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Endocrine-Disrupting Properties Of Pharmaceuticals And Personal Care Products (ppcps): An Evaluation Using Aquatic Model Organisms

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ENDOCRINE-DISRUPTING PROPERTIES OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS (PPCPS): AN EVALUATION USING AQUATIC MODEL ORGANISMS

by

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THESIS

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_____

Advisor Date

Dedication

Dedicated to

My Parents, My Husband, My Siblings, and My Little Princess.

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Sincere and special thanks to my parents for teaching me the value of knowledge, encouraging me daily to pursue all my dreams including higher education, and care. Many thanks go to my siblings who were there every time I needed one of them during my whole master's journey.

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Table of Contents

List of Tables

List of Figures

Abbreviations

DDT: Dichloro-diphenyl-trichloro-ethane

EPA: Environmental Protection Agency

POP: Persistent Organic Pollutant

CECs: Contaminants of Emerging Concern

USGS: United States Geological Survey

PPCPs: Pharmaceuticals and Personal Care Products

EDCs: Endocrine Disrupting Chemicals

WWTPs: Wastewater Treatment Plants

TCS: Triclosan

TCC: Triclocarban

GC-MS: Gas Chromatography/Mass Spectrometry

LC-MS: Liquid Chromatography/Mass Spectrometry

D. pulex: *Daphnia pulex*

D. rerio: *Danio rerio*

DPF: Days Post fertilization

HPF: Hours Post Fertilization

Chapter 1: Introduction

Environmental contamination has become the hallmark of human presence, particularly since the industrial revolution. It has been estimated that as many as 1500 new chemical entities are synthesized each year ("Key Issues: Toxic Chemicals - High Risk Issue," 2017). Many of these chemicals find their way into the environment either through deliberate disposal or haphazardly as chemical waste. Concern about the impact of this chemical pollution in the USA reached a higher level of consciousness in the early 1960s, when Rachel Carson published her famous book "*Silent Spring.*" This book focused particularly on the use of pesticides such as dichloro-diphenyl-trichloro-ethane (DDT) in agriculture. The increased awareness of the potential biological impact of these chemicals eventually lead to the creation of the Environmental Protection Agency (EPA) in 1970 by the Nixon administration. The monitoring and study of the impact of chemical pollutants has since become increasingly sophisticated, and has been associated with the development of its own nomenclature, such as the term, persistent organic pollutant or "POP." Recently a relatively new term is increasingly found in the research literature, *contaminants of emerging concern* or CECs. The United States Geological Survey (USGS) has provided a relatively concise definition of CECs. CEC definition according to USGS,

"Emerging contaminants" can be broadly defined as any synthetic or naturally - occurring chemical or any microorganism that is not commonly monitored in the environment but has the potential to enter the environment and cause 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. In other cases,

synthesis of new chemicals or changes in use and disposal of existing chemicals can create new sources of emerging contaminants" (Kolpin, 2017).

For a chemical to be classified as a CEC, two key criteria include that it is: (1) not commonly monitored, and (2) known or suspected to cause adverse ecological and/or human health effects. The CECs can be further subdivided into other categories based on their utility or their biological impact. There are CECs that are used either therapeutically or as personal care products and these have been referred to as PPCPs, which stands for pharmaceuticals and personal care products. So, a simple division among the compounds considered to be PPCPs, could be made to divide CECs into PPCPs and non-PPCPs. Another subdivision could be made that divides CECs into endocrine disrupting chemicals (EDCs) and CECs that are not endocrine disrupting (CECs non-EDCs).

CEC Sources

CECs, including PPCPs, have been found in wastewater and surface waters around the world at levels of up to micrograms per liter (Grassi, Rizzo, & Farina, 2013; Niemuth, Jordan, Crago, Blanksma, Johnson, & Klaper, 2015). The sources of water contamination include the discharge of raw sewage and wastewater from sewage treatment plants, ground water contamination from landfills, and runoff from agricultural and urban landscapes. The contaminants can include both human and veterinary pharmaceuticals that find their way into ground water and surface water, such as rivers and lakes (Grassi et al., 2013; D. W. Kolpin, E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber, & H. T. Buxton, 2002; Kummerer, 2009a, 2009b); (Grassi et al., 2013); (Niemuth et al., 2015).

