DANIO RERIO*

By

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_____

Advisor Date

DEDICATION

I would like to dedicate this work to my parents, Ravinder R. Alla and Vani Alla, and my grandparents who have always supported me.

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I would like to thank my Advisor Dr. David K. Pitts who has been very supportive and encouraging throughout my journey of my Masters. I would consider myself very lucky to work under guidance. His guidance helped me in the laboratory and in writing my thesis. I would like to extend my gratitude to my committee members Dr. Randall L. Commissaris and Dr. Donna Kashian who have provided me support and encouragement.

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Chapter 1: INTRODUCTION

Conserving and protecting our water resources has become an imperative environmental issue of this century. Two factors that limit the availability of water suitable for human use can be identified as scarcity and quality. In order to provide for sustainable water use in the presence of an expanding population, the demand for the planet's finite fresh water supply must be in balance with the best management and regulatory practices. This study focuses on the issue of water quality and examines the toxicity of known water contaminates on aquatic model animals.

There is increasing concern about the production, use and disposal of various chemicals and their transport into water systems from numerous sources, such as wastewater treatment plants and agricultural fields, and the significant detrimental effects on ecosystems and human health. Some of these chemicals unintentionally find their way into the environment (e.g., pharmaceuticals), whereas others are accidentally distributed into the environment (e.g., pesticides). Numerous chemicals coming from residential, business, hospital, and industrial sources can find their way into wastewater treatment systems that are not designed to process them, and therefore may enter into surface water via the wastewater effluent. (Kolpin et al. (2002);Yang et al. (2012)). The conventional activated sludge process utilized in many U.S. wastewater treatment plants is often not effective in removing the chemicals from wastewater influent and could be replaced by more effective activated charcoal adsorption processes (Grassi et al. (2013))

Chemicals that originate as human and veterinary medicines, surfactants and pesticides that serve useful purposes are found as contaminants in the aquatic environment and affect non-target organisms (Fent et al. (2006)). The chemical

contaminants found in water that are not routinely monitored or regulated are now most commonly referred as Chemicals of Emerging Concern(CECs). According to U.S. Geological survey (USGS; https://toxics.usgs.gov/investigations/cec/index.php), these CECs can be broadly defined as:

"Any synthetic or naturally occurring chemical or any microorganism which are not commonly monitored in the environment but have the potential to penetrate the environment resulting in known or suspected adverse ecological and(or) human health effects. In some cases, release of emerging chemical or microbial contaminants to the environment has likely occurred for a long time, but may not have been recognized until new detection methods were developed. On the other hand, synthesis of new chemicals or changes in use and disposal of existing chemicals can create new sources of emerging contaminants."

A subset of the CECs is considered to be known or suspected endocrine disrupting chemicals (EDCs) due to their endocrine disrupting properties. According to the NIHEHS (https://www.niehs.nih.gov/health/topics/agents/endocrine/index.cfm):

"EDCs are chemicals that may interfere with the body's endocrine system and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife."

The sources of EDCs and CECs are wide-ranging and disparate. EDCs can be either natural or synthetic chemicals, which would include pharmaceuticals, dioxin and dioxin-like chemicals, polychlorinated biphenyls, pesticides (e.g., DDT) and plasticizers (e.g., bisphenol-A). Endocrine disruptors can act through four distinctive mechanisms, they can: (1) mimic or partially mimic naturally occurring hormones at hormone receptors, (2) bind to hormone receptors and block hormone action, (3) interfere synthesis or degradation of hormones or hormone receptors, and (4) alter the regulation and/or timing of hormone release.

The main concern about finding EDCs in lakes and streams is their impact on environmental and human health at potentially very low concentrations. For example, male fish have been shown to be feminized by very low concentrations of estrogens (Jobling et al. (2006)) and many other synthetic chemicals with estrogen-like properties (X. Li et al. (2017)). Male frogs have been shown to be completely feminized by exposure to the herbicide, atrazine, at environmentally relevant concentrations Hayes (2010) and Agopian et al. (2013) have demonstrated a correlation between ground water concentrations of atrazine and the incidence of hypospadias, a malformation of the human male penis.

A primary concern about of EDCs in the environment is influenced by their individual chemical properties and their interaction with water, solid substrates, sunlight, and other chemical processes. As a subset of CECs, EDCs, can be pharmaceuticals, surfactants, pesticides, and other synthetic chemicals that are found in surface water and/or ground water. The concentration of these chemicals detected in the environment can range from nanograms (ng) per liter to a hundred micrograms (μg) per liter (Kolpin et al. (2002)). Although these chemicals are most often found in relatively low concentrations, their capacity to disrupt endocrine function and cause developmental abnormalities in organisms is further exacerbated by such processes as bioconcentration, bioaccumulation, and biomagnification (Montanes et al. (1995); Welling and Devries (1992).

There are numerous reports of the estrogenic activity of synthetic chemicals, (Anderson et al. (2002); Nishihara et al. (2000); Zerulla et al. (2002)) one approach to estimating the estrogenic activity of these chemicals is the use of a yeast two-hybrid

assay, as reported by Nishihara et al. (2000), where hormone activity is detected by the ligand interaction with a hormone receptor and a coactivator that stimulates B-galactosidase activity. Nishihara (2000) have reported that a diverse group of synthetic chemicals such as bisphenol-A, estrone, 4-nonylphenol, exhibit estrogenic activity in their assay system. Exposure of fish to synthetic estrogen was demonstrated to cause a collapse in the population due to exposure in Lake Ontario (Kidd et al. (2007)). One of the best characterized examples of endocrine disruption in aquatic systems results from exposure to estrogenic or anti-androgenic chemicals that can cause feminization of males, alterations in sex ratio (increase proportion of females), and increased vitellogenin expression. Male fish downstream of a wastewater treatment plant in northwestern Ontario have been shown to have an increased level of vitellogenin and early stage eggs in their testis as result of effluent exposure (Kidd et al. (2007)). Many of these estrogenic chemicals have been found in wastewater effluent and downstream of wastewater treatment plants where intersex fish have been found. In tests of wastewater effluent from Boulder, Colorado, Vajda et al. (2011) found that exposure to effluent produced evidence of intersex male fish and reproductive disruption in fathead minnows. Intersex white perch from the Lower Great Lakes have been shown to have increased vitellogenin levels, which suggests that there is likely an endocrine disrupting effect present in waters of this region Kavanagh et al. (2004).

EDC Project

In order to increase our understanding of EDCs that can cause effects such as feminization of fish or changes in sex ratio due to their estrogenic or anti-androgencic properties, a strategy was developed to screen for behavioral toxicity, lethality, and

changes in morphology in preparation for an assessment of chemically induced alterations in gene expression. The design is divided into 3 parts, the effects of known or suspected EDC on: (1) *Daphnia pulex* and *Danio rerio* swimming behavior and survival, (2) developmental effects expressed as changes in morphology or sex ratio, and (3) gene expression as assessed by EnDseq. An Initial list of 9 chemicals were selected based on the literature that reports their presence in the environment and biological effects consistent with potential estrogenic or anti-androgenic activity. This study focuses on the non-PPCPs selected from of the list of 9 chemicals. Results from this study and a parallel study examining PPCPs will be used to determine the chemical concentrations of interest for developmental and morphological studies and studies on gene expression. These studies will also provide the context for evaluating the outcome of gene expression studies. The overall hypothesis of this project is that behavioral and morphological outcomes, and the profile of genomic responses observed in these two model organisms (vertebrate and invertebrate), can be used to develop a molecular identification model capable of detecting estrogenic and anti-androgenic activity.

Two aquatic organisms were selected to evaluate EDC activity, *Daphnia pulex* (waterflea), and *Danio rerio* (zebrafish). *D. pulex* is a fresh water crustacean, key stone species, and NIH model organism that that is well established in ecology, and is often used in the evaluation of water quality. The genome has been sequenced by Colbourne et al. (2011), and epigenetic mechanisms associated with responses to stress, reproduction and adaptation to predators have been well described in Altshuler et al. (2011);Eads, Andrews, and Colbourne (2008);Harris, Bartlett, and Lloyd (2012). Since there is significant homology with the human genome, *daphnia* can also be useful in risk

assessment pertaining to human health (Colbourne et al. (2011)).

Danio rerio (zebrafish) is a small tropical freshwater fish of South Asia. As vertebrates, zebrafish have physiology similar to humans and they share a wide-variety of hormone systems common with all vertebrates including humans Dia et al. (2012); Hill and Janz (2003).. *Danio rerio* are considered an NIH model organism and are ideal for studying development because they are translucent at an early age and morphology can be easily observed and they exhibit a developmental pattern that is similar to humans. When combined into one study these two model organisms provide a way to examine chemical responses of both an invertebrate and vertebrate model organism with relevance to understanding potential effects on ecosystems and on human health.

The CECs can be divided into Pharmaceuticals and personal care products (PPCPs) and non-PPCPs. This thesis focuses on non-PPCPs, and includes a diverse array of chemicals, whose wide-spread use has resulted in significant environmental contamination.

Chlorpyrifos(CPF):

Chlorpyrifos is an organophosphate insecticide, which inhibits the enzyme, acetylcholinesterase. It acts on the cholinergic nervous system of the organism by inhibiting the degradation of the neurotransmitter, acetylcholine (ACh), through enzyme inhibition and thereby increasing levels of ACh. CPF exposure has been shown to significantly affect the reproductive system of adult male albino rats. This can be observed as reduced testicular weight, decreased sperm count, motility and viability with respect to controls (*Alaa-Eldin* et al., *2016*). CPF also affects the both the nervous system and

reproduction in aquatic life. CPF increased the vitellogenin (VTG) in male fish and reduced serum 11B-estradiol in female fish. CPF also caused structural damage of both the gonads in zebrafish (Manjunatha and Philip (2016)). CPF alters the expression of estrogen responsive gene VTG and ER∝ receptor in the embryos of zebrafish, it also affects the hatching rate of the embryos as the concentration of the exposed chlorpyrifos increases (Yu et al. (2015)). The residential usage was stopped in 2000 and completely banned from public usage in 2012 (https://www.epa.gov/chlorpyrifos). EPA denied a petition asking to remove all pesticides charges and cancel registrations on chlorpyrifos in 2017.

Dieldrin:

Dieldrin is an organochloride originally produced as an insecticide. This chemical caused concern because it is a persistent organic pollutant (POP) It does not break down very easily. This compound has been reported to disrupt the endocrine system, can say has estrogenic properties. There is evidence that dieldrin acts antagonist of $(GABA)_A$ receptor, blocking the synaptic transmission (Martynuik et al. (2013)). Dieldrin has been detected both in the sediment as well as the fish muscle of *Tilapia guineensis* in Nigeria aquatic systems. There is evidence that the luteinizing hormone (LH), follicle-stimulating hormone (FSH), 11-ketotestosterone (11-KT) and 17β-estradiol (E2) increases, significantly in male fish Adeogun, Ibor, Adeduntan, and Arukwe (2016). Dieldrin also decreased the estradiol and 11-ketotestosterone in largemouth bass (males and females). It also altered steady state mRNA expression levels of a set of genes which represent three possible mechanisms of endocrine disruption: (1) direct interaction with soluble sex steroid

receptors, (2) biosynthesis of endogenous sex hormones, and (3) metabolism of endogenous hormones Garcia-Reyero, Barber, Gross, and Denslow (2006). Except for controlling termites, the usage of dieldrin was banned by EPA in 1974, and it was completely banned in 1987.

Toxaphene:

Toxaphene is an insecticide that in the United States during the 1960s and 1970s. Exposure to toxaphene results in neurotoxic effects and causes changes in the behavior. It was also found to be weakly estrogenic *in vitro* systems. de Geus et al. (1999) Toxaphene has been detected in the tissue of the fish at low levels (McMaster et al. (2006)). Toxaphene is a potential endocrine disrupting agent in *Daphnia* since it increases the production of males and decreases fecundity (Kashian and Dodson (2002)). Toxaphene might elicit its endocrine disrupting effect in part by modulating prolactin mRNA in GH_3 cells, where growth hormone is generated faster than normal cells (Graham et al. (2003)). Scott and Jones (2000) demonstrated that toxaphene alters the lipid composition of the liver of yellowtail flounder, which could alter lipid metabolism and reproduction. The EPA banned toxaphene on March 1st, 1990.

Atrazine:

Atrazine is a herbicide (weed killer) and is a member of the triazine chemical class. Atrazine prevents plant growth by blocking photosynthesis. It is transported through the cuticle into the cell and chloroplast and binds to the Q_B protein and prevents it from accepting and transferring electrons to the plastoquinone (PQ) pool in the photosystem II

and the process of photosynthesis is therefore inhibited. Atrazine is considered an endocrine disrupting agent since it alters the reproductive function in zebrafish by increasing the progesterone levels and decreases the spawning rate in embryos (Wirbisky et al. (2016)). Atrazine exposure has also been reported to cause the complete feminization of male frogs, which are able to mate and have offspring like fully functioning females (Hayes et al. (2010)). Atrazine also increased the expression of Zcyp19a1 which encodes for aromatase and increased the ratio of female to male fish (Suzawa and Ingraham (2008)). Atrazine alters the expression of genes which are associated with adult males and females during embryogenesis providing origins of endocrine disrupting properties of Atrazine (Wirbisky and Freeman (2017)). An increase in the incidence of a rare birth defect in human infants called hypospadias, the malformation of the male penis, has been reported to be associated with exposure to atrazine through ground water (Agopian et al., 2013)

4-Nonylphenol:

4-Nonylphenol belongs to a class of organic compounds called as the alkylphenols. These compounds are associated with the manufacturing of detergents, emulsifiers and solubilizers. The mechanism for the toxic effects of 4-nonylphenol is not clearly understood, but it might exhibit endocrine disrupting property by binding to the sex hormone-binding globulin (Adeogun et al. (2016); Sheikh et al. (2017)). Watanabe et al. (2017), evaluated 4-nonylphenol using medaka. Chemical exposure caused an increase in the VTG (vitellogenin) in male fish in a concentration-dependent manner (F_o generation) and effected the hatching and survival rate of embryos (F_1 generation). Barber et al. (2015) collected and analyzed the fish and found widespread occurrence of

alkylphenols and biomarkers indicating a long term exposure of endocrine disrupting agents. The EPA proposed "New Use Rule" to be reviewed by the Agency before a manufacturer starts or resumes use of 15 nonylphenols (NPs).

Bisphenol-A

Bisphenol-A(BPA) is an organic synthetic compound which is utilized in the manufacturing of plastic and epoxy resins. Bisphenol-A has been reported to have estrogenic activity (Nishihara et al., 2000). The mechanism of action of Bisphenol A is not completely understood, but it may show low affinity for genomic ER when compared to estradiol and is as active as estradiol biologically. It also has affinity for the androgen receptor (Negal and Bromfield, 2013). Evidence for the presence of BPA in the tissues of fish has been reported as well as contamination of two sediment sites in Italy Errico et al. (2017); Nicolucci et al. (2017). An alteration of the structure of testis tissue and abnormal gene expression related to the testicular steroidogenesis has been reported X. Li et al. (2017). BPA has been banned from use in certain children's food and bottle containers in some states in the United States and legislation has proposed nationwide ban (https://www.fda.gov/food/newsevents/constituentupdates/ucm360147.htm)

HYPOTHESIS:

The goals for this thesis is to assess the effects of potential endocrine disrupting chemicals on the behavior and survival of *D.pulex* and *D. rerio* and to identify concentrations of interest to screen for changes in development and/or gene expression in subsequent studies. The overall hypothesis for the project is that known or suspected

EDCs have detectable behavioral effects, and that the characterization of these behavioral effects in combination with developmental and gene expression data will provide a mathematical model that enables the identification of chemicals contributing to the estrogenicity or ant-androgenic qualities of contaminated water. Furthermore, the study of the impact of EDCs on the behavior of two different aquatic organisms, one invertebrate and one vertebrate (Daphnia pulex and Danio rerio) will increase the discriminating power of the behavioral results and broaden the application of these results to the assessment of potential ecological impact. These behavioral effects constitute one biological level that can be used to differentiate the chemical identity of PPCPs.

Chapter 2: MATERIALS AND METHODS

Behavioral assay: This assay uses digital photography and image analysis software was used to track the sub-lethal effects of selected chemicals on the swimming behavior of two aquatic organisms, *Daphnia pulex* (waterflea) and *Danio rerio* (zebrafish). The method for optical tracking of *D. pulex* and *D. rerio* was adapted from (Zein et al, (2014)) and modified as described below.

Animals: The animals used in the behavioral assay were *D. pulex* and *D. rerio.*

D. pulex

D. pulex were collected from a pond at the Michigan State University Kellogg Biological Station in 2008 grown and the cultures have been maintained in the WSU laboratories (Dr. Kashian's laboratory for *daphnia*). The *D.pulex* are maintained at a temperature in an incubator at 21 ± 0.5°C in 4L jars with a light/dark cycle of 16/8 hours. The animals were fed 3 times a week with 50/50 mixture of *ankistrodesmus falcatus* and *Desmodesmos*. The culture jars were cleaned once a week and the COMBO culture medium was replaced. The COMBO media (composition described below) is specifically designed to support the growth of zooplankton. Kilham, Kreeger, Lynn, Goulden, and Herrera (1998).

D. rerio

Zebrafish embryos at the 4-day post-fertilization stage of development (4dpf) were obtained from the laboratory of Dr. Tracie Baker (Institute of Environmental Health

Sciences, WSU). *D. rerio* are maintained in artificially prepared fish media (see Chemicals, Drugs and Solvents, below). The animals were maintained at 28-30°C and fed 2 times a day. The fish media was continuously exchanged while the animals were in culture. *D. rerio* were maintained under a 14/10 light dark cycle throughout the fish husbandry. At the end of behavioral experiments, the fish were euthanized under the guidelines of an Institutional Animal Care and Use Committee protocol using tricaine metasulfate.

24 well plate setup: Prior to the experiment *D. pulex* were removed from the incubator and filtered through plastic mesh so that all the animals used in the experiment were adults (> 1.4mm in length). Zebrafish used in the experiments were 4-day post-fertilization embryos and were approximately around 2.4mm in length. The selected animals are placed into the isolated wells of the translucent 24-well plate (Falcon polystyrene 24-well plate, Fisher Scientific) using a plastic water dropper. The *D. pulex* were transferred into the plates at room temperature (~ 20°C). Zebrafish embryos and the fish media were maintained at a temperature of 28°C on a warming pad prior to placing the animals into a 24-well plate. Excess media, which was present in the wells due to animal transfer, was removed to prevent the dilution of the added test solutions. The excess media was removed using a glass pipette. The test solutions were randomly placed in the 24 well plate. This entire procedure requires about 15-20mins.