Environmental Impact

Wastewater treatment plants (WWTPs) were not initially created to remove bio-solids and pathogens from wastewater, not CECs (such as pharmaceuticals and household chemicals). Moreover, very little is known about the impacts of these chemicals on environment health or human health. Although many new analytical methods have been developed that can detect widespread water contamination, and the transport and environmental fate of these chemicals is an area of active research, very little is known about the impact on ecosystem function. There is a growing body of data on the biological effects resulting from exposure to individual chemicals on aquatic life, but the shear number of chemicals that have already been released into the environment and the additional complexity of addressing the issue of mixture toxicology dwarf even this effort.

The clearest example of the potential for pharmaceuticals to have an impact on ecosystem function comes from the reports that the pharmaceutical, diclofenac, when used to treat animals in an agricultural setting, caused a severe decline in the white vulture population (Green, Newton, Shultz, Cunningham, Gilbert, Pain, & Prakash, 2004; Shultz, Baral, Charman, Cunningham, Das, Ghalsasi, Goudar, Green, Jones, Nighot, Pain, & Prakash, 2004). The decline in the vulture population resulted from feeding on animal carcasses that were tainted with diclofenac. When the vultures ingested the flesh, they died from kidney damage due to a form of gout they developed because of inadequate diclofenac metabolism (Oaks, Gilbert, Virani, Watson, Meteyer, Rideout, Shivaprasad, Ahmed, Chaudhry, Arshad, Mahmood, Ali, & Khan, 2004). The decline in the vulture population had a cascading effect on the spread of pathogens that resulted in

a rise in rabies that threatened the human population in the Indian subcontinent (Balmford & Bond, 2005).

Impact on Human health

Atrazine is a well- known pesticide with endocrine disrupting properties. Between 1999-2008, a case control study was done connecting prenatal atrazine exposure to male genital malformation. They found that medium level exposure to atrazine during gestation in humans were at a significantly high risk of developing hypospadias, cryptorchidism, and small penis compared to low level exposure. High risk of cancers, reproduction impairment, and antibiotic resistance are major possibilities of the presence of these compounds in the environment (Agopian, Lupo, Canfield, & Langlois, 2013).

Organophosphate insecticides in humans are found in amniotic fluid and can cross the placenta. Chlorpyrifos, an organophosphate insecticide, has been widely used as an insecticide. Prenatal exposures to chlorpyrifos as detected by cord blood, and exposure to other organophosphate pesticides have been linked to smaller head size, lower birth weight, attention problems, neurodevelopmental deficits, and reduction in childhood IQ in preschool-aged children (Rauh, Perera, Horton, Whyatt, Bansal, Hao, Liu, Barr, Slotkin, & Peterson, 2012).

Endocrine disrupting chemicals

One sub-category of CECs that can be identified as the endocrine disrupting chemicals (EDCs). These have been defined as: "*An exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior.*" (EPA 1998). There are many CECs that are known or

suspected of being EDCs (Dana W. Kolpin, Edward T. Furlong, Michael T. Meyer, E. Michael Thurman, Steven D. Zaugg, Larry B. Barber, & Herbert T. Buxton, 2002). Since the vertebrate endocrine system by design is very sensitive to endogenous hormones, any exogenous chemical that can mimic or enhance hormone signaling can elicit powerful effects on developing or mature organisms at very low levels of exposure (Vandenberg, Colborn, Hayes, Heindel, Jacobs, Lee, Shioda, Soto, vom Saal, Welshons, Zoeller, & Myers, 2012); (Lee, Jeung, Cho, Kim, Leung, & Choi, 2013); (Preciados, Yoo, & Roy, 2016). Given that there are many CECs that may be considered EDCs, that they may bio-accumulate, and that there is the potential for additive or synergistic effects from complex mixtures, biological effects may be expected to occur at relatively low exposure levels.

Problem

A complete picture of the environmental impact of EDCs is not well developed, especially when considering the different levels of biological organization, from individual organisms to communities, to ecosystems. Although EDC effects have been reported in the literature (Dana W. Kolpin et al., 2002), the number of newly released and existing chemicals in the environment underscores the need for better EDC detection tools. These new detection tools can also be used to evaluate the scope of the problem, potential water treatment options, and the effectiveness of remediation efforts. Some of the most commonly observed evidence for endocrine disruption in the environment comes from the observation of feminized male fish or altered sex ratios with fewer males downstream of wastewater effluent (Rempel & Schlenk, 2008). Evidence strongly suggests that these EDC effects are due to the estrogenic and/or anti-androgenic influence of chemical contaminants in the water (Rempel & Schlenk, 2008).

Hypothesis

We propose that aquatic model organisms can be used to detect the estrogenicity and anti-androgenic effects of known or suspected PPCPs 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 anti-angdrogenic 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.

This study is a part of a larger project which is divided up into several parts. One part focuses on the analysis of chemicals coming into the Waterworks GLWA drinking water plant to characterize chemicals present in the source water. The biological component is being conducted in three phases with the behavior and lethality assessed in the first phase. In the second phase, the developmental consequences of exposure will be assessed in morphological studies. In the third phase, EnD-seq will be used to characterize genomic responses to chemical exposure in concentration ranges determined by phase 1. Finally, a mathematical model will be developed based on the biological data, and qPCR customizable array plates will be used to test water samples from GLWA drinking water and wastewater plants that likely contain EDCs. This thesis

focuses on the first phase of the biological studies, which evaluates the effects of specific EDCs on the two-aquatic species, *Daphnia pulex* and *Danio rerio*. More specifically, this study focuses on a sub-category of EDCS, pharmaceuticals and personal care products (PPCPs), that are suspected to have endocrine disrupting properties.

This part of the phase 1 study focuses on four PPCPs that are widely found in wastewater and surface waters around the world: triclosan (TCS), triclocarban (TCC), metformin, and estrone. Results from this study and a parallel study examining non-PPCPs will be used to determine the chemical concentrations of interest for developmental, morphological and gene expression studies.

Triclosan(TCS) and Triclocarban(TCC)

Triclosan(TCS) and triclocarban(TCC) are polychlorinated antimicrobials used worldwide for killing microorganisms rapidly and by nonspecific action. These agents are both a boon and threat to human health since their utility in healthcare settings is acknowledged. However, the high-volume use of these antimicrobials by humans caused widespread contamination in the environment. Accordingly, the concern about microbes resistant to these antibiotics has arisen, and has triggered the need for regulation of antimicrobial usage. TCS, which is a bacteriostatic and fungicide, was detected through gas chromatography/mass spectrometry (GC-MS) as an environmental contaminant in U.S. wastewater and sediment (Halden, 2014). A nationwide preliminary research, revealed that TCS was among the top seven detected compounds in U.S. streams (Dana W. Kolpin et al., 2002). When TCS goes under photolysis degradation, a major by-product is identified in wastewater samples which is 2,7/2,8-dibenzodichloro-p-dioxin. Since dioxins are more toxic than TCS, this finding points to the immediate need to improve the

conventional water treatment techniques to eliminate compounds like TCS (Mezcua, Gómez, Ferrer, Aguera, Hernando, & Fernández-Alba, 2004). TCC, which is also a fungicide and bacteriostatic, has showed activity against methicillin-resistant staphylococcus aureus (MRSA) and vancomycin-resistant enterococci (VRE). Unlike TCS, TCC was documented to be a CEC much later because it was only detectable through liquid chromatography/mass spectrometry (LC-MS). Through this method, TCC was detected in Baltimore, Maryland, U.S. in its streams, groundwater, drinking water, wastewater, and sewage sludge (Halden, 2014).