Figure1: A 24-well plate with freely swimming *D. pulex* in test solutions.

The Old Setup (visible light): The assay setup is as shown below in Figure 1. The 24-well plate was placed on a clear plexiglas table with an LED light plate source below the table (Art Light). 56cm above the 24-well plate an Infinity 2-1M digital monochrome camera (Model 2.1M, Lumenera Corporation, Ottawa, Ontario) was used along with a telecentric lens (Opto Engineering, Houston, TX) to record swimming behavior. With the exception of the addition of the telecentric lens, this setup is similar to that used by Zein et al. (2014, 2015) for analyzing the swimming behavior of *D. pulex* and was used in preliminary experiments.

The Improved Setup (infrared light): The above assay system was improved to in order to establish a better regulated 24-well plate environment with adjustable temperature that could be operated independently of visible light levels. The modifications necessary to use the assay system described above for two different aquatic animals required a well-regulated adjustable environmental temperature for the two different aquatic animals: 21°C for *D. pulex* and 28°C for zebrafish. The following innovations were

made to update the behavioral assay system. (1) In order to prevent evaporation of the solutions from the wells and prevent fogging from condensation, a heated Plexiglass top was added to cover the 24-well plate (see Figure 2). (2) The LED light panel was replaced with an infrared light panel (Edmund Scientific) in order to track animal movement without the need to direct visible light through the bottom of the 24-well plate (Figure 2). (3) A new digital camera (Model 3S, Lumenera Corporation, Ottawa, Ontario) with greater sensitivity in the IR spectrum and equipped with an infrared filter (850 nM band pass, Advanced Illumination) was connected to the telecentric lens (Figure 3). (4) A temperature control flow cell was manufactured (WSU engineering machine shop) to warm or cool the bottom of the 24-well plate as necessary to achieve a constant temperature within the media of the wells that was appropriate for each of the aquatic animals (Figure 2). (5) A Polysci water bath circulator (Figure 3) perfused Polysci coolant through the temperature flow cell at the appropriate temperature to achieve culture media temperatures within the wells of the 24-well plates at $\pm$ 0.2 °C of the appropriate animal target temperature (see above). (6) The entry of the bubbles into the temperature flow cell made tracking of animal subjects more difficult due to the challenges of discriminating bubbles from animals. Therefore, a bubble trap made out of PVC plastic pipe was placed in the coolant line to prevent introduction of bubbles into the temperature flow cell (Figure 4).

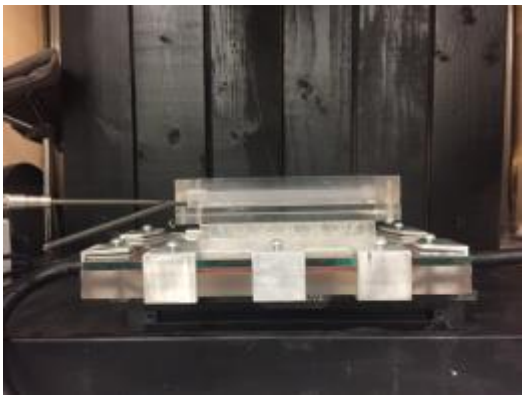

Figure2A: The 24-well plate setup under the camera and lens

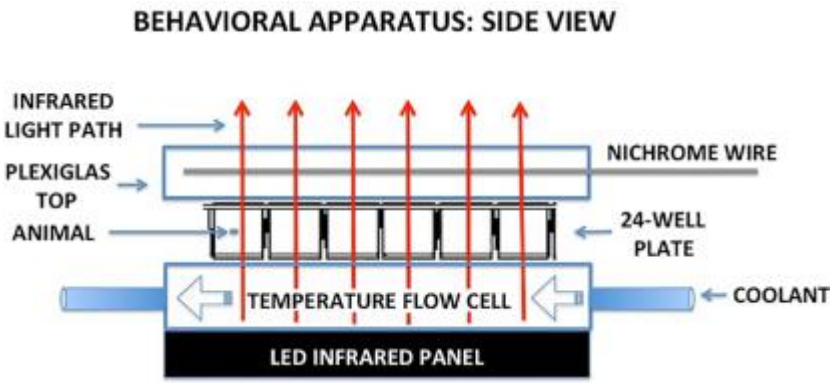

Figure 2B: Diagramatic represenation of the 24-well plate setup under the camera and lens.

Figure3: the circulating bath for maintaining optimum temperatures.

Figure4: The camera, lens and the 24-well plate setup for the swimming behavior of *daphnia pulex* and *danio rerio.*

Optical Tracking: A Lumenera 3S camera provided a resolution of 1280 x 1024 which is used to capture videos in AVI format. The videos were analyzed using Image pro premier 9 software (Media Cybernetics, Rockville, MD). The 2-D tracking model was calibrated for a linear distance in mm. Prior to the analysis of the videos were adjusted for contrast (flattened) to the videos to remove background lighting variation and then the image was sharpened to enhance the fine details of the videos. The two main parameters which were analyzed were the maximum accumulated distance and change in angle. The accumulated distance is calculated by summing the total distance moved by the *daphnia* in 148 frames (Figure 6). The change in angle is measured by the change in the vectors between frames (Figure 6). For the measurement of the distance, a minimum of 2 frames are required (Figure 6). Maximum accumulated distance represents the sum of distance

measurements over the duration of the recording (5sec for *D. pulex*). For the measurement of angular change, a minimum of 3 frames are required (Figure 6). Measurements of maximum accumulated distance (mm) and angular change (degrees) were exported in an Excel spreadsheet for each video (Figure 6). Mean angular change was calculated by an Excel macro from the raw angular change values which ranged from -180 (left) to +180 degrees (right) from the directional vector (Figure 6). The following formula was used to convert changes in angle into positive numbers and calculate a mean angular change as an index of the average amount of turning away from the straight-line vector:

=IF(OR(E93="",F93=""),"",IF(E93*F93>0,ABS(E93F93),IF((ABS(E93)+ABS(F93))<180,(ABS(E93)+ABS(F93)),360-(ABS(E93)+ABS(F93)))))

The terms, E93 and F93, refer to examples of successive columns (video frames) within an Excel spreadsheet of raw data from Image Pro Plus.

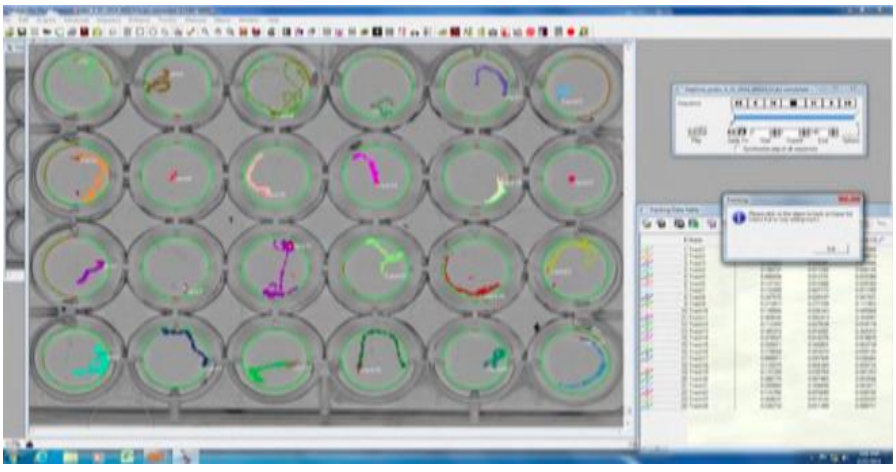

Figure5: A video file tracked for the movement of *daphnia.*

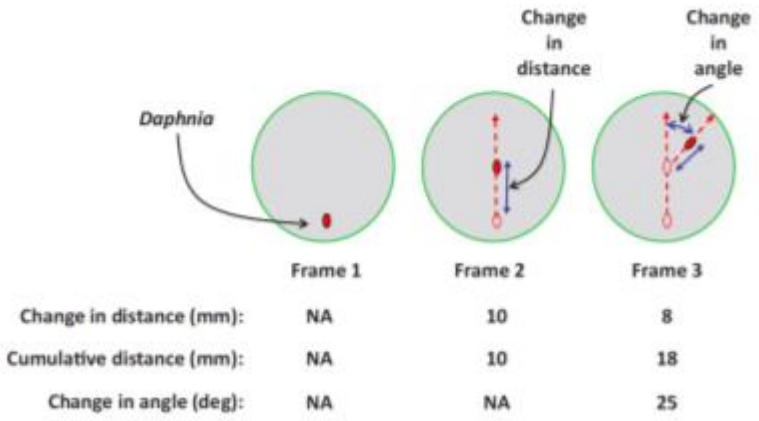

Figure6: Example of the quantification of the maximum accumulated distance and mean angle. Adapted from Zein et al. (2014)

Recordings: For *D. pulex* experiments, 5 second videos were taken every 10 mins for 3 hours, then every 30mins until the end of the 24-hour recording period. A total of 65 videos are recorded per experiment. Due to the very large data set, we used only 29 of these videos in our behavioral analysis. The sample of videos used were: every 10 mins for the first 3 hours, then every hour for the next 3 hours, then every 2 hours for the next 6 hours, and then every 4 hours for the next 12 hours. The 5 second videos consisted of 148 frames which were exported as maximum accumulated distance and angular change to an Excel spreadsheet. An Excel macro was used to combine the 29 Excel spreadsheets from a full 24-hour experiment into one spreadsheet. The macro calculated the maximum accumulated distance for every animal in each video/time point and the mean angular change for every animal in each video/time point as described above. The *D. pulex* behavior was further quantified by Statistica software (Version10, Tulsa, OK, USA), which provided graphs of mean values plus or minus standard error for the two variables (see Statistics, below).

Since zebrafish were generally less active than *D. pulex* in the control situation, recordings for zebrafish experiments used 20 seconds videos instead of 5 seconds. This generated approximately 592 frames per recording. The remainder of quantitative design remained the same as described above for *D. pulex*.

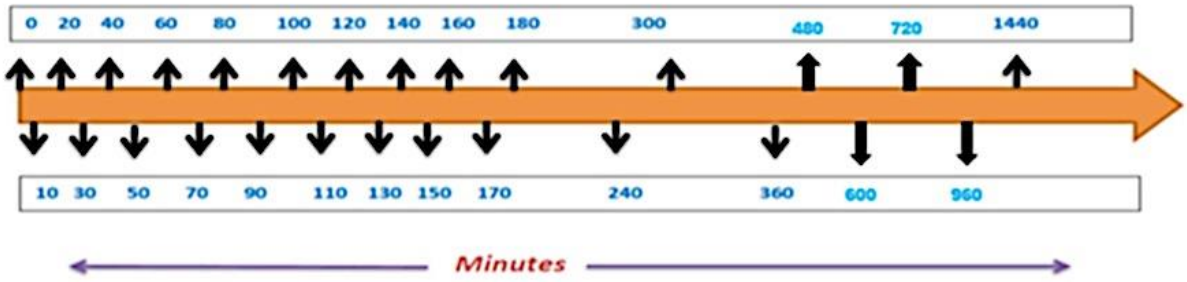

Figure7: The time points at which the recordings are tracked.

Statistical Analysis: All the statistical analysis was executed using Statistica (Version10, Tulsa, OK, USA). The dependent variables were the maximum accumulated distance (millimeters) and mean angle (degrees). These parameters were collected at regular intervals during the 24-hour experiment. The independent variables were the time (0-1440), concentration, well-number, chemical and the plate-number. Analysis of variance (ANOVA) with repeated measures (time) was used to evaluate changes in the dependent variables over 24 hours (maximum accumulated distance and mean angle) following exposure to the different chemicals. The Least Significant Difference test (LSD) was used as the post hoc test to compare the means of different concentrations across the same time-points.

Drugs, chemicals and solutions: All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All the stock solutions were made in acetone and used for

2 days in the refrigerator. The concentration of the stock solution was generally 10mM except for Atrazine, which was 100mM. The concentration of acetone was not more than 0.05% in the highest concentration used for all chemicals evaluated except for atrazine which was 0.1% of acetone for the highest concentration was used. All the stock solutions were made as 10mM or 100mM (atrazine) concentrations, and were then serially diluted with the appropriate media (COMBO or fish media) to make the test concentrations.

Chapter 3A: Behavioral Studies on *D.pulex*

Chlorpyrifos (Lower concentrations)

Distance: Although the mean accumulated distance for the highest concentration (5 nM) was higher than control chlorpyrifos did not show a significant concentration dependent effect on maximum accumulated distance (P> 0.50), as shown in the Figure 1. Chlorpyrifos did not show significant time x concentration interaction effect on maximum accumulated distance (P>0.50). Figure 2 depicts the time course for the effects of the highest concentration of chlorpyrifos and the control. There was no evidence of stimulation of swimming in terms of increased maximum accumulated difference at any of the concentrations (0.31, 0.62, 1.25, 2.5, 5nM) over time. In addition, there was no significant stimulation of swimming by any concentration of chlorpyrifos (P>0.50) at 24hrs.

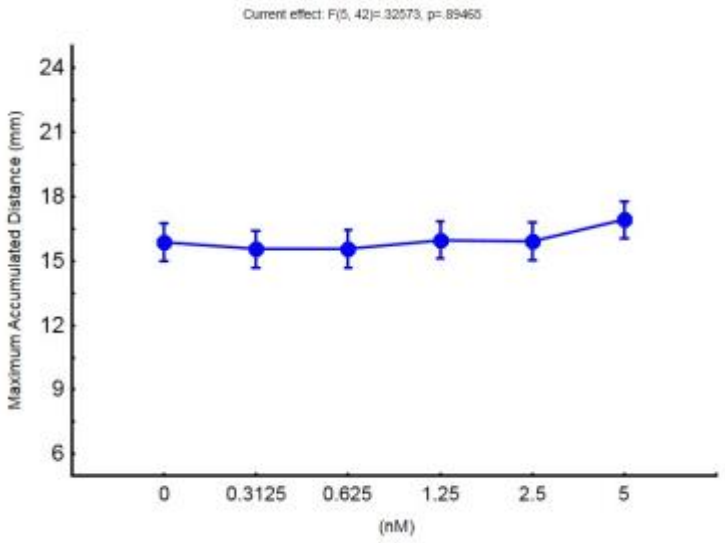

Figure 8: The effect of 5 different chlorpyrifos concentrations on maximum accumulated distance by *Daphnia pulex.*

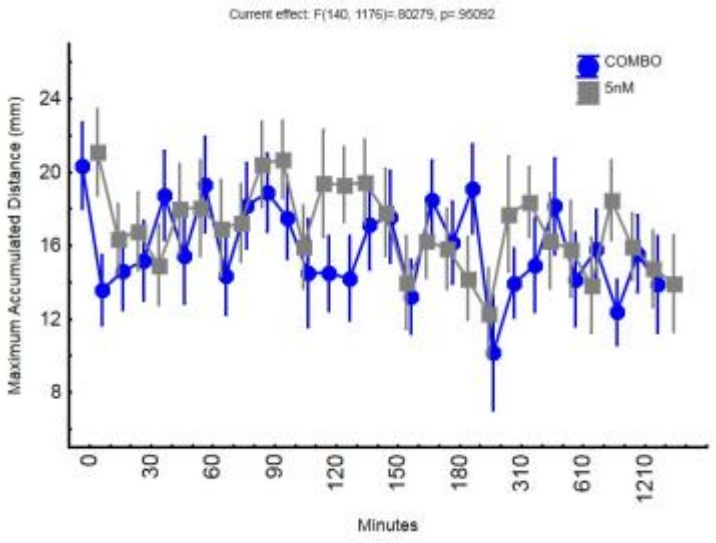

Figure 9: Time-dependent effect of five different chlorpyrifos concentrations (0.31, 0.62, 1.25, 2.5, 5nM) on maximum accumulated distance by *Daphnia pulex.*

Angle: As can be seen in Figure 3, the mean angle did not change significantly at any concentration of chlorpyrifos (P>0.50). The effect of concentration on the mean angle was not dependent on time (P>0.05; Figure 4).

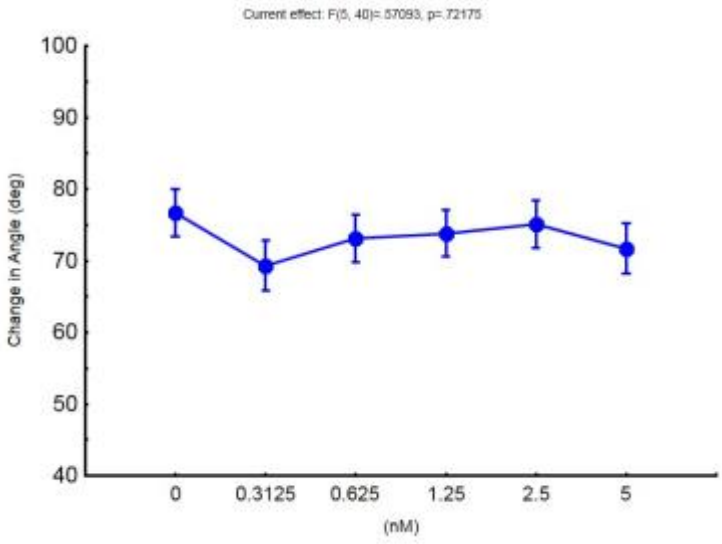

Figure 10: The effects of five different chlorpyrifos concentrations (0.31, 0.62, 1.25, 2.5, 5nM) on mean Angle by *Daphnia pulex.*

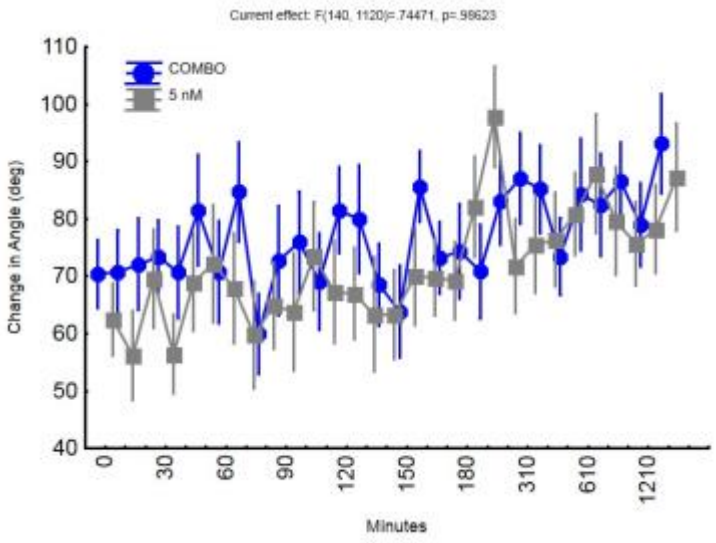

Figure 11: The effect of two different concentrations of chlorpyrifos on mean angle over time by *Daphnia pulex.*

4- Nonylphenol

Distance: 4- Nonylphenol elicited a significant concentration-dependent effect on maximum accumulated distance ($P<0.05$), as shown in Figure 5. The distance for concentration 2uM and 4uM is lower than the control ($P<0.05$, LSD for 2 concentrations). Although, the value for the 0.25uM concentration was also lower than the control this was not significant. Although 4- nonylphenol decreased the maximum accumulated distance, there was not a significant time X concentration interaction effect on maximum accumulated distance ($P>0.20$), indicating that the effect tended to be sustained across the time course. The onset of the decrease in the maximum accumulated distance in the time course can be seen within the first thirty minutes. Concentrations 2um and 4um showed a significant decrease in maximum accumulated distance at 24 hours ($p<0.05$).