Metformin

The biguanide, metformin, has expanded in global use as the first line treatment in type II diabetes. It is also a potential treatment for polycystic ovarian syndrome (PCOS), and some types of cancers. Moreover, metformin is one of the most ubiquitous pharmaceuticals found in the environment. Metformin passes through human body unchanged, and it is difficult to remove from the effluent by conventional wastewater treatment plants (WWTPs). It is found in WWTP effluent, in Milwaukee, Wisconsin, USA, at concentrations of 1 µg/L to 47 µg/L (0.006 µM – 0.285 µM) and in surface water at a concentration of 0.06 µg/L to 3 µg/L (0.0003 µM – 0.018 µM) (Niemuth et al., 2015) (Bradley, Journey, Button, Carlisle, Clark, Mahler, Nakagaki, Qi, Waite, & VanMetre, 2016). A recent study detected metformin in 89% of samples and 97% of sites along Piedmont USA streams (Bradley et al., 2016).

Estrone

Estrogen hormones are divided into two types. First, endogenous estrogens, 17b-estradiol, estrone, and estriol are found in the urine of premenopausal women. Second, synthetic estrogen hormones, like 17 α -ethinylestradiol, are present in the urine of women taking birth control pills or hormone replacement therapy. Both types were detected in Germany and Canada sewage treatment plants (STPs) effluents in nano-gram per liter concentrations (Metcalfe, Metcalfe, Kiparissis, Koenig, Khan, Hughes, Croley, March, & Potter, 2001). One of the most common aquatic hormone contaminants is estrone, a major metabolite of 17α-ethinylestradiol. Individual estrogen compounds are found in low concentrations; however, these compounds are known to have additive effects (Notch & Mayer, 2013).

Chapter 2: Materials and Methods

Behavioral Assay

Animal Care

Daphnia pulex

The animal culture was maintained in two- to four-liter glass jars at 21ºC in an incubator. The culture was fed 3 times weekly on a 50/50 algae mixture of Ankistrodesmus falcatus and Desmodesmus. The COMBO media (Kilham, Kreeger, Lynn, Goulden, & Herrera, 1998) in the culture jars was changed weekly. Adult *D. pulex* used in experiments were obtained by filtering the culture through a plastic mesh that captured any animal of 1.4 mm or larger (Zein, McElmurry, Kashian, Savolainen, & Pitts, 2014).

Danio rerio

We receive 26-30 four days' post fertilization (dpf) zebrafish from the laboratory of Dr. Tracie Baker in the Institute of Environmental Health Sciences, WSU. They set a spawn 5 days prior to a planned experiment. At noon, they start a spawn by netting one male and two females in a small fish tank. In the following day, between 8 am to 10 am lights go on, the spawning happens during that period. Females lay between 50-200 eggs. Most eggs die and they are left with approximately 30 zebrafish of 4^{th} dpf. Zebrafish embryos were maintained in petri dishes in an incubator set between 27ºC -30ºC. The fish culture media was composed of 1.32 g of Instant Ocean Salts (Tetra) and 1.08 g sodium bicarbonate per 20 L of RO water. The animals were maintained in 14-h/10-h light/dark cycle. Four-day old zebrafish embryos were utilized in all behavioral bioassays.

At the end of the 24-hour experiment the animals were euthanized with Tricaine methansulfonate (1.67 mg/ml) (Baker, Peterson, & Heideman, 2013).

Behavioral bioassay: *D. pulex and D. rerio*

24 - well plates were loaded with animals by placing one adult *D. pulex* female or one 4-day-old *D. rerio* in each well. Each well was filled with a specific chemical solution. In a typical concentration-response experiment examining the effects of one chemical, the 24-well plate was divided up into 6 treatments with 4 replicates. The treatments included 5 different concentrations of a specific chemical dissolved in COMBO plus one control (COMBO alone) and these treatments were randomly placed in the wells using a randomization program.