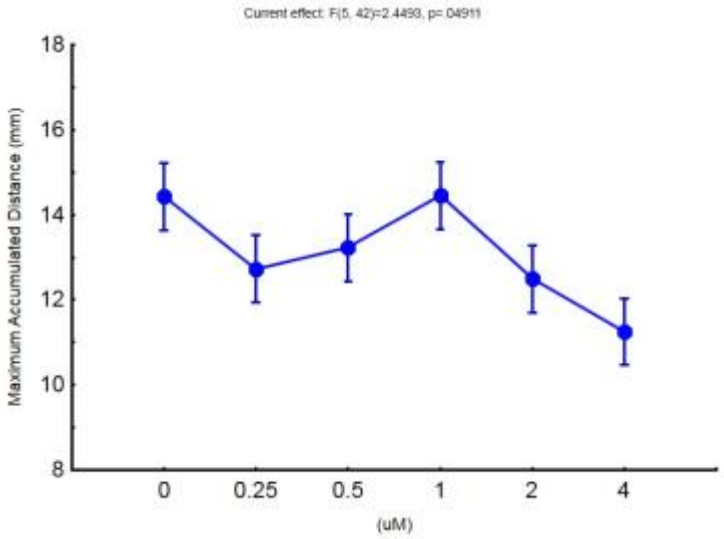

Figure 12: Concentration-dependent effect of 4-nonylphenol on maximum accumulated distance by *Daphnia pulex.* The LSD test indicated a significant difference between 4-nonylphenol treated animals and controls. (* P<0.05)

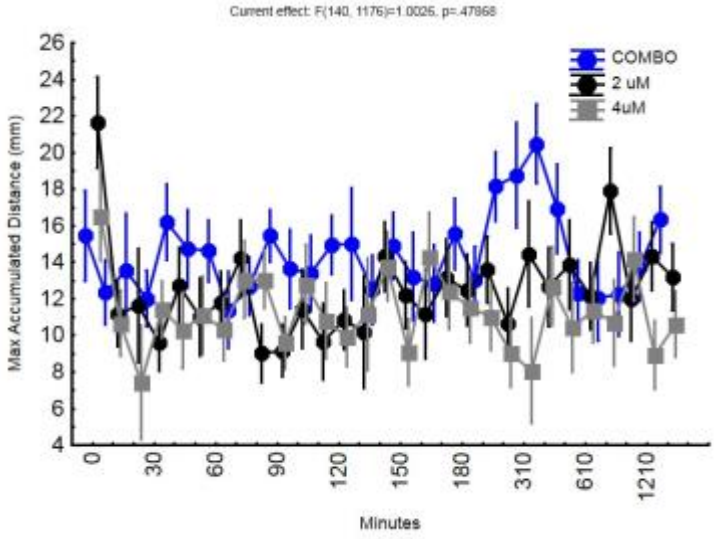

Figure 13: Time- dependent effect of 4-nonylphenol on maximum accumulated distance by *Daphnia pulex.*

Angle: There was a very significant concentration-dependent increase in mean angle elicited by 4- Nonylphenol (P<0.001) as shown in Figure 7. The mean angle for all of concentrations was higher than the control (P<0.05, LSD in all cases). Figure 8, shows the significant concentration-dependent effect of 4-nonylphenol on mean angle over time (P< 0.001), The fast response of mean angle to the 2 and 4uM is apparent within thirty minutes and becomes a maximal response for the duration of the time course. The increase in the mean angle for concentrations 2 and 4um is associated with the decrease in maximum accumulation distance (p<0.05).

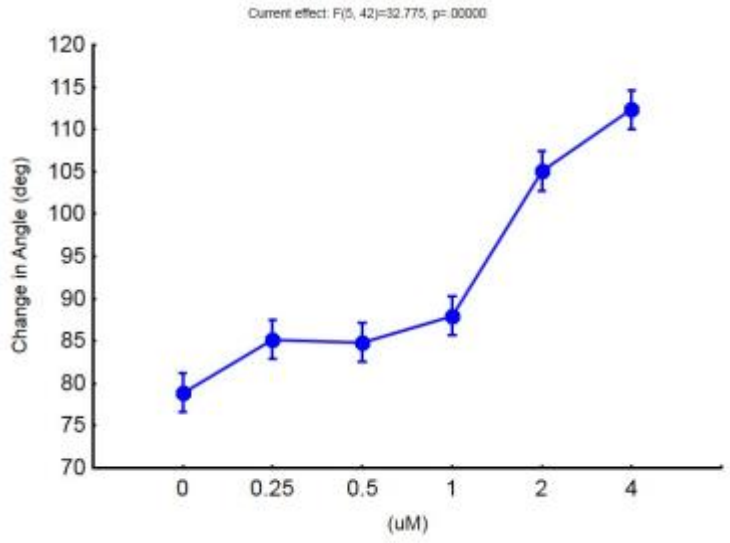

Figure 14: Concentration-dependent effects of 4-nonylphenol on mean angle by *Daphnia pulex.* The LSD test indicated a significant difference between 4-nonylphenol treated animals and controls. (* P<0.05)

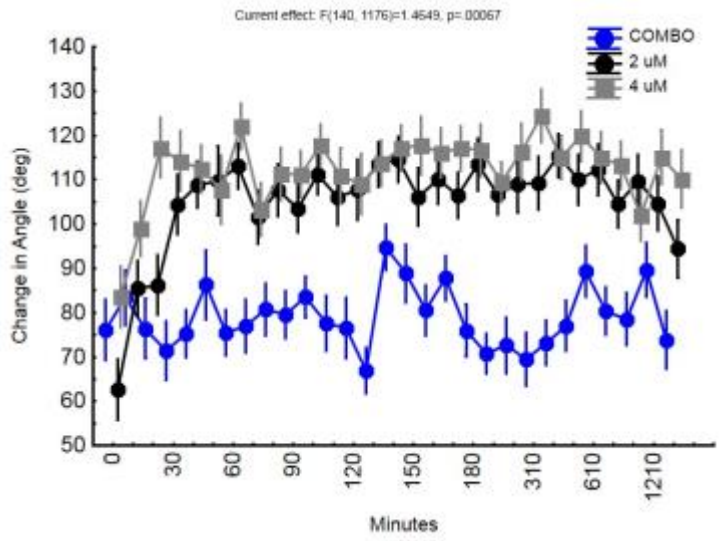

Figure 15: Time-dependent effects of 4-nonylphenol on mean angle by *Daphnia pulex.* The LSD test indicated a significant difference between 2 and 4 uM 4-nonylphenol treated animals and controls at the corresponding time point. (* P<0.05)

Bisphenol-A

Distance: As shown in Figure 9, bisphenol A did not significantly affect maximum accumulated distance at any concentration studied (P>0.20). The maximum accumulated distance for the higher concentration, 64uM, was lower than control (P>0.05, LSD in 64uM case) suggesting that it might inhibit swimming behavior at higher concentrations. There was no significant effect of Bisphenol-A on maximum accumulated distance over time (time x concentration effect, P>0.50, Figure 10). A decrease in maximum accumulated distance by 64uM was observed throughout 24 hours.

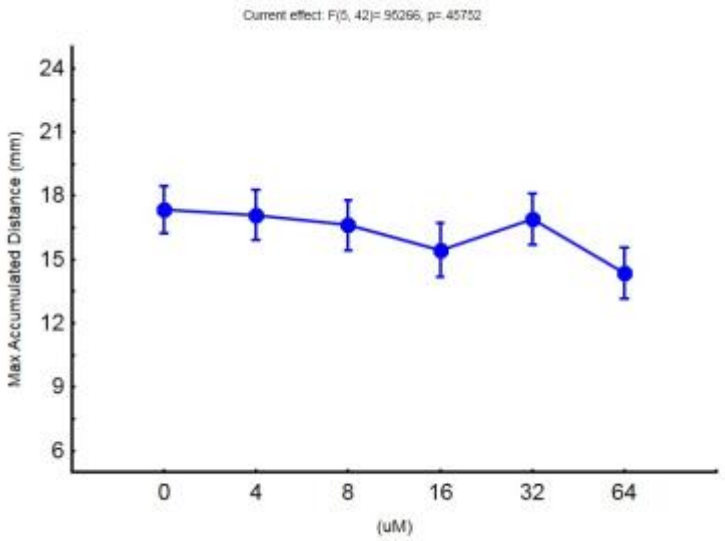

Figure 16: Effect of bisphenol-A (4 to 64 uM) on maximum accumulated distance by *Daphnia pulex.*

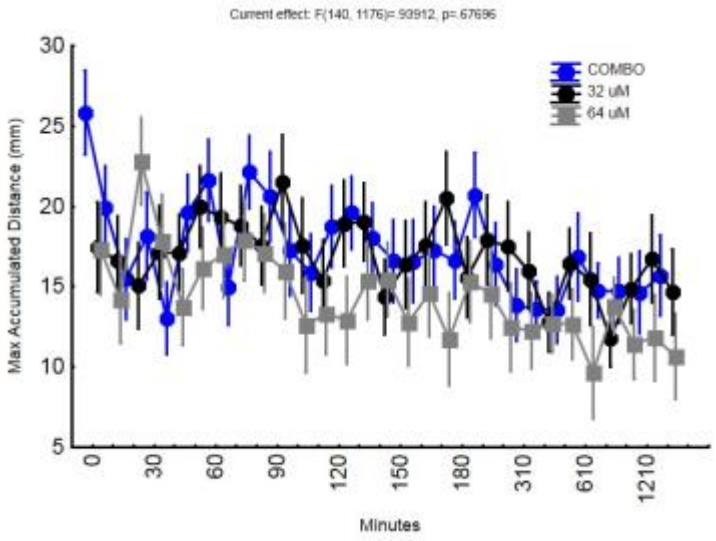

Figure 17: Effects of 32 and 64 uM bisphenol A on maximum accumulated distance over time by *Daphnia pulex.*

Angle: As shown in Figure 11, bisphenol-A did not significantly change mean angle at any concentration(P>0.50). Figure 12 shows the effects of bisphenol-A over time. The time x concentration interaction for the effects bisphenol-A was also not significant (P>0.10). In

addition, the effect of concentration does not show a significant change on mean angle ($p>0.05$).

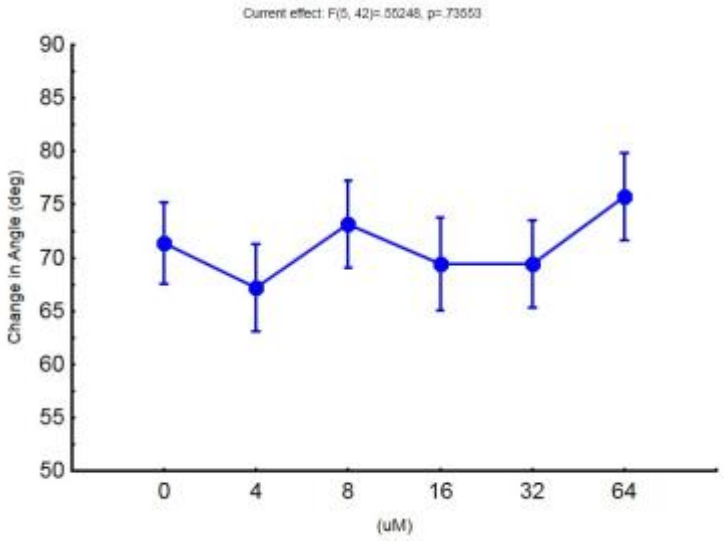

Figure 18: Effect of bisphenol A (4 to 64 uM) on mean angle by *Daphnia pulex.*

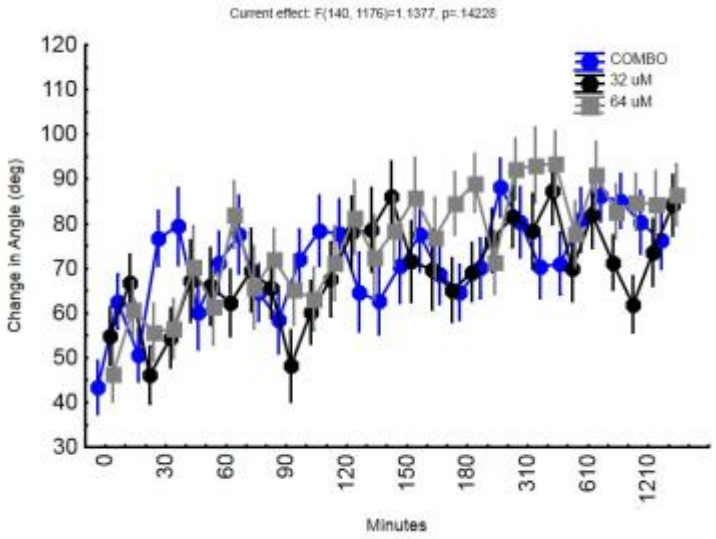

Figure 19: Effect of 32 and 64 uM bisphenol A on mean angle over time by *Daphnia pulex.*

Dieldrin:

Distance: Dieldrin showed a significant concentration dependent effect on maximum accumulated distance (P<0.01). The higher concentrations (2.5uM, 12.5uM, 62.5uM) significantly stimulated swimming behavior and showed an increase in maximum accumulated distance (P<0.01, LSD for 2.5 uM, 12.5 uM, 62.5 um). The effects of concentration on maximum accumulated distance was not dependent on time (P>0.50,). The time course for the higher concentrations, 12.5uM and 62.5uM, are shown in Figure 14. The overall effect of dieldrin on maximum accumulated distance was not dependent on time (P>0.50).

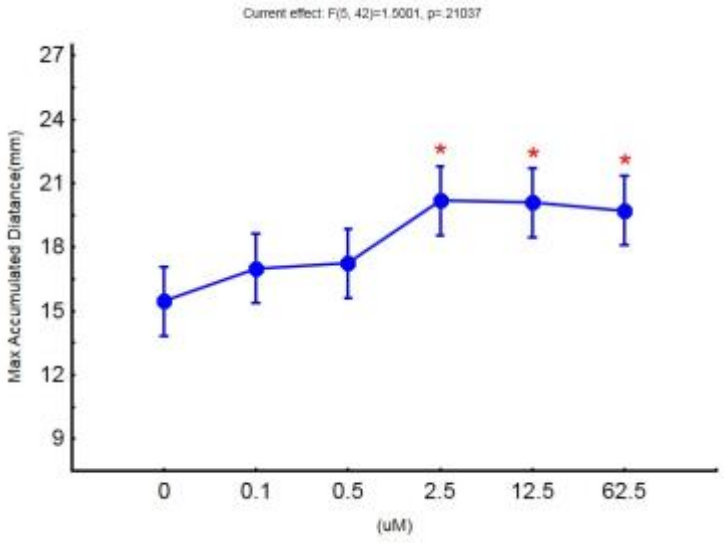

Figure 20: Concentration-dependent effect of Dieldrin on Maximum Accumulated Distance by *Daphnia pulex.* The LSD test indicated a significant difference between dieldrin treated animals and controls. (*P<0.05)

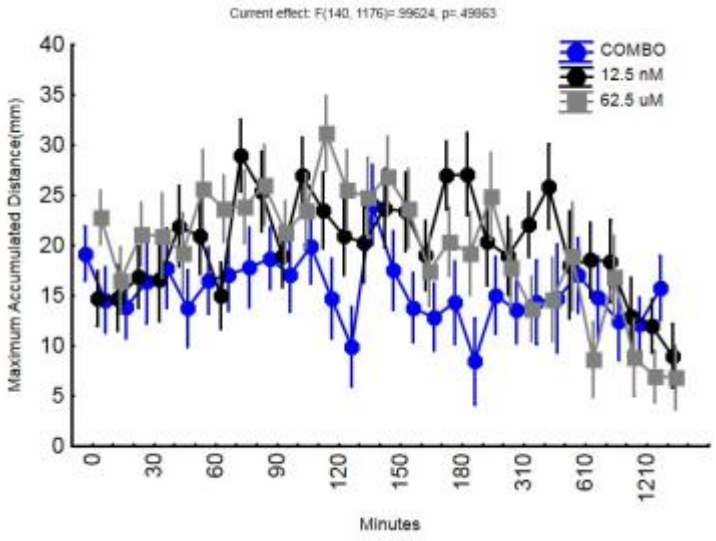

Figure 21: Time-dependent effect of dieldrin on maximum accumulated distance by *Daphnia pulex.*

Angle: Mean angle was not significantly affected by dieldrin when considering concentration alone (P>0.50). However, the concentration x time interaction effect for mean angle was significant (P<0.005). The concentration-dependent effect for 62.5 uM was observed after 210 minutes (P<0.05).

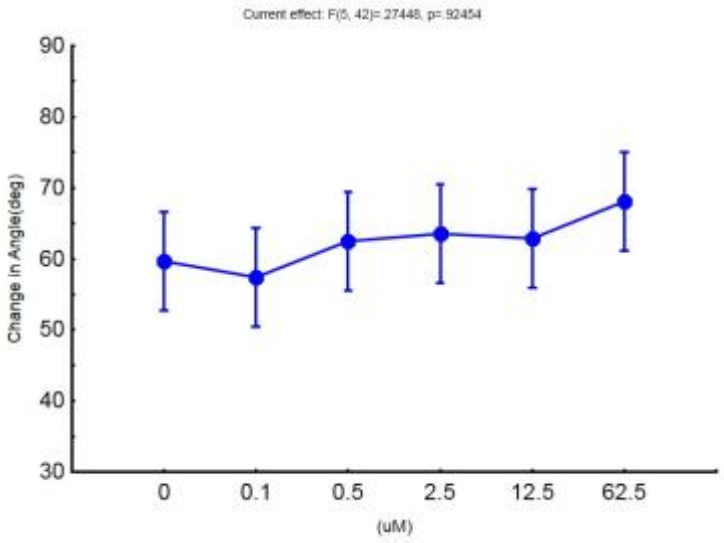

Figure 22: The effect of dieldrin (0.1 to 62.5uM) on mean angle by *Daphnia pulex.*

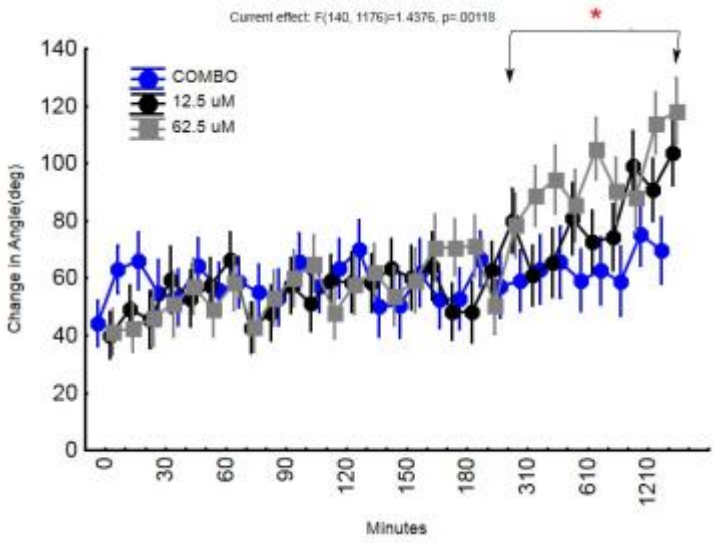

Figure 23: The time-dependent effects of dieldrin on mean angle by *Daphnia pulex.* There was a significant difference between dieldrin treated animals and controls at the corresponding time points as indicated by the bracket (Contrast analysis, * P<0.05).

Chlorpyrifos (Medium Range):

Distance: Chlorpyrifos elicited a significant concentration-dependent effect on maximum accumulated distance (p<0.001) as shown in the Figure 15. The maximum accumulated distance for the highest concentrations (62.5, 125.4um and 250nm) was lower than the control. There was a dramatic decrease in maximum accumulation distance over time with increasing concentration (concentration x time interaction, P<0.05). Immobility was observed at the highest concentrations, 125nM and 250nM. The 250 nM concentration produced immobility approximately 2 hours after exposure and the12uM concentration produced immobility after approximately 3hrs.

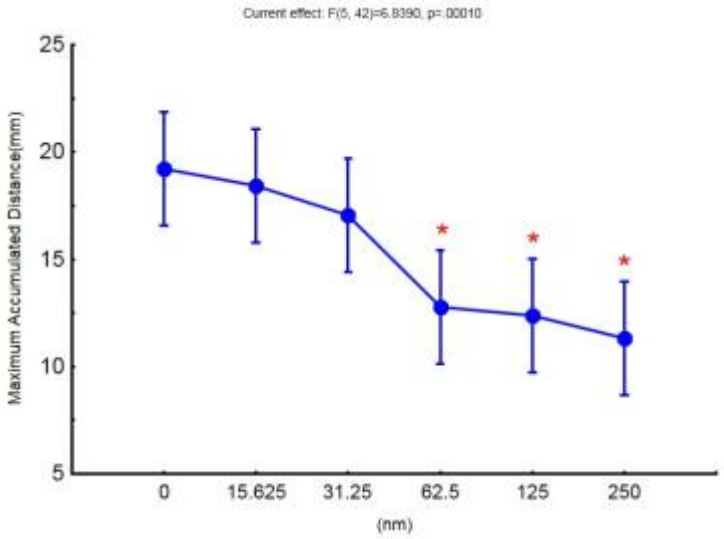

Figure 24: Concentration-dependent effects of chlorpyrifos (15.6 to 250 nM) on maximum accumulated distance by *Daphnia pulex*. The LSD test indicated a significant difference between chlorpyrifos treated animals and controls (* P<0.05).

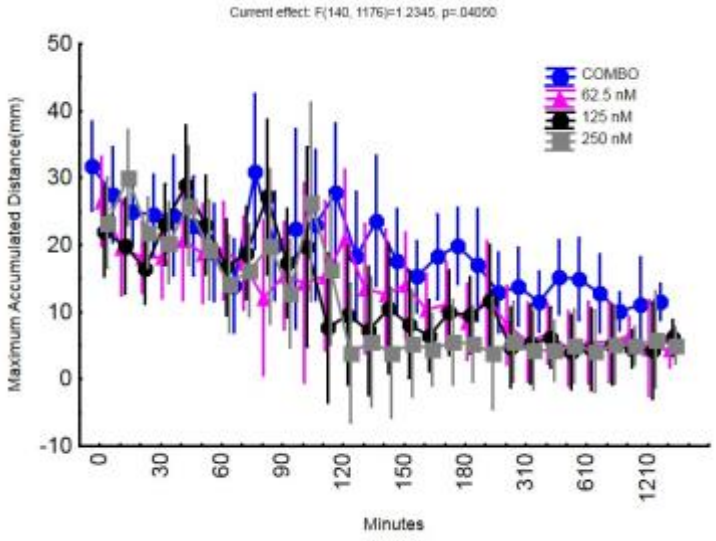

Figure 25: The time-dependent effect of chlorpyrifos on maximum accumulated distance by *Daphnia pulex*. The LSD test indicated a significant difference between chlorpyrifos treated animals and controls at the corresponding time point (* P<0.05).

Angle: Chlorpyrifos had a significant concentration-dependent effect on mean angle as shown in Figure 17($p<0.001$). Mean angle increased progressively in a linear fashion with increasing concentrations. The effect of chlorpyrifos on mean angle over time was significant (concentration x time interaction $P<0.001$). The animals exposed to 125 nM and 250 nM exhibited a plateau at maximum values of mean angle and were immobilized after 310 min.

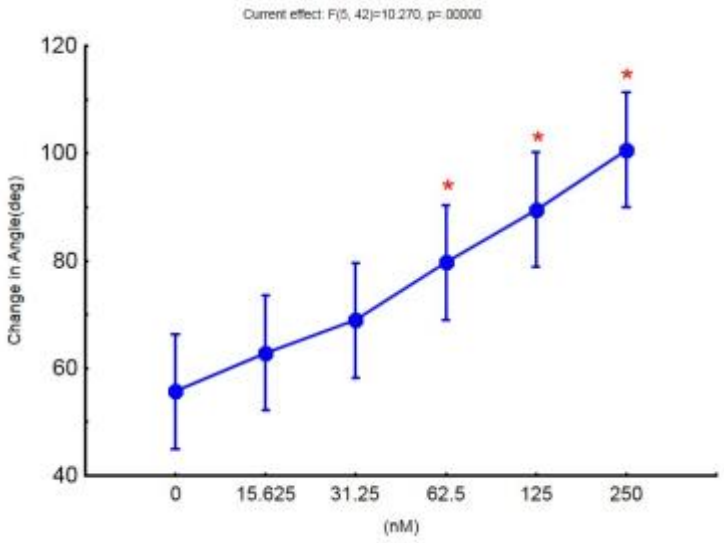

Figure 26: The concentration-dependent effect of chlorpyrifos on mean angle by *Daphnia pulex.* The LSD test indicated a significant difference between chlorpyrifos treated animals and controls (* $P<0.05$).

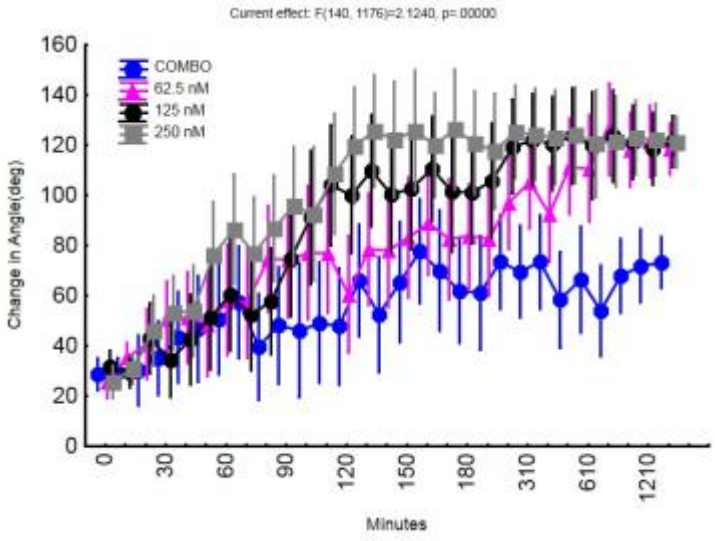

Figure 27: The time-dependent effect of chlorpyrifos on mean angle by *Daphnia pulex*. The LSD test indicated a significant difference between chlorpyrifos treated animals and controls at the corresponding time point (* P<0.05).

Chlorpyrifos (High Range):

Daphnia were immobilized immediately as soon as we administer the chemical with a concentration higher than 0.25uM.

Atrazine:

Distance: As shown in Figure 19, atrazine elicited a significant concentration-dependent effect on maximum accumulated distance(P<0.001). The effects of atrazine on maximum accumulated distance was not found to be dependent on time (concentration x time interaction) P>0.50. The behavioral stimulation resulting in an increase in maximum

accumulated distance was seen within 30 minutes for the two highest concentrations, 20 □M and 100 □M, and tended to be elevated throughout the 24hr period of exposure.

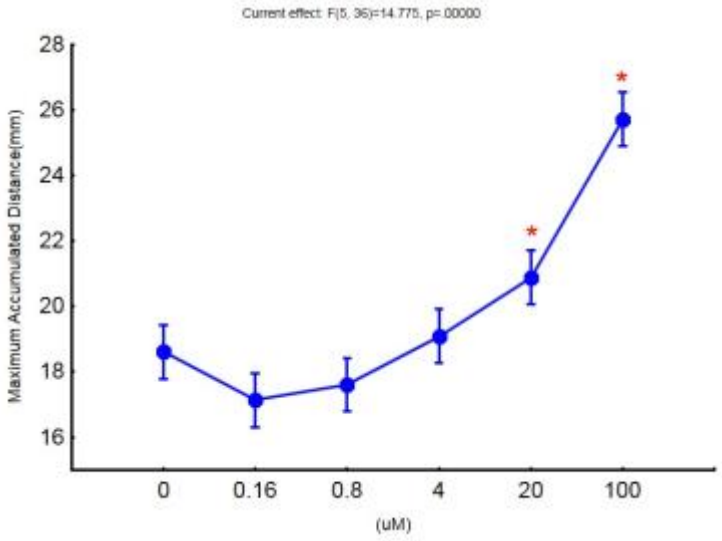

Figure 28: Concentration-dependent effect of Atrazine on Maximum Accumulated Distance by *Daphnia pulex.* The LSD test indicated a significant difference between atrazine treated animals and controls at the corresponding time point (* P<0.05).

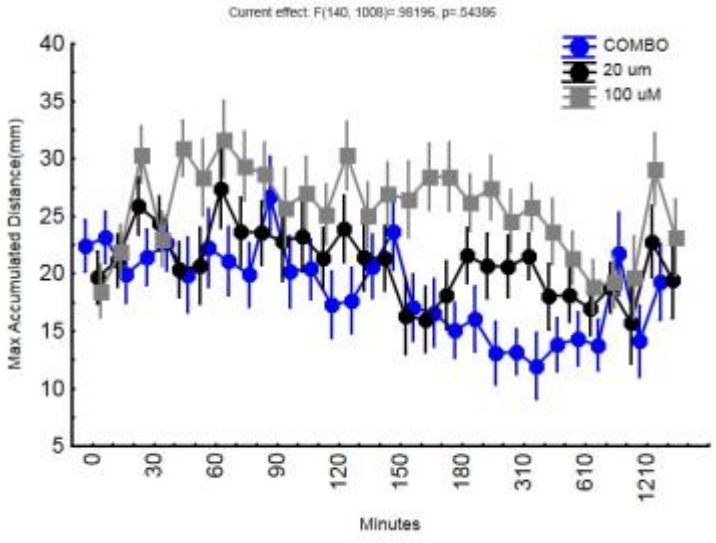

Figure 29: Time-dependent effect of Atrazine on Maximum Accumulated Distance by *Daphnia pulex.*

Angle: Atrazine The did not show any significant concentration-dependent effect on mean angle, as shown in Figure 21. In addition, there was no significant effect of atrazine over on mean angle over time (concentration x time interaction effect, P>0.50).

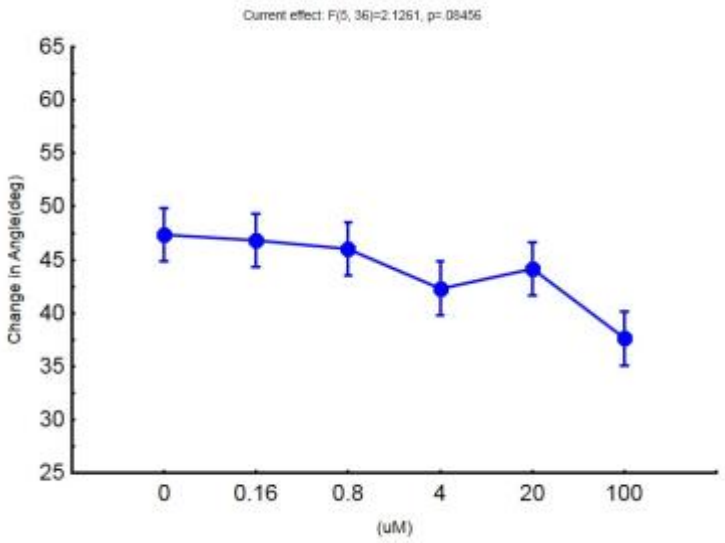

Figure 30: Concentration-dependent effect of Atrazine on Mean Angle by *Daphnia pulex.*

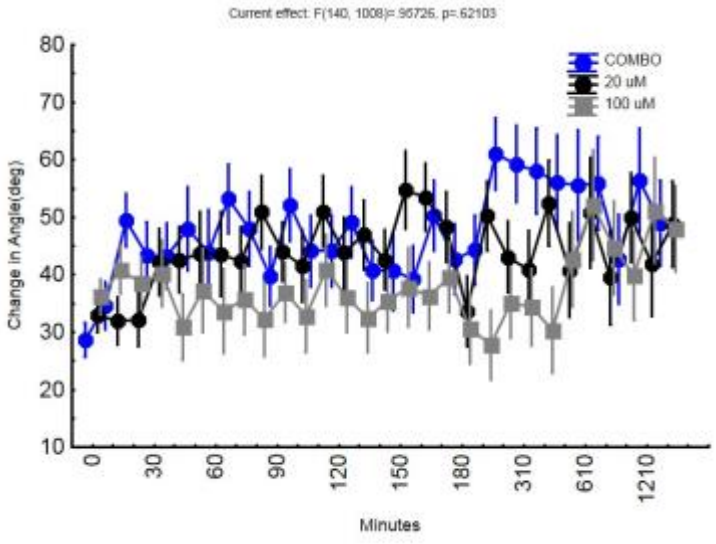

Figure 31: The effects of atrazine on mean angle over time by *Daphnia pulex.*

Toxaphene:

Distance: Toxaphene elicited a significant concentration-dependent effect on maximum accumulated distance ($P<0.05$), as shown in Figure 23. The maximum accumulated distance for the two highest concentrations, 1.2uM and 2.4uM, was significantly higher than the control (LSD test, $P<0.05$ in both cases compared to 0.15 and 0.3 nM). The effect of toxaphene maximum accumulated distance over time was not significant (concentration x time interaction effect, $P>0.20$). Figure 24 illustrates the time course for the effects of toxaphene over time. The peak level of stimulation is observed at the highest concentration (2.4uM), after 30 minutes, before it subsides, and then again after 180 minutes.

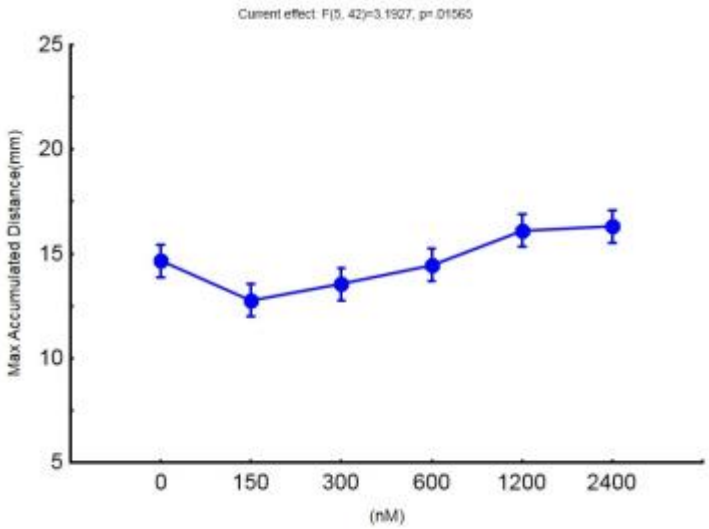

Figure 32: The concentration-dependent effect of toxaphene on maximum accumulated distance by *Daphnia pulex.* The LSD test indicated a significant difference between 150 nM toxaphene treated animals and higher concentration of toxaphene at the corresponding time point (* $P<0.05$).