Figure 1, Figure 2 and Figure 3 depict the behavioral setup used to monitor behavior. The 24-well plate was placed on top of clear flow cell (described below) and an infrared LED panel (Edmund Scientific, Model: 100x200 mm Backlight (880 nm)). Constant temperature control was maintained by using a flow cell made of Plexiglas and glass plate underneath the 24-well plate that was able to transmit infrared light from the LED panel below. In addition, a heated Plexiglas top covered the 24-well plate in order to prevent evaporation and provide additional temperature control.

Coolant (Polyscience, Polycool HC-50) was circulated through the flow cell at constant temperature by a Polyscience (model: AD07R-20-A11B) water bath circulator. The LED panel transmitted infrared light through the bottom of the flow-cell and up through the 24-well plate. To prevent fogging, the Plexiglas top covering the 24-well plate was heated by a nichrome wire that was controlled by a Wattlow HI WATT temperature controller. The temperature of the circulator and the Wattlow controller were adjusted to

maintain *D. pulex* at 21 and *D. rerio* at 28 degrees centigrade and avoid fogging of the Plexiglas cover during the entire course of the 24-hour experiment. An inline bubble trap was used to prevent the formation of bubbles by the circulator inside the flow cell. The addition of the bubble trap improved the efficiency of animal behavior tracking by avoiding the miss-identification of bubbles as animals by the software.

Behavior was automatically recorded at specific time intervals using a digital camera (Lumenera digital camera, Model: INFINITY 35-1URM) and Image Pro Premier software (Mediacybernetics) over 24 hours.

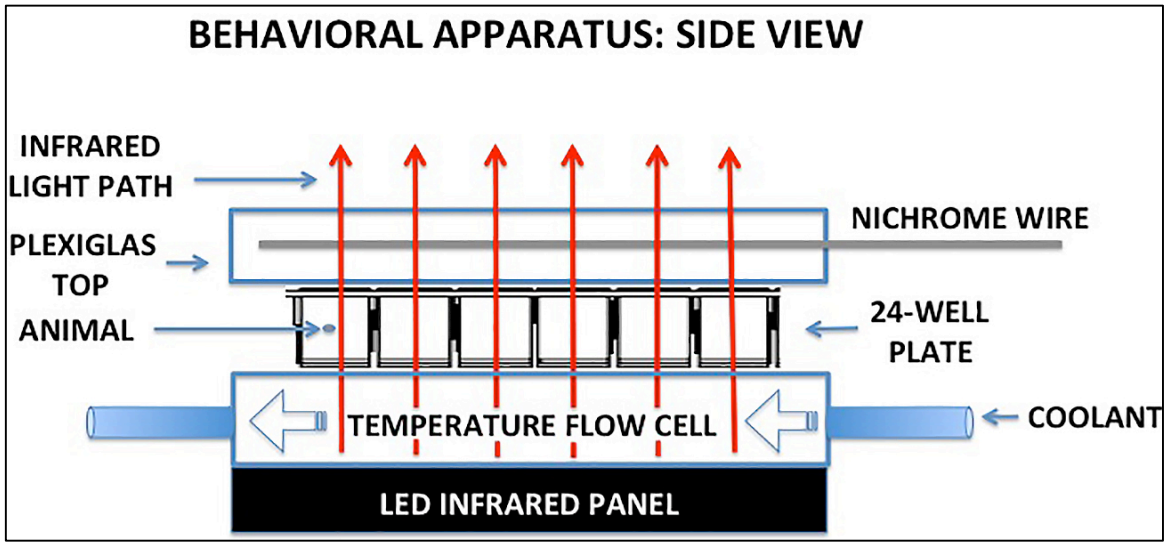

Figure 1: Diagram of behavioral assay setup for tracking *D. pulex* and *D. rerio* with temperature control.