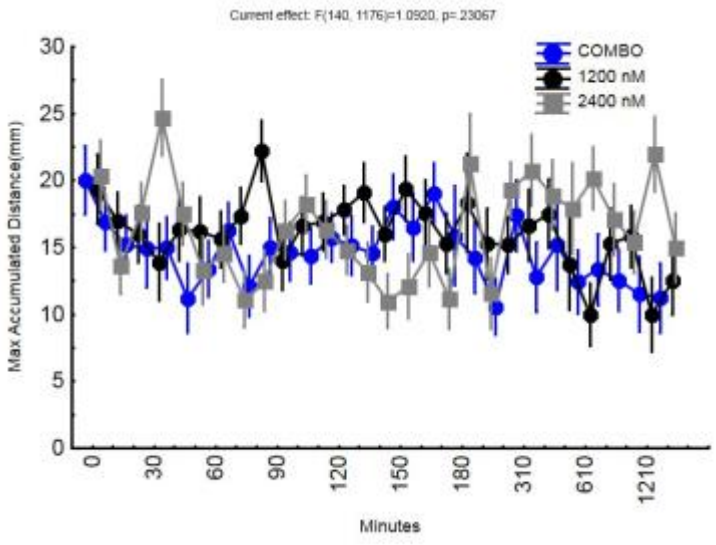

Figure 33: The effect of toxaphene on maximum accumulated distance over time by *Daphnia pulex.*

Angle: Toxaphene did not show any significant concentration-dependent effect on mean angle (P>0.50). There was no significant time-dependent effect of toxaphene on mean angle (concentration x time interaction effect, P>0.20). In contrast to the effects of some of the other chemical agents that affect swimming behavior, a significant increase in maximum accumulated distance was not associated with a corresponding decrease in mean angle.

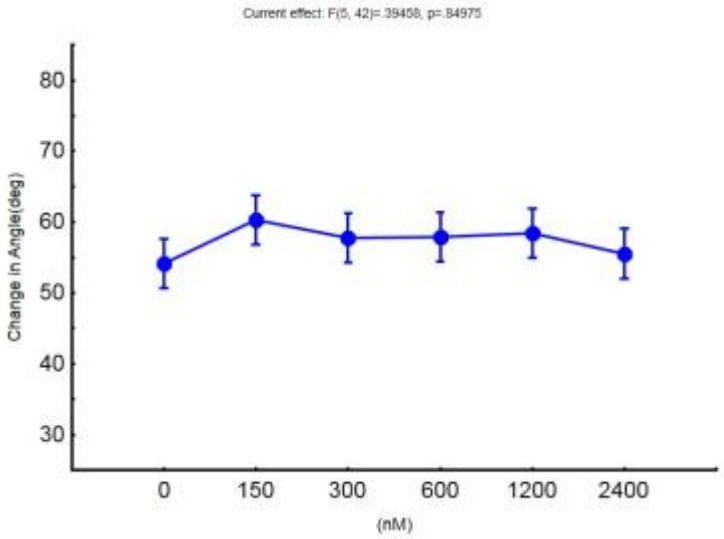

Figure 34: The effect of toxaphene on mean angle by *Daphnia pulex.*

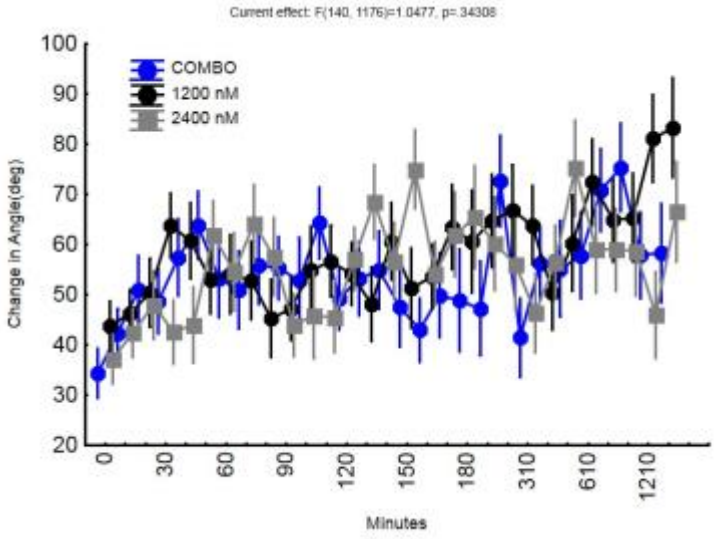

Figure 35: The effect of toxaphene on mean angle over time by *Daphnia pulex.*

Chapter 3B: Behavioral studies on Zebrafish

Chlorpyrifos

Distance: Chlorpyrifos did not elicit a significant concentration-dependent effect on maximum accumulated distance (P>0.50). Figure 28 depicts the time course for the effect of the 2 highest concentrations of chlorpyrifos (500, 1000nM) along with the control. Although there was an increase in maximum accumulated distance by 1000nM relative to control after 310 minutes, chlorpyrifos did not elicit a significant time x concentration interaction effect on maximum accumulated distance (P>0.50).

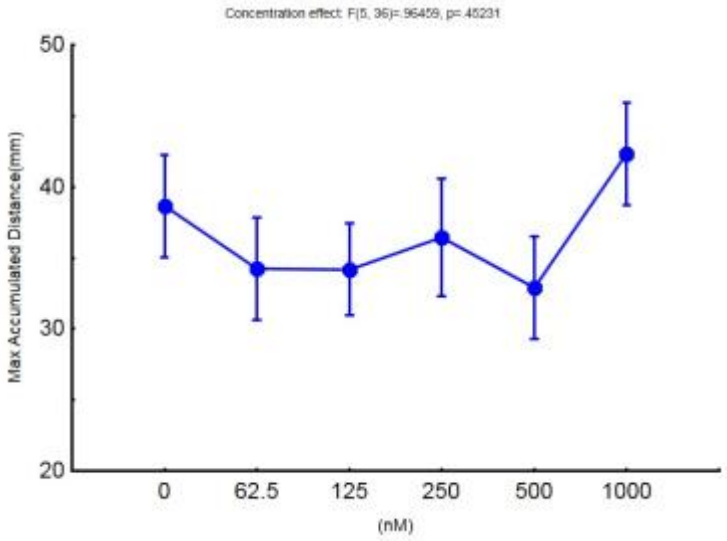

Figure 36: The effect of 5 different chlorpyrifos concentrations on maximum accumulated distance by *Danio rerio.*

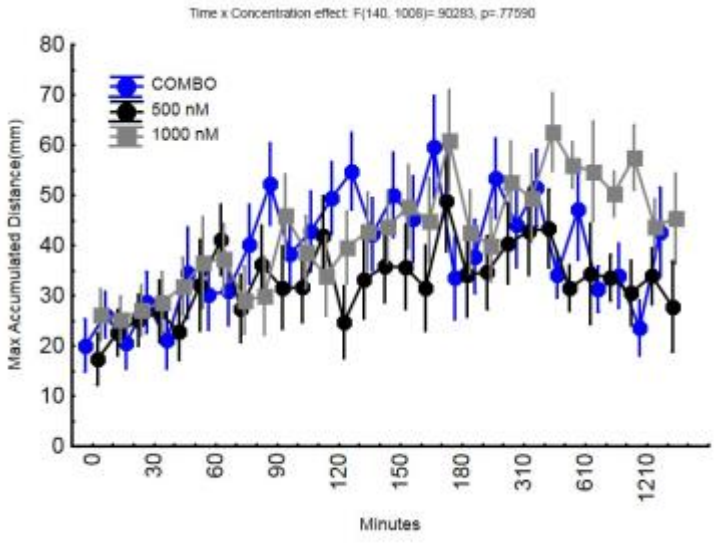

Figure 37: Time-dependent effect of two chlorpyrifos concentrations (500 and 1000nM) on maximum accumulated distance by *Danio rerio.*

Angle: The effect of 5 concentrations of chlorpyrifos (62.5, 125, 250, 500, 1000nM) on mean angle was not significant (P>0.20) as shown in Figure 29. The effect of chlorpyrifos on mean angle over time was not significant (time x concentration effect, P>0.20). However, the increased value for mean angle at the highest 1000nM concentration is consistent with the elevated maximum accumulated distance suggesting that the effects of the 1000nM concentration on swimming behavior may become significant with a larger sample size Figure 30 shows the time course for the two highest concentrations of chlorpyrifos (500, 1000nM) along with control.

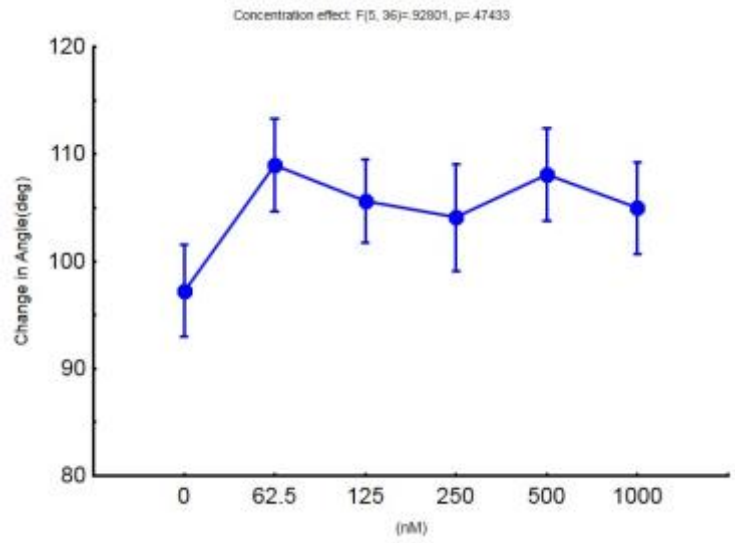

Figure 38: The effect of 5 different chlorpyrifos concentrations on mean angle by *Danio rerio.*

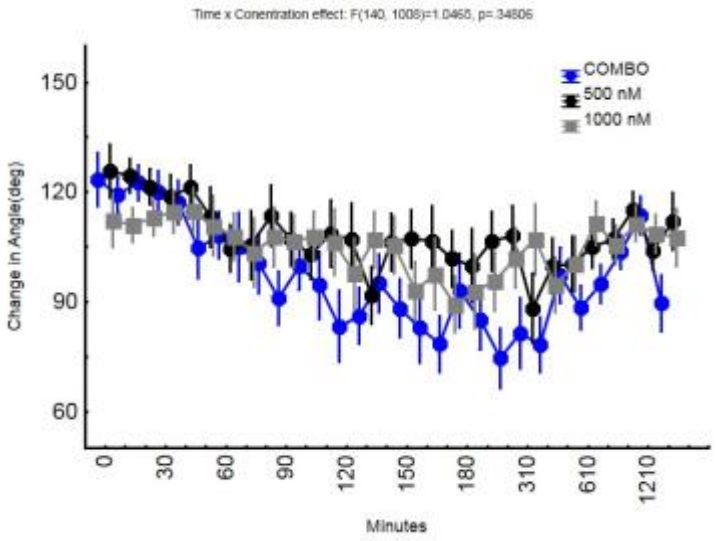

Figure 39: Time-dependent effect of two chlorpyrifos concentrations (500 and 1000nM) on mean angle by *Danio rerio.*

Dieldrin

Distance: Dieldrin induced a significant concentration-dependent effect on the maximum accumulated distance (P<0.001) as shown in Figure 31. A slight decrease in maximum

accumulated distance at the concentration of 0.0625uM and a steep increase in maximum accumulated distance at higher concentrations was noted. The dieldrin effect on maximum accumulated distance was also found to be significant (time x concentration interaction, P<0.001, Figure 32) . Behavioral stimulation occurred earlier in the time-course at higher concentrations than it did for the lower concentrations. For example, the peak stimulation for 1uM was seen at approximately 40 minutes, 40 to 50 minutes for 0.5uM, and approximately 100 minutes for 0.25uM.

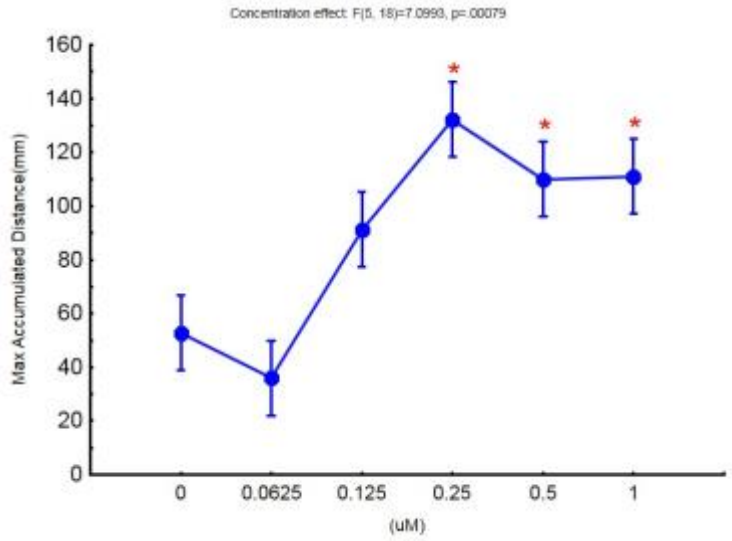

Figure 40: The effect of 5 different dieldrin concentrations on maximum accumulated distance by *Danio rerio.* The LSD test indicated a significant difference between dieldrin treated animals and controls (*P<0.05).

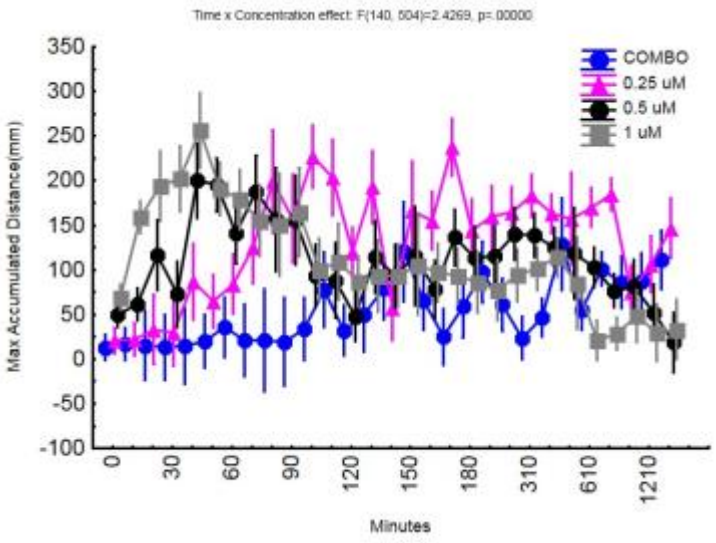

Figure 41: Time-dependent effect of three dieldrin concentrations (0.25, 0.5 and 1uM) on maximum accumulated distance by *Danio rerio.*

Angle: The mean angle show a significant concentration-dependent effect (P<0.001) as shown in Figure 33. The mean angle increased at the lower concentration (0.0625nM) and then higher concentrations (0.25 – 1.0 μM) causing a decrease in the mean angle. The effect of concentration on mean angle was dependent on time (concentration x time interaction, P<0.05). Figure 34 illustrates the time course of the effects of the 3 higher concentrations on mean angle along with control. The highest concentration (1uM), shows a decrease in mean angle within 10 minutes, whereas the lower concentrations, 0.5 and 0.25uM, show a decrease in the mean angle later in the time course.

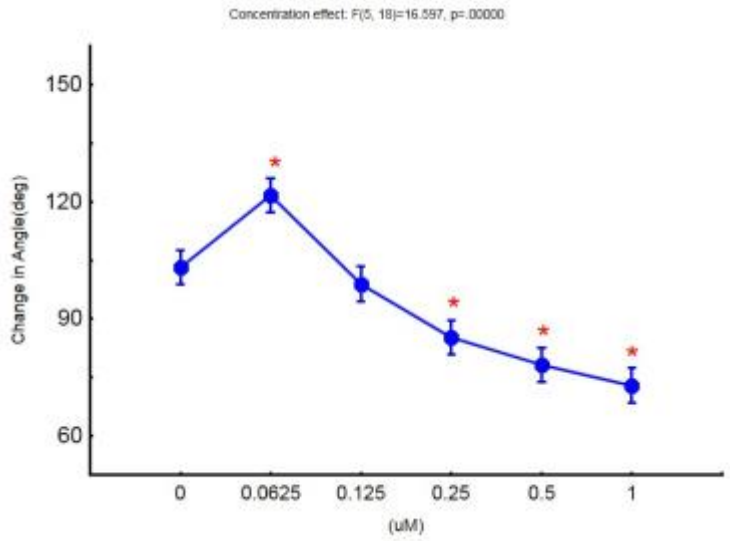

Figure 42: The effect of 5 different dieldrin concentrations on mean angle by *Danio rerio.* The LSD test indicated a significant difference between dieldrin treated animals and controls (* P<0.05).

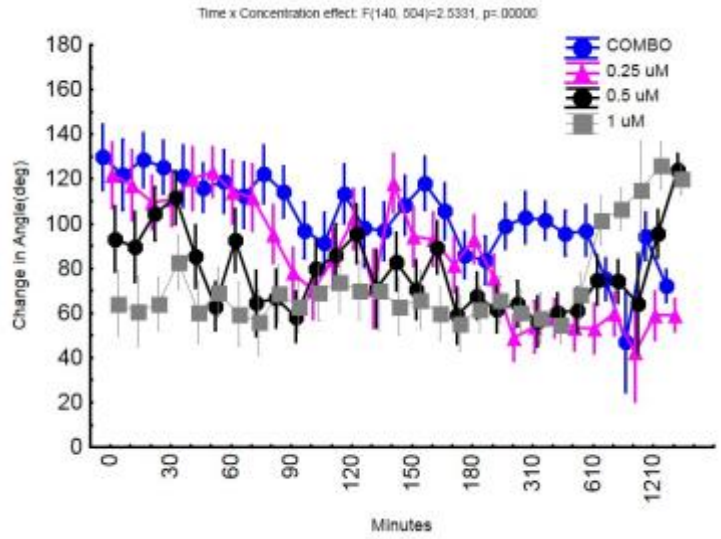

Figure 43: Time-dependent effect of three dieldrin concentrations (0.25, 0.5 and 1uM) on mean angle by *Danio rerio.*

4-Nonylphenol

Distance: The relationship between the maximum accumulated distance and concentration appeared to be non-monotonic, however, there was no significant concentration-dependent effect on maximum accumulated distance (P>0.10) when time was not included as a factor as shown in Figure 35. The mean values were lower for concentrations of 0.25 and 0.5nM begin to increase and approach the control level at the higher concentrations (1, 2, and 4nM). 4-nonylphenol did elicit a significant effect of on maximum accumulated distance when time was included as a factor (time x concentration interaction, P<0.001).. The highest concentration, 4μM exhibited a periodic stimulatory effect on maximum accumulated distance over the first two hours before subsiding to control levels. The 0.25 uM concentration reduced maximum accumulated distance below control levels over the first two to three hours.