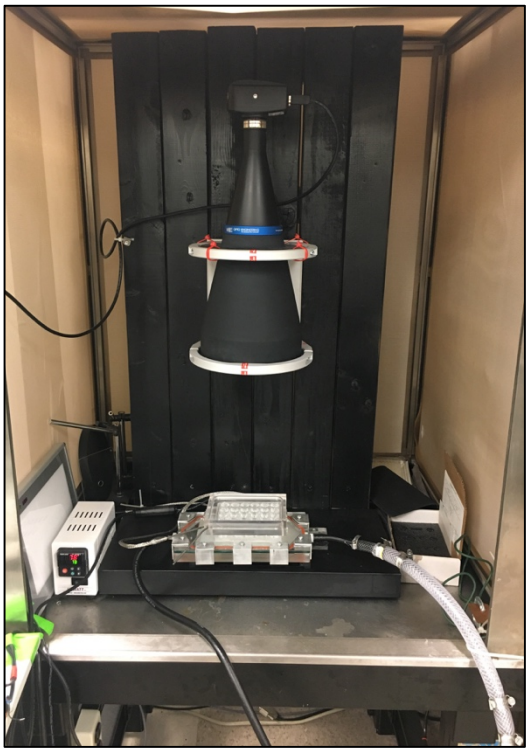

Figure 2: Photograph of behavioral assay setup including telecentric lens and digital camera.

Figure 3: Circulating water bath

Video Recording and Optical Tracking

A Lumenera digital camera (Model: INFINITY 35-1URM) with an Opto-engineering telecentric lens (Model TC 12 120) was used and was able to record videos of the entire 24-well plate within the field of view with minimal distortion. The duration of the video recordings was based on the relative level of baseline activity of the animals, and was 5 secs for *D. pulex* and 20 secs duration for *D. rerio* (generally had lower level of activity). The camera exposure rate was 28 frames per second, which generated 148 frames for *daphnia pulex* and 592 frames for *Danio rerio* per video. Image Pro Premier software was used to generate standard variables describing motion and it provided an Excel spreadsheet as output for each recording.

The 2D-tracking feature of Image Pro Premier software was used to analyze the motion of the animals in the videos. Distance was measured by Image Pro Premier software across 2 successive frames. Angular change was measured by the software as the change in angle occurring in the third frame out of set of three successive frames as shown in Figure 4 (Zein et al., 2014). These two swimming variables were quantified and compiled for 29 spreadsheets through the use of the 2D-tracking module and an Excel macro respectively (see design, below). The macro provided a measure of both maximum accumulated distance and mean angular change for each video. These two animal behavior parameters have been previously utilized to quantify *D. pulex* swimming behavior (Zein et al., 2014) and were also used to quantify *D. rerio* swimming behavior in the present study.

Experimental Design and Statistics

Each experiment included of a minimum of two separate 24-hour runs of a single plate. When just one chemical was examined there would be 6 concentrations per plate

including a control (concentration of 0), and this would generate 4 replicates per concentration (n=4 animals). The combination of two 24-well plates generated 8 replicates for each concentration studied. 29 video recordings were made over the 24-hour period. The intervals were as follows: every 10 minutes for the first 190 minutes, then every hour over the next 3 hours, then every 2 hours for the next 6 hours, and finally every 4 hours for the last 12 hours.

The typical design for an experiment examining the effects of one chemical for either *D. pulex* or *D. rerio* was a factorial ANOVA with repeated measures. The factors were: Concentration (6 levels including control of 0), Plate number (typically two different plates), and Time (29 time points over the 24-hour period). The Least Significant Difference test was used for specific planned comparisons where appropriate. A P-value of less than 0.05 was considered significant.