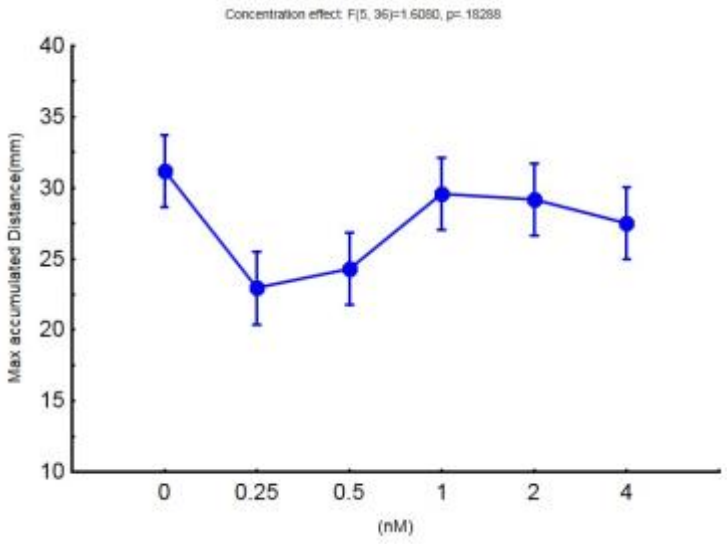

Figure 44: The effect of 5 different 4-nonylphenol concentrations on maximum accumulated distance by *Danio rerio.*

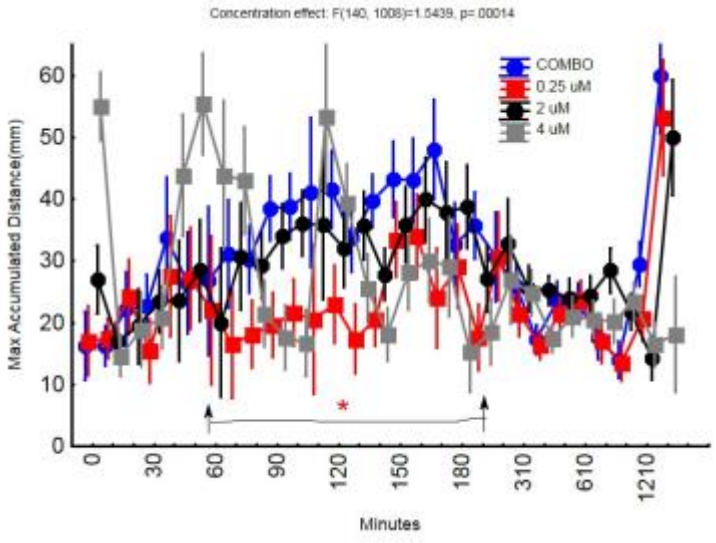

Figure 45: Illustrates the time-dependent effect of three 4-nonylphenol concentrations (0.25, 2 and 4uM) on maximum accumulated distance by *Danio rerio.* here was a significant difference between 4-nonylphenol treated animals and controls at the corresponding time points as indicated by the bracket (Contrast analysis, * P<0.05).

Angle: As can be seen in Figure 37, there was a non-significant trend for 4-nonylphenol to have a concentration-dependent mean angle effect of (0.05<P<0.10) that was consistent with the pattern of effects on maximum accumulated distance (above). There was a significant time-dependent effect of 4-nonylphenol on mean angle (P<0.001; Figure 38), with the mean values for 0.25 and 4.0 μM were generally elevated in relationship to controls between 70 and 300 minutes.

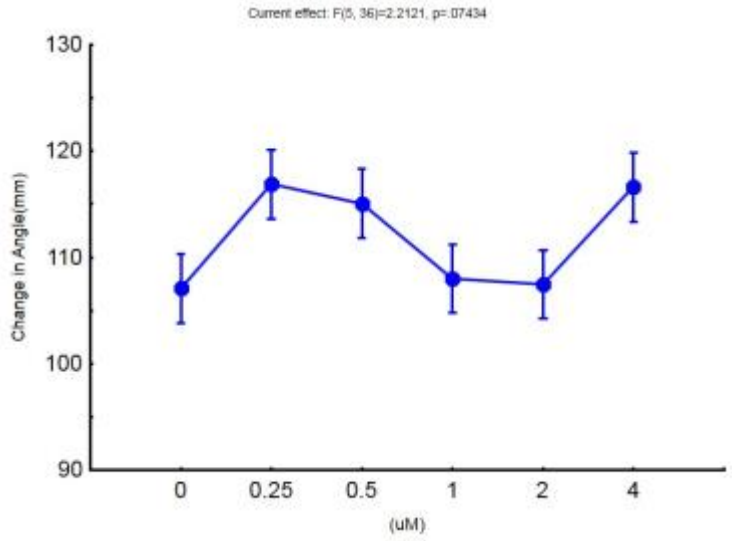

Figure 46: The effect of 5 different 4-nonylphenol concentrations on mean angle by *Danio rerio.*

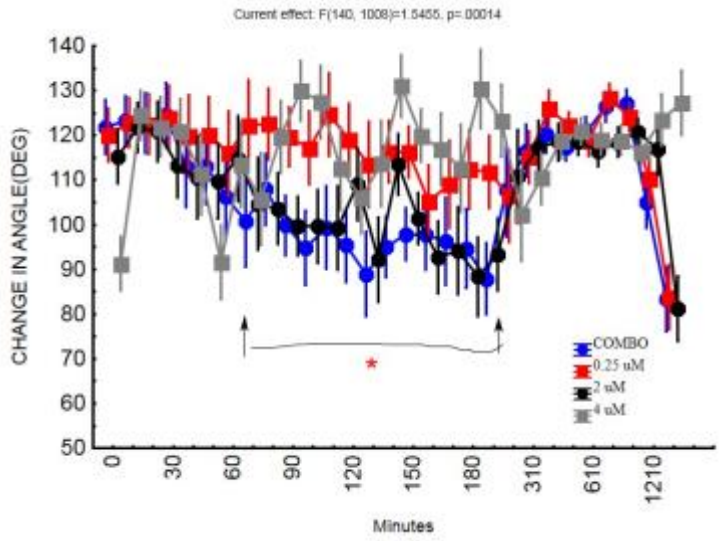

Figure 47: Time-dependent effect of three 4-nonylphenol concentrations (0.25, 2 and 4uM) on mean angle of *Danio rerio.* here was a significant difference between 4-nonylphenol treated animals and controls at the corresponding time points as indicated by the bracket (Contrast analysis, * P<0.05).

Bisphenol-A

Distance: Bisphenol-A did not produce a significant concentration-dependent effect on maximum accumulated distance (P>0.10) as shown in Figure 39. The 4uM shows a decrease in maximum accumulated distance when compared with control. The time x concentration interaction of Bisphenol-A was not significant (P>0.20; Figure 40).

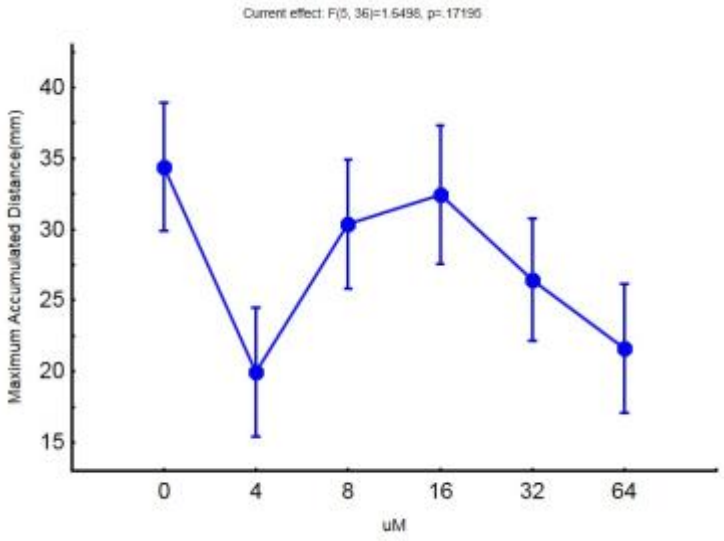

Figure 48: The effect of 5 different Bisphenol-A concentrations on maximum accumulated distance by *Danio rerio.*

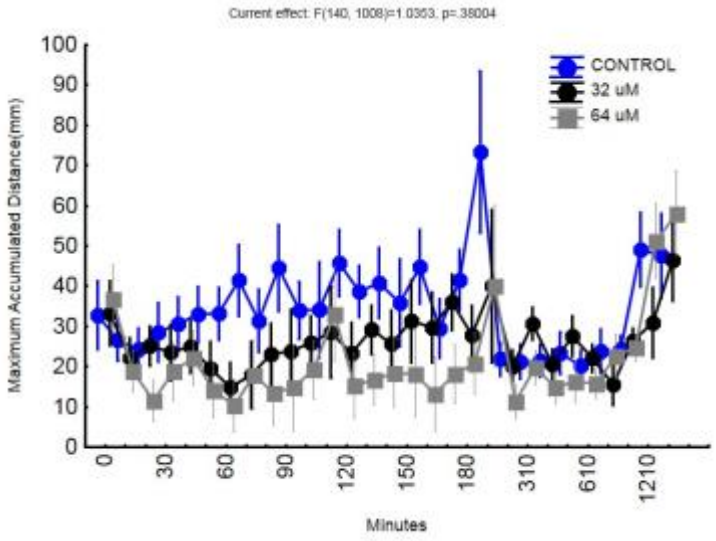

Figure 49: Time-dependent effect of two Bisphenol-A concentrations (32 and 64uM) on maximum accumulated distance by *Danio rerio.*

Angle: As shown in Figure 41, there was a non-significant trend for Bisphenol-A to elicit concentration-dependent effect on mean angle ($0.05<P<0.10$) and non-significant trend for a time-dependent effect of concentration (time x concentration interaction, $0.05<P<0.10$; Figure 42).

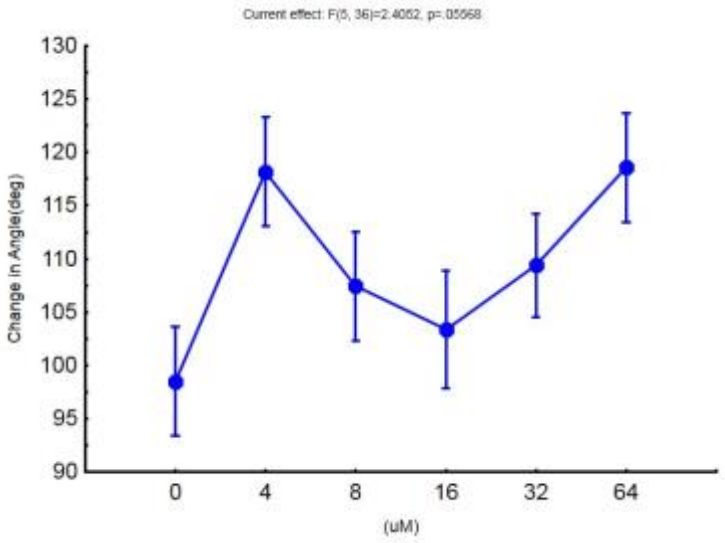

Figure 50: The effect of 5 different Bisphenol-A concentrations on mean angle by *Danio rerio.*

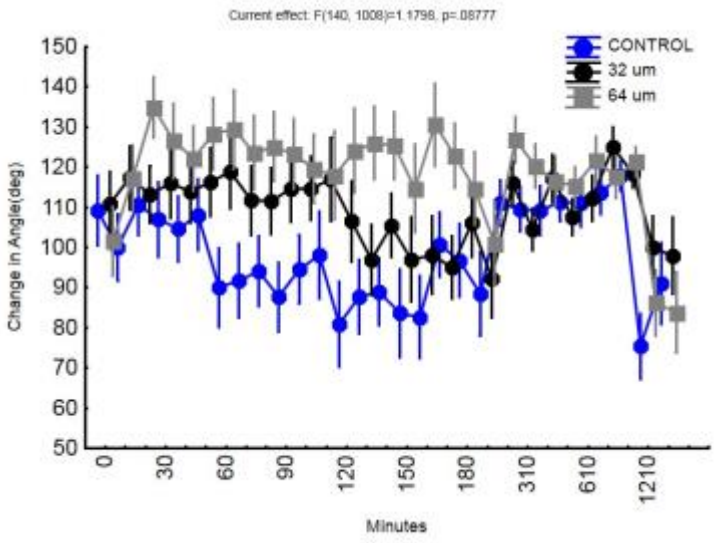

Figure 51: Time-dependent effect of two Bisphenol-A concentrations (32 and 64uM) on mean angle by *Danio rerio.*

Atrazine

Distance: Although atrazine did not elicit a significant concentration-dependent effect on maximum accumulated distance a ($P>0.20$; Figure 43), there was a significant time-dependent effect of concentration on maximum accumulated distance (time x concentration, $P<0.01$; Figure 44). The higher concentrations of atrazine (20 and 100 uM) produced higher mean values relative to controls and stimulated swimming behavior within the first 90 minutes of exposure and this effect subsided later in the time course.

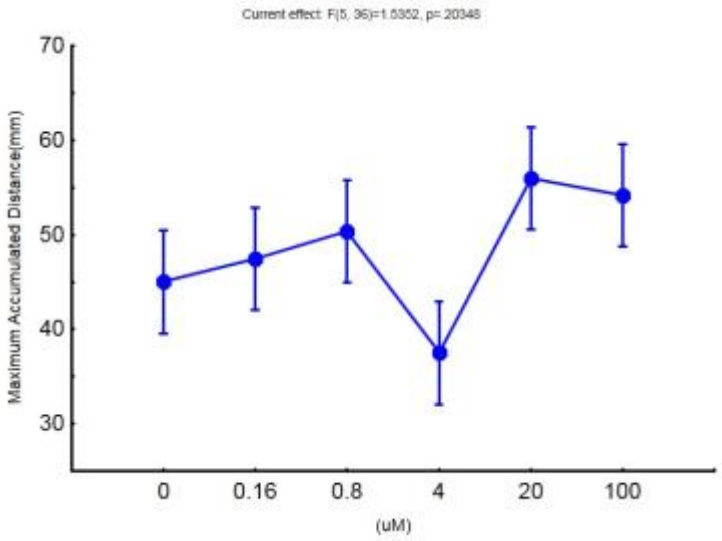

Figure 52: The effect of 5 different Atrazine concentrations on maximum accumulated distance by *Danio rerio.*

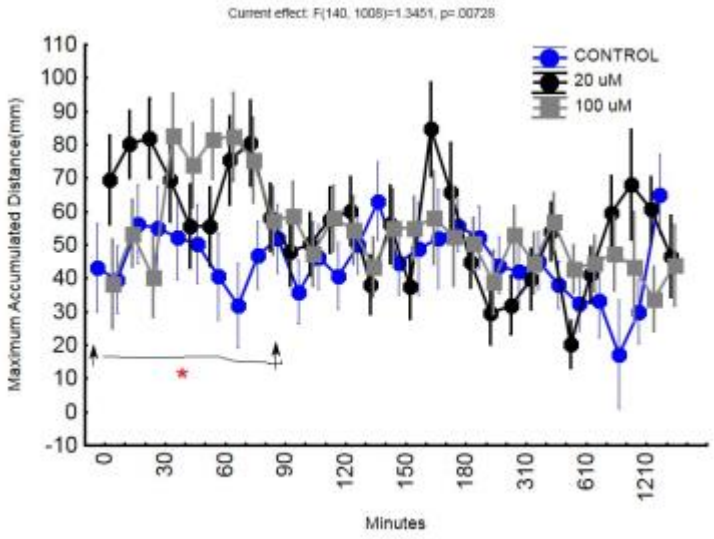

Figure 53: Time-dependent effect of two Atrazine concentrations (20 and 100uM) on maximum accumulated distance by *Danio rerio.* here was a significant difference between atrazine treated animals and controls at the corresponding time points as indicated by the bracket (Contrast analysis, * P<0.05).

Angle: Atrazine did not elicit a significant concentration-dependent effect on mean angle (P<0.50; Figure 45). However, when time was included as a factor there was a significant concentration x time interaction effect for mean angle. (P<0.005; Figure 46).

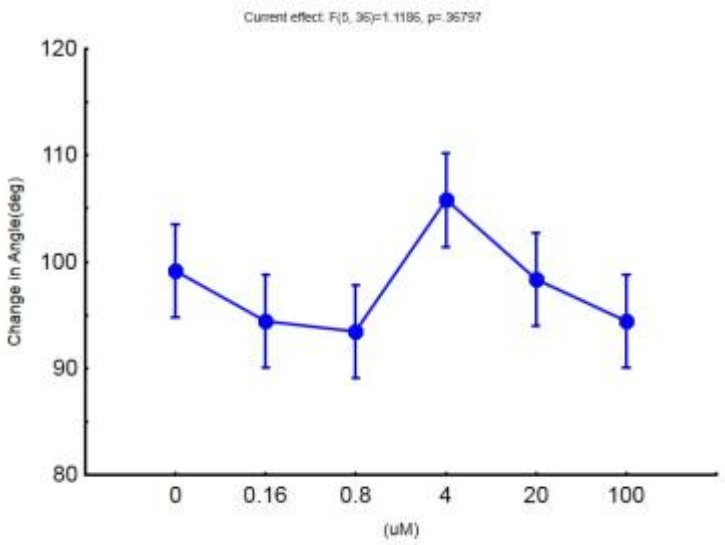

Figure 54: The effect of 5 different Atrazine concentrations on mean angle by *Danio rerio.*

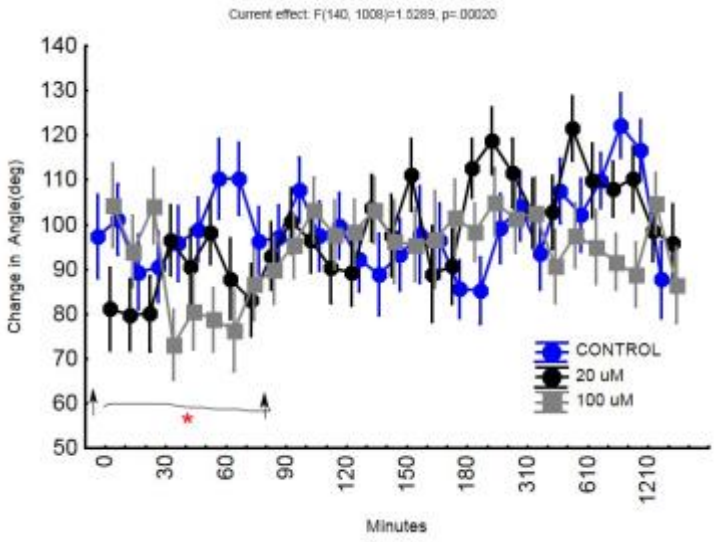

Figure 55: Time-dependent effect of two Atrazine concentrations (20 and 100uM) on mean angle by *Danio rerio.* here was a significant difference between atrazine treated animals and controls at the corresponding time points as indicated by the bracket (Contrast analysis, * P<0.05).

CEC(Concentration)	***D. pulex***	***D. rerio***
Chlorpyrifos		
(0.3125-5nM)	0/0	
(15-250nM)	-/+	
(62.5-1000nM)		0/0
(0.25-4uM)	*	
4-Nonylphenol		
(0.25-4uM)	-/+	0/+?
BPA		
(4-64uM)	0/0	0/+?
Dieldrin		
(0.0625-1uM)		+/-
(0.1-62.5uM)	+/0	*(2.5,12.5,62.5)

TABLE 1: Summary of the results of *D. pulex* and *D.rerio*

Atrazine		
(0.16-100uM)	+/-?	0/0
Toxaphene		
(150-2400nM)	+/0	

Maximum Accumulated Distance / Mean Change in Angle

(+): Increase

(-): Decrease

(?): Non-significant trend [0.05<P<0.10]

(*): Immobilized/Dead

Comparison of chemical responses in *D.pulex* and *D.rerio*

The qualitative behavioral effects differed across chemicals. Some had stimulatory effects on swimming behavior (dieldrin, atrazine, and toxaphene), some had predominately inhibitory effects on behavior (chlorpyrifos, 4-nonylphenol), and some did not exhibit very much effect on behavior even at high concentrations (bisphenol-A). The behavioral responses to these CECs showed some similarities across species and there also appeared to be differences in sensitivity to the toxic effects of specific chemicals. Chlorpyrifos elicited behavioral effects in *D. pulex* at lower concentrations than *D. rerio*. In the higher concentration ranges, chlorpyrifos caused immobility in *D. pulex*, but not *D. rerio*, indicating that It was more toxic to *D. pulex* than *D. rerio*. *D. pulex* appeared to be

more sensitive to 4-nonylphenol. Dieldrin was highly stimulatory to *D. rerio*, and although the stimulatory effect in *D. pulex* was significant, *D. pulex* were less sensitive to dieldrin. Atrazine was found to be stimulatory to *D. pulex* in a concentration range that did not affect *D. rerio*. Toxaphene was only studied in *D. pulex* and was found to stimulate swimming behavior at relatively low concentrations. Dieldrin found to be more sensitive for than *D. rerio*, it showed significant decrease in maximum accumulated distance and increase in angle for *D. pulex* and close to significant effect on angle for *D. rerio*. Bisphenol-A was found to be more sensitive on *D. rerio* than on

D. pulex, it showed closed to significant effect on mean angle for *D. rerio* but non-significant effect for *D. pulex*. Dieldrin was found to be more sensitive on *D. rerio* than *D. pulex*, it showed significant effect on distance but not on angle for *D. pulex* but on *D. reri*o significant effect on maximum accumulated distance as well as on mean angle was seen at lower concentration range. Atrazine was found to be more sensitive on *D. pulex* than *D. rerio*. It showed significant effect on distance and close to significant effect on angle for *D. pulex* whereas for *D. rerio* it was not significant.

Chapter 4: DISCUSSION

The effects of EDC exposure on the swimming behavior of these two aquatic animals can provide the essential information needed to: (1) assess behavioral responsiveness over a specific range of concentrations, (2) ascertain the nature of the behavioral change observed (stimulation, inhibition, or alteration of turning behavior), (3) evaluate survival over a 24-hr period of exposure, (4) compare the similarities or differences in response across chemicals, and (5) compare the similarities or differences in response across species. The results obtained are critical for the other phases of the larger EDC project. The second phase focuses on EDC effects on development (morphological changes), and the third phase assesses the effects of specific chemicals on gene expression. These three phases of the project are tightly connected by the concentration ranges selected for study. The characterization of EDC-induced behavioral changes will help identify potential neurotoxic actions, and provide the context for a functional evaluation of significant alterations in gene expression. For example, if similar gene pathways are associated with similar outcomes for two chemicals as a result of EDC exposure (e.g., feminization of males), it may be easier to differentiate the chemical identity of EDCs when there are contrasting behavioral responses (e.g., presence of stimulated swimming behavior for certain pesticides).

This aspect of the first phase of the EDC project focused on the behavioral effects of contaminants that would not be classified as pharmaceuticals or personal care products (PPCPs), but instead focused on the remaining CECs such as the pesticides, chlorpyrifos, dieldrin, and toxaphene, the detergent metabolite, 4-nonylphenol, the plasticizer, bisphenol-A, and the herbicide, atrazine.

Chlorpyrifos

Chlorpyrifos is an organophosphate insecticide which inhibits the enzyme acetylcholinesterase (AChE). Acetylcholine (ACh) and ACh receptors are found in both vertebrates and invertebrates Pezzementi and Chatonnet (2010);Thany and Tricoire-Leignel (2011); Venter et al. (1988). Previous studies of *D. pulex* by our lab suggested that chlorpyrifos may not have the same ability to stimulate swimming behavior as was previously demonstrated for two other AChE inhibitors, physostigmine (Zein et al., 2014) and diazinon (Zein et al., 2015). However, the concentration range of chlorpyrifos studied by Zein et al. (2014) was limited to 0.016 to 0.25 μM. In the present study, concentrations above and below this range for chlorpyrifos were explored without discovering any evidence for stimulation of swimming behavior. In the present study, chlorpyrifos did not show stimulation in the concentration range of 0.3125nM to 250nM, but higher concentrations 62.5nM, 125nM, and 250nM caused a decrease in the mobility. Any concentration higher than 250nM caused immediate immobilization of *D. pulex*. Therefore, this study reinforces the findings of the previous study by Zein et al. (2014), and suggests that there are some fundamental differences in the properties of chlorpyrifos relative to physostigmine or diazinon. Zein et al. (2014) had previously suggested that the differences between the ability of physostigmine and diazinon to stimulate swimming behavior relative to the lack of stimulatory effect by chlorpyrifos may be due to toxicokinetic differences among the three AChE inhibitors. However, there could also be other unknown pharmcodynamic differences between chlorpyrifos and the other two AChE inhibitors, such as the possibility of additional drug targets for chlorpyrifos.

D. rerio were exposed to similar concentrations as described for *D. pulex* above, but the fish did not show any significant change in swimming behavior within a concentration range of 15.625nM to 1μM. Bonansea, Wunderlin, and Ame (2016) exposed *J. multidentata* to chlorpyrifos (0.4 and 4ug/L) for 24 hours and found that it decreased swimming activity. They also found that AChE in muscles was more sensitive to the inhibitory effect of chlorpyrifos than AChE in brain, and concluded that the inhibition of swimming activity correlated more with the inhibition of AChE in muscle than brain. Jin, Liu, Peng, and Fu (2015) evaluated the swimming behavior of 96-hour post fertilization *D. rerio* when exposed to chlorpyrifos (10 to 300 mg/L) and concluded that the swimming behavior of the animals decreased at the higher concentrations (100 and 300 mg/L). Chlorpyrifos also decreased the swimming behavior of *D. rerio* embryos during the dark phase of their light/dark cycle, and this was used as a positive control for evaluating the other organophosphates Dishaw, Hunter, Padnos, Padilla, and Stapleton (2014). The fact that we did not identify any stimulatory effect of chlorpyrifos on swimming behavior in *D. rerio* is consistent with these other studies. However, the present study did not detect any inhibition of swimming activity in *D. rerio* at chlorpyrifos concentrations up to 1 μM, whereas the other fish studies described above have observed inhibition of swimming activity. The lack of stimulation of swimming behavior across fish studies and *D. pulex* appears to indicate some similarity in the responses of *D. pulex* and fish, however, *D. pulex* were more sensitive to the toxic effects of chlorpyrifos on swimming behavior, exhibiting a decrease in maximum accumulated distance and increase in mean angle at relatively low concentrations.

Dieldrin

Dieldrin is an organochloride insecticide which has been banned since the 70s, but is still found in sediment, surface water, and ground water. It is considered to be one a persistent organic pollutant (POP) because it does not readily degrade in the environment. The mechanism of action was evaluated by Kluver et al. (2015), and they reported that dieldrin inhibits GABA receptors and causes neurotoxicity. They also reported that there is a significant decrease in the *D. rerio* locomotive response after exposure to dieldrin for 96 hours. In the present study *D. rerio* and *D. pulex* showed a stimulatory response following exposure to dieldrin. The swimming behavior was stimulated in both of these animals at relatively low concentrations. *D. rerio* was more sensitive, responding to lower concentrations, and showed greater stimulation of swimming behavior than *D. pulex*. To the best of our knowledge this is the first description of the behavioral effects of dieldrin.

Toxaphene

The behavioral effects of toxaphene were only studied in *D. pulex*. Toxaphene exposure has previously been shown to cause a significant change in the sex ratio of *D. pulex*, with a significantly larger number of males produced only during the developmental stage (where the eggs are still in the oviduct of the mother) (Kashian et al., 2011). Toxaphene was included in this study because of its potential endocrine disrupting effects in *D. pulex*, however its effects on *D. rerio* were not examined because there were already two pesticides selected to be included in the second and third phase of biological evaluation and it was therefore excluded. Toxaphene elicited a significant increase in the

distance travelled but no change in mean angle. To the best of our knowledge there are no other studies of the behavioral effects of toxaphene in daphnia.

4-Nonylphenol

4-nonylphenol is a detergent metabolite that is widely found in surface water (Kolpin et al., 2002). M. H. Li (2008) evaluated the effect of 4-nonylphenol (10 to 300 ug/L) on cholinesterase activity in the male guppies for 7 days, and concluded that 4-nonylphenol inhibited cholinesterase in muscle significantly (at 60 and 150 ug/L). 4-nonylphenol has also been shown to inhibit the cholinesterase activity in the planarian and other aquatic organisms (M. H. Li, 2008). The swimming behavior of *D. pulex* was significantly affected by exposure to 4-nonylphenol (0.25-4 uM). 4-nonylphenol significantly inhibited swimming activity, which was seen as a decrease in the maximum accumulated distance and an increase in mean angle in *D. pulex*. 4-nonylphenol showed non-monotonic behavior when The effects on *D. pulex* were very similar to those previously reported Zein et al. (2014), a significant increase in the mean angle and non-significant decrease in the maximum accumulated distance. It is of interest to point out that this is another chemical that has been reported to inhibit acetylcholinesterase, which does not appear to stimulate swimming behavior like physostigmine (Zein et al., 2014) or diazinon (Zein et al., 2015). The behavioral effect is more like chlorpyrifos. However, it is quite possible that there is more than one toxic mechanism associated with 4-nonylphenol and that inhibition of acetylcholinesterase may not be the only mechanism by which it influences behavior.

Bisphenol-A

Bisphenol-A is a plasticizer which used in manufacturing of many different products including plastic bottles. The swimming behavior of *D. pulex* and *D. rerio* was not significantly affected, even by relatively large concentrations of bisphenol A. . D*. rerio* exposed to bisphenol A at a relatively low concentration (0.22nM) for 7 weeks showed significant changes in locomotive behavior during courtship. The females preferred the control animals over the animals given low dose of the chemical. This affected reproduction and population size of the zebrafish. (X. Li et al. (2017))

Atrazine

Atrazine is a herbicide which is commonly used in agriculture to eliminate weeds before planting corn. Atrazine significantly stimulated swimming behavior of *D. pulex*, but not *D. rerio*. In *D. pulex* the maximum accumulated distance increased, but not mean angle. Liu et al. (2016) exposed zebrafish larval for 5 days to atrazine (30,100,300 ug/L) and observed that the swimming behavior was significantly disturbed and that acetylcholinesterase was inhibited. Atrazine and its metabolites are said to be neuroendocrine disruptors that inhibit the expression of neurotoxicity related genes (Liu et al., 2016)

Conclusions

This series of experiments examined the effects of five chemicals on the swimming behavior of two different aquatic species, *D. rerio* (vertebrate) and *D. pulex* (invertebrate), and has demonstrated significant concentration-dependent differences in responses across the series of chemicals, between species for a given chemical (chlorpyrifos,

atrazine), and similarities in response to a chemical by both species (dieldrin). In the future phase of the EDC project, morphological effects resulting from exposure to these two chemicals will be examined. This effort will be mostly focused on *D. rerio* because the ecdysteroid endocrine system of *D. pulex* is significantly different from the vertebrate steroid system. (LeBlanc, Mu, and Rider (2000)). However, there will also be an evaluation of selected chemicals such as dieldrin in *D. pulex*. These behavioral studies in conjunction with the planned morphological evaluation of development will provide the foundation for interpreting the effects of EDCs on gene expression, and the creation of the initial prototype of a mathematical model to predict the nature of the chemical entities contributing to the estrogenic or anti-androgenic qualities of water samples. The identification of key sets of genes representing the pathways associated with such EDC activity will enable the creation of tools to assess the endocrine disrupting quality of water samples taken from surface water, ground water or water infrastructure. This new bioassay approach will compliment and expand the power of existing analytical chemistry techniques and enable the evaluation of the complex issues associated with the contamination of aquatic systems by CECs.

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Appendix A

Stock Solution Calculations

Chlorpyrifos

Stock solution of 10mM

$\text{Molecular weight of Chlorpyrifos} = 350.59 \text{ g/mol}$

$$10 \text{ mM} = \frac{4\text{mg}}{0.350\text{mg/ml}} \times \frac{1}{\text{volume (ml)}}$$

Volume= 1.14 ml of acetone

Concentrations studied:

[0, 0.3125, 0.625, 12.5, 2.5, 5 nM] – *Daphnia pulex*

[15.625, 31.25, 62.5, 125, 250 nM] – *Daphnia pulex*

[62.5, 125, 250, 500, 1000 nM] – *Danio rerio*

4-Nonylphenol

Stock solution of 10mM

$\text{Molecular weight of } 4-\text{nonylphenol} = 220.35 \text{ g/mol}$

$$10 \text{ mM} = \frac{4 \text{ mg}}{0.22035\text{mg/ml}} \times \frac{1}{\text{Volume (ml)}}$$

Volume= 1.81 ml of acetone

Concentrations studied:

[0, 0.25, 0.5, 1, 2, 4 μM] - *Daphnia pulex*, *Danio rerio*

Dieldrin

Stock solution of 10 mM

$$\text{Molecular weight of dieldrin} = 380.91\ \text{g/mol}$$

$$10\ \text{mM} = \frac{4\ \text{mg}}{0.38091\ \text{mg/ml}} \times \frac{1}{\text{Volume (ml)}}$$

Volume= 1.050 ml of acetone

Concentrations studied:

[0, 0.1, 0.5, 2.5, 12.5, 62.5 μM] – *Daphnia pulex*

[0, 0.0625, 0.125, 0.25, 0.5, 1 μM] – *Danio rerio*

Atrazine

Stock solution of 10 mM

$$\text{Molecular weight of atrazine} = 215.68\ \text{g/mol}$$

$$10\ \text{mM} = \frac{5\ \text{mg}}{0.21568\ \text{mg/ml}} \times \frac{1}{\text{Volume (ml)}}$$

Volume= 2.31 ml of acetone

Concentrations studied:

[0, 0.16, 0.8, 4, 20, 100 nM] *Daphnia pulex*, *Danio rerio*

Bisphenol A

Stock solution of 10mM

$\text{Molecular weight of BPA} = 228.29$ g/mol

$$10\ \text{mM} = \frac{4\ \text{mg}}{0.22829\ \text{mg/ml}} \times \frac{1}{\text{Volume (ml)}}$$

Volume= 1.75 ml of acetone

Concentrations studied:

[0, 4, 8, 16, 32, 64 μM] - *Daphnia pulex*, *Danio rerio*

Appendix B

Effect	Repeated Measures Analysis of Variance: Chlorpyrifos *Danio rerio* Dependent variable: Maximum accumulated distance				
	SS	Degree of Freedom	MS	F	p
Intercept	1809497	1	1809497	599.5142	0.000000
conc	14557	5	2911	0.9646	0.452311
Plate Position	27944	1	27944	9.2583	0.004359
conc *Plate Position	15873	5	3175	1.0518	0.402898
Error	108658	36	3018		
TIME	70600	28	2521	7.3796	0.000000
TIME*conc code	43186	140	308	0.9028	0.775900
TIME*Plate Position	33278	28	1188	3.4785	0.000000
TIME*conc code*Plate Position	49092	140	351	1.0263	0.406674
Error	344407	1008	342		

Effect	Repeated Measures Analysis of Variance: Chlorpyrifos *Danio rerio* Dependent variable: Mean angle				
	SS	Degree of Freedom	MS	F	p
Intercept	14965487	1	14965487	3468.733	0.000000
conc code	20019	5	4004	0.928	0.474329
Plate Position	18958	1	18958	4.394	0.043153
conc code*Plate Position	10912	5	2182	0.506	0.769858
Error	155318	36	4314		
TIME	90801	28	3243	10.222	0.000000
TIME*conc code	46481	140	332	1.047	0.348064
TIME*Plate Position	27039	28	966	3.044	0.000000
TIME*conc code*Plate Position	40600	140	290	0.914	0.746458
Error	319781	1008	317		

Effect	Repeated Measures Analysis of Variance: Dieldrin *Danio rerio* Dependent variable: Maximum accumulated distance				
	SS	Degree of Freedom	MS	F	p

Intercept	5496275	1	5496275	243.0927	0.000000
conc	802570	5	160514	7.0993	0.000794
Error	406976	18	22610		
TIME	240708	28	8597	1.9171	0.003536
TIME*conc	1523569	140	10883	2.4269	0.000000
Error	2260050	504	4484		

Effect	Repeated Measures Analysis of Variance: Dieldrin *Danio rerio* Dependent variable: Mean angle				
	SS	Degree of Freedom	MS	F	p
Intercept	6064993	1	6064993	2639.436	0.000000
conc	190692	5	38138	16.597	0.000003
Error	41361	18	2298		
TIME	78656	28	2809	4.732	0.000000
TIME*conc	210522	140	1504	2.533	0.000000
Error	299186	504	594		

Effect	Repeated Measures Analysis of Variance: 4-nonylphenol *Danio rerio* Dependent variable: Maximum accumulated distance				
	SS	Degree of Freedom	MS	F	p
Intercept	1048512	1	1048512	700.6144	0.000000
conc	12032	5	2406	1.6080	0.182882
Plate Position	100878	1	100878	67.4069	0.000000
conc *Plate Position	6266	5	1253	0.8373	0.532057
Error	53876	36	1497		
TIME	76298	28	2725	9.0559	0.000000
TIME*conc	65039	140	465	1.5439	0.000145
TIME*Plate Position	43591	28	1557	5.1738	0.000000
TIME*conc *Plate Position	52084	140	372	1.2364	0.040978
Error	303309	1008	301		

	Repeated Measures Analysis of Variance: 4-nonylphenol *Danio rerio* Dependent variable: Mean angle

Effect	SS	Degree of Freedom	MS	F	p
Intercept	17416102	1	17416102	7212.146	0.000000
conc	26710	5	5342	2.212	0.074340
Plate Position	123216	1	123216	51.025	0.000000
conc code*Plate Position	18804	5	3761	1.557	0.196982
Error	86934	36	2415		
TIME	100344	28	3584	9.685	0.000000
TIME*conc	80064	140	572	1.545	0.000140
TIME*Plate Position	107758	28	3848	10.400	0.000000
TIME*conc *Plate Position	65315	140	467	1.261	0.028640
Error	373004	1008	370		

Effect	Repeated Measures Analysis of Variance: 4-nonylphenol *Daphnia pulex* Dependent variable: Maximum accumulated distance				
	SS	Degree of Freedom	MS	F	p
Intercept	238787.8	1	238787.8	1615.871	0.000000
conc code	1751.1	5	350.2	2.370	0.058691
Plate	37.6	1	37.6	0.254	0.617123
conc *Plate	648.1	5	129.6	0.877	0.506210
Error	5320.0	36	147.8		
TIME	1545.6	28	55.2	1.729	0.010977
TIME*conc	4500.9	140	32.1	1.007	0.464553
TIME*Plate	1005.6	28	35.9	1.125	0.298632
TIME*conc* Plate	4532.6	140	32.4	1.014	0.442732
Error	32172.7	1008	31.9		

Effect	Repeated Measures Analysis of Variance: 4-nonylphenol *Daphnia pulex* Dependent variable: Mean angle				
	SS	Degree of Freedom	MS	F	p
Intercept	11884041	1	11884041	12100.85	0.000000
conc code	201760	5	40352	41.09	0.000000
Plate	11021	1	11021	11.22	0.001907

conc*Plate	5333	5	1067	1.09	0.384619
Error	35355	36	982		
TIME	45020	28	1608	7.12	0.000000
TIME*conc code	50099	140	358	1.58	0.000060
TIME*Plate	19581	28	699	3.10	0.000000
TIME*conc *Plate	39954	140	285	1.26	0.027639
Error	227741	1008	226		

Effect	Repeated Measures Analysis of Variance: Atrazine *Daphnia pulex* Dependent variable: Maximum accumulated distance				
	SS	Degree of freedom	MS	F	p
Intercept	548050.9	1	548050.9	3488.635	0.000000
conc code	11605.4	5	2321.1	14.775	0.000000
Plate Position	25.1	1	25.1	0.160	0.691721
conc*Plate Position	2069.0	5	413.8	2.634	0.039567
Error	5655.5	36	157.1		
TIME	8311.7	28	296.8	4.993	0.000000
TIME*conc	8173.8	140	58.4	0.982	0.543860
TIME*Plate Position	2685.2	28	95.9	1.613	0.023408
TIME*conc *Plate Position	7537.4	140	53.8	0.905	0.769098
Error	59933.0	1008	59.5		

Effect	Repeated Measures Analysis of Variance: Atrazine *Daphnia pulex* Dependent variable: Mean angle				
	SS	Degree of Freedom	MS	F	p
Intercept	2702118	1	2702118	1845.566	0.000000
conc	15565	5	3113	2.126	0.084562
Plate Position	2494	1	2494	1.703	0.200153
conc *Plate Position	11035	5	2207	1.507	0.211905

Error	52708	36	1464		
TIME	26210	28	936	3.049	0.000000
TIME*conc	41150	140	294	0.957	0.621032
TIME*Plate Position	14579	28	521	1.696	0.013731
TIME*conc *Plate Position	38614	140	276	0.898	0.787297
Error	309508	1008	307		

Effect	Repeated Measures Analysis of Variance: Bisphenol A *Daphnia pulex* Dependent variable: Maximum accumulated distance				
	SS	Degree of Freedom	MS	F	p
Intercept	366709.4	1	366709.4	2639.737	0.000000
Conc	1329.0	5	265.8	1.913	0.116266
Plate Position	8321.3	1	8321.3	59.900	0.000000
Conc*Plate Position	556.5	5	111.3	0.801	0.556283
Error	5001.1	36	138.9		
TIME	3629.4	28	129.6	3.046	0.000000
TIME*Conc	5731.3	140	40.9	0.962	0.606832
TIME*Plate Position	3362.2	28	120.1	2.821	0.000002
TIME*Conc*Plate Position	4914.6	140	35.1	0.825	0.925173
Error	42902.3	1008	42.6		

Effect	Repeated Measures Analysis of Variance: Bisphenol A *Daphnia pulex* Dependent variable: Mean angle				
	SS	Degree of Freedom	MS	F	p
Intercept	6976373	1	6976373	4658.549	0.000000
Conc	11710	5	2342	1.564	0.195115
Plate Position	97292	1	97292	64.968	0.000000
Conc*Plate Position	7580	5	1516	1.012	0.424716
Error	53912	36	1498		
TIME	93026	28	3322	10.397	0.000000
TIME*Conc	52620	140	376	1.176	0.091819
TIME*Plate Position	21982	28	785	2.457	0.000043
TIME*Conc*Plate Position	50943	140	364	1.139	0.143092

Error	322113	1008	320		

Effect	Repeated Measures Analysis of Variance: toxaphene *Daphnia pulex* Dependent variable: Maximum accumulated distance				
	SS	Degree of Freedom	MS	F	p
Intercept	298214.0	1	298214.0	2108.444	0.000000
conc code	2251.5	5	450.3	3.184	0.017591
Plate Position	96.1	1	96.1	0.680	0.415105
conc *Plate Position	735.9	5	147.2	1.041	0.409028
Error	5091.8	36	141.4		
TIME	1703.6	28	60.8	1.385	0.088691
TIME*conc	6859.2	140	49.0	1.115	0.184628
TIME*Plate Position	1940.2	28	69.3	1.577	0.029247
TIME*conc *Plate Position	6538.3	140	46.7	1.063	0.303492
Error	44286.2	1008	43.9		

Effect	Repeated Measures Analysis of Variance: toxaphene *Daphnia pulex* Dependent variable: Mean angle				
	SS	Degree of Freedom	MS	F	p
Intercept	4576651	1	4576651	1982.940	0.000000
conc code	5567	5	1113	0.482	0.787033
Plate Position	20977	1	20977	9.089	0.004692
conc code*Plate Position	14442	5	2888	1.251	0.305950
Error	83088	36	2308		
TIME	55851	28	1995	4.998	0.000000
TIME*conc code	59571	140	426	1.066	0.295196
TIME*Plate Position	21991	28	785	1.968	0.002042
TIME*conc code*Plate Position	53316	140	381	0.954	0.630322
Error	402289	1008	399		

	Repeated Measures Analysis of Variance: Dieldrin *Daphnia pulex* Dependent variable: Maximum Accumulated Distance

Effect	SS	Degree of Freedom	MS	F	p
Intercept	465361.6	1	465361.6	2014.969	0.000000
conc	4553.5	5	910.7	3.943	0.005927
Plate Position	13248.8	1	13248.8	57.366	0.000000
conc*Plate Position	3934.8	5	787.0	3.407	0.012714
Error	8314.3	36	231.0		
TIME	13115.4	28	468.4	5.156	0.000000
TIME*conc	13173.2	140	94.1	1.036	0.378919
TIME*Plate Position	5155.3	28	184.1	2.027	0.001318
TIME*conc*Plate Position	14341.0	140	102.4	1.128	0.161859
Error	91576.4	1008	90.8		

Effect	Repeated Measures Analysis of Variance: Dieldrin *Daphnia pulex* Dependent variable: Mean angle				
	SS	Degree. of Freedom	MS	F	p
Intercept	5411223	1	5411223	2279.896	0.000000
conc code	15308	5	3062	1.290	0.289777
Plate Position	353946	1	353946	149.127	0.000000
conc*Plate Position	29079	5	5816	2.450	0.052044
Error	85444	36	2373		
TIME	182216	28	6508	13.635	0.000000
TIME*conc	99085	140	708	1.483	0.000529
TIME*Plate Position	27683	28	989	2.071	0.000938
TIME*conc*Plate Position	70177	140	501	1.050	0.337731
Error	481102	1008	477		

Effect	Repeated Measures Analysis of Variance: Chlorpyrifos(HR) *Daphnia pulex* Dependent variable: Maximum accumulated distance				
	SS	Degree of Freedom	MS	F	p
Intercept	321491.1	1	321491.1	1407.802	0.000000
conc	13665.6	5	2733.1	11.968	0.000001
Plate Position	8133.3	1	8133.3	35.616	0.000001
conc *Plate Position	430.4	5	86.1	0.377	0.861165

Error	8221.1	36	228.4		
TIME	44999.8	28	1607.1	16.125	0.000000
TIME*conc	18298.8	140	130.7	1.311	0.012891
TIME*Plate Position	8437.2	28	301.3	3.023	0.000000
TIME*conc *Plate Position	15607.6	140	111.5	1.119	0.178223
Error	100467.0	1008	99.7		

Effect	Repeated Measures Analysis of Variance: Chlorpyrifos(HR) *Daphnia pulex* Dependent variable: Maximum accumulated distance				
	SS	Degree of Freedom	MS	F	p
Intercept	8086184	1	8086184	1803.287	0.000000
conc	333630	5	66726	14.880	0.000000
Plate Position	90921	1	90921	20.276	0.000068
conc *Plate Position	20544	5	4109	0.916	0.481542
Error	161429	36	4484		
TIME	760809	28	27172	53.833	0.000000
TIME*conc	166782	140	1191	2.360	0.000000
TIME*Plate Position	49496	28	1768	3.502	0.000000
TIME*conc *Plate Position	101325	140	724	1.434	0.001413
Error	508777	1008	505		

Effect	Repeated Measures Analysis of Variance: Chlorpyrifos (LR) *Daphnia pulex* Dependent variable: Maximum accumulated distance				
	SS	Degree of Freedom	MS	F	p
Intercept	337705.6	1	337705.6	2018.863	0.000000
conc	345.1	5	69.0	0.413	0.836663
Plate	1188.0	1	1188.0	7.102	0.011693
conc *Plate	309.6	5	61.9	0.370	0.865459
Error	5687.4	34	167.3		
TIME	2200.2	28	78.6	1.992	0.001725
TIME*conc	4688.9	140	33.5	0.849	0.888528
TIME*Plate	1374.2	28	49.1	1.244	0.179209
TIME*conc*Plate	5778.5	140	41.3	1.047	0.348707
Error	37546.8	952	39.4		

Effect	Repeated Measures Analysis of Variance: Chlorpyrifos (LR) *Daphnia pulex* Dependent variable: Mean angle				
	SS	Degree of Freedom	MS	F	p
Intercept	7124582	1	7124582	4751.045	0.000000
conc	5454	5	1091	0.727	0.607756
Plate	36676	1	36676	24.457	0.000020
conc *Plate	12239	5	2448	1.632	0.178041
Error	50986	34	1500		
TIME	43935	28	1569	3.609	0.000000
TIME*conc	48932	140	350	0.804	0.948045
TIME*Plate	16548	28	591	1.359	0.101663
TIME*conc *Plate	57514	140	411	0.945	0.658050
Error	413875	952	435		

Effect	Repeated Measures Analysis of Variance: Atrazine *Danio rerio* Dependent variable: Maximum accumulated distance				
	SS	Degree of Freedom	MS	F	p
Intercept	3262443	1	3262443	478.2056	0.000000
conc	52367	5	10473	1.5352	0.203479
Plate Position	27298	1	27298	4.0014	0.053050
conc*Plate Position	67274	5	13455	1.9722	0.106474
Error	245601	36	6822		
TIME	67839	28	2423	3.1480	0.000000
TIME*conc	144940	140	1035	1.3451	0.007280
TIME*Plate Position	46301	28	1654	2.1485	0.000518
TIME*conc *Plate Position	103192	140	737	0.9577	0.619685
Error	775800	1008	770		

Effect	Repeated Measures Analysis of Variance: Atrazine *Danio rerio* Dependent variable: Mean angle				
	SS	Degree of Freedom	MS	F	p
Intercept	13263250	1	13263250	2991.101	0.000000

conc	24801	5	4960	1.119	0.367967
Plate Position	90430	1	90430	20.394	0.000065
conc*Plate Position	48423	5	9685	2.184	0.077537
Error	159633	36	4434		
TIME	39942	28	1426	3.909	0.000000
TIME*conc	78119	140	558	1.529	0.000201
TIME*Plate Position	20131	28	719	1.970	0.002011
TIME*conc*Plate Position	73342	140	524	1.435	0.001373
Error	367890	1008	365		

Effect	Repeated Measures Analysis of Variance: Bisphenol A *Danio rerio* Dependent variable: Maximum accumulated distance				
	SS	Degree of Freedom	MS	F	p
Intercept	1044284	1	1044284	219.0939	0.000000
conc	39318	5	7864	1.6498	0.171954
Plate Position	74325	1	74325	15.5936	0.000350
conc*Plate Position	44516	5	8903	1.8679	0.124413
Error	171590	36	4766		
TIME	79252	28	2830	7.1176	0.000000
TIME*conc	57640	140	412	1.0353	0.380043
TIME*Plate Position	58004	28	2072	5.2093	0.000000
TIME*conc*Plate Position	55684	140	398	1.0002	0.486541
Error	400847	1008	398		

Effect	Repeated Measures Analysis of Variance: Bisphenol A *Danio rerio* Dependent variable: Mean angle				
	SS	Degree of Freedom	MS	F	p
Intercept	16432059	1	16432059	2700.810	0.000000
conc	73167	5	14633	2.405	0.055680
Plate Position	24032	1	24032	3.950	0.054522
conc *Plate Position	69447	5	13889	2.283	0.066866
Error	219028	36	6084		
TIME	86217	28	3079	8.250	0.000000
TIME*conc	61644	140	440	1.180	0.087771

TIME*Plate Position	92367	28	3299	8.839	0.000000
TIME*conc*Plate Position	59499	140	425	1.139	0.143056
Error	376202	1008	373		

Abstract

AN EVALUATION OF ENDOCRINE DISRUPTING EFFECTS OF EMERGING CONTAMINANTS USING *DAPHNIA PULEX* AND *DANIO RERIO*

By:

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Major: Pharmaceutical Sciences

Degree: Master of Science

The limit of the availability of water is based on two factors, scarcity and quality. Conserving and protecting our water resources one of the most critical issues facing humanity as we struggle to deal with contaminated lakes and rivers and climate change. The main aim of this study is to examine toxicity of known water contaminants on aquatic model organisms and be able to develop methodology that will enable the characterization of endocrine disrupting potential of water samples with unknown contaminants. Both the category of water contaminants known as pharmaceuticals, personal care products that are known as PPCPs and other non-PPCP contaminants such as pesticides, plasticizers, flame retardants, combustion products, and herbicides are being detected in surface water and ground water. Some of the main sources of these

contaminants are agricultural runoff, industrial sources and wastewater effluent. These compounds can be found in very low concentrations and may alter the physiological processes and have long term developmental impacts when animals are exposed. This exposure can alter the endocrine system, and may also be associated with other toxic properties (e.g., neurotoxic). The contaminants found to alter endocrine function have become known as endocrine disrupting chemicals. There is evidence of EDC activity on wildlife and increasing concern about EDC effects on humans. Some of the best evidence to date for EDC activity suggests that there is significant estrogenic and ant-androgenic activity in some of our surface water, especially downstream from wastewater effluent outflows.

This study is part of a large EDC project that focuses on the behavioral effects of EDCs as one part of a triad of behavioral assays to characterize estrogenic and anti-androgenic activity in water. The overall goal for the EDC project is to develop a mathematical model for estimating the estrogenicity and anti-androgenic properties of contaminants contributing to EDC – like activity in water. The hypothesis is that known or suspected EDCs have detectable behavioral effects, and that the characterization of these behavioral effects in combination with developmental and gene expression data will provide a mathematical model that enables the identification of chemicals contributing to the estrogenicity or ant-androgenic qualities of contaminated water. Two model organisms, *Daphnia pulex* and *Danio rerio,* used to evaluate the sub-lethal effects of the chemicals known or suspected to be EDCs*.* The behavioral assay evaluated the swimming behavior of these aquatic animals using a novel optical tracking system and measured the maximum distance travelled (mm) and mean change in angle (degrees)

over a 24-hour period of exposure. This series of experiments has demonstrated significant concentration-dependent differences in responses across the series of chemicals, between species for a given chemical (chlorpyrifos, atrazine), and similarities in response to a chemical by both species (dieldrin).

AUTOBIOGRAPHICAL STATEMENT

I was born and brought up in Hyderabad, a city in India. I always enjoyed chemistry and biology throughout my education and realized that pharmaceutical sciences would be the best program for me. I graduated from Osmania University with a Bachelors' of Pharmacy degree and wanted to pursue my higher studies in United States. I researched many programs in many universities and found Wayne State University has the best Master's program and it is interdisciplinary. After doing rotations, I joined in Dr. Pitts' Laboratory and learnt a lot of methods which involve pharmaceutical sciences in an environmental perspective.

The journey of my masters has not only made a better in education but also made me a better person. I thank everyone who has helped me throughout this journey. I am very excited to be graduated and pursue my future goals.