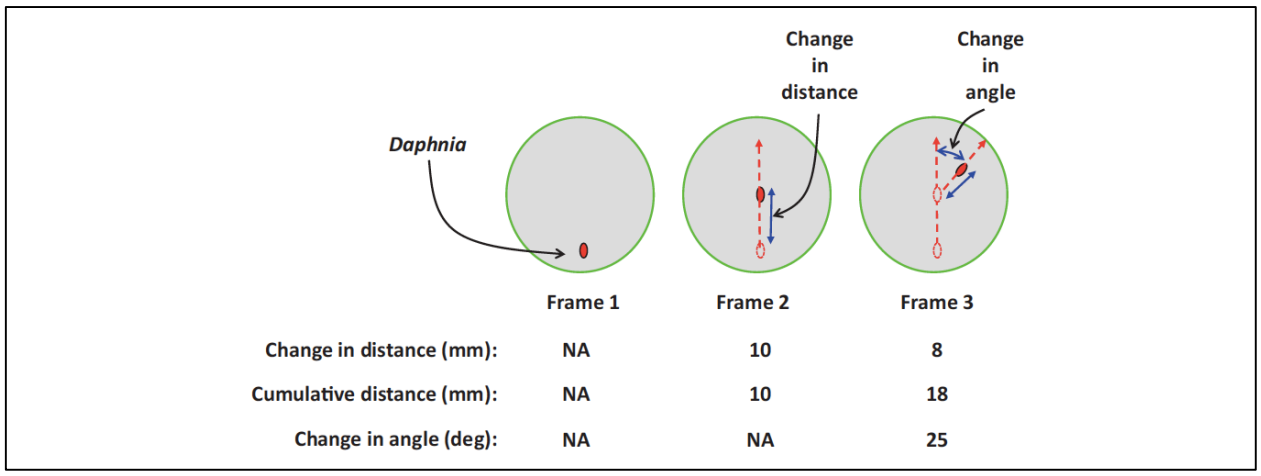

Figure 4: Example of maximum accumulated distance and mean angle quantification from Zein et al. (2014).

Chemicals

All chemicals were purchased from Sigma-Aldrich. Acetone was used as a solvent as necessary to make stock solutions of the chemicals tested and the various concentrations ranges used in behavioral tests were generated by serial dilution with either COMBO freshwater media or fish freshwater media as appropriate for *D. pulex* or *D. rerio*, respectively. The concentration range selected for each chemical studied was based on literature that reported LC50 values or behavioral effects in *D. pulex* or *D. rerio* or related species.

Triclosan (TCS) was prepared by dissolving it in acetone with a final concentration of 50mM.TCS stock solution was then diluted to 8, 4, 2, 1, and 0.5 µM with freshwater media. Likewise, acetone was used as the solvent for triclocarban (TCC) with a stock concentration of 50mM and then diluted to a concentration range of 8, 4, 2, 1, and 0.5 µM or 100, 50, 25, 12.5, and 6.25 nM with freshwater media. Estrone was dissolved in acetone as a 1mM stock concentration and then diluted to 4, 2, 1, 0.5, and 0.25 nM with freshwater media. Ultrapure water (Type 1) was used to dissolve Metformin with a stock solution concentration of 10 mM that was diluted to a range of either 400, 200, 100, 50, and 25 µM or 100, 10, 1.0, 0.1, 0.01 µM with freshwater media. Freshwater media was used as the control in the chemical test assays. The final concentration of acetone for the highest concentration of the test chemicals was 0.030%. All the remaining acetone concentrations used in the behavioral bioassay were less than 0.030%. This range of acetone concentrations was not found to significantly affect the behavior of *D. pulex* (Zein et al., 2014); (Zein, McElmurry, Kashian, Savolainen, & Pitts, 2015) or *D. rerio*.

Chapter 3: EDCs Effects on the Behavior of Daphnia pulex

Results

Triclosan (TCS)

Distance

TCS elicited a significant concentration-dependent effect on maximum accumulated distance ($P<0.05$) in *D. pulex* as shown in Figure 5. The maximum accumulated distance for concentrations 0.5 µM to 4 µM was significantly higher than control ($P<0.05$, LSD in all cases) indicating that TCS stimulated swimming behavior. There was a significant time x concentration effect of TCS on maximum accumulated distance ($P<0.05$).

Figure 6 shows the time-course for the effects of selected concentrations of TCS. The time period when peak stimulation of swimming occurred varied according to concentration, with the 2 µM concentration showing the greatest distance at approximately 60 minutes, and the 8 µM concentration showing the greatest distance at approximately 190 minutes. At 24-hours there was no significant stimulation of swimming by any concentration of TCS ($P>0.05$ in all cases).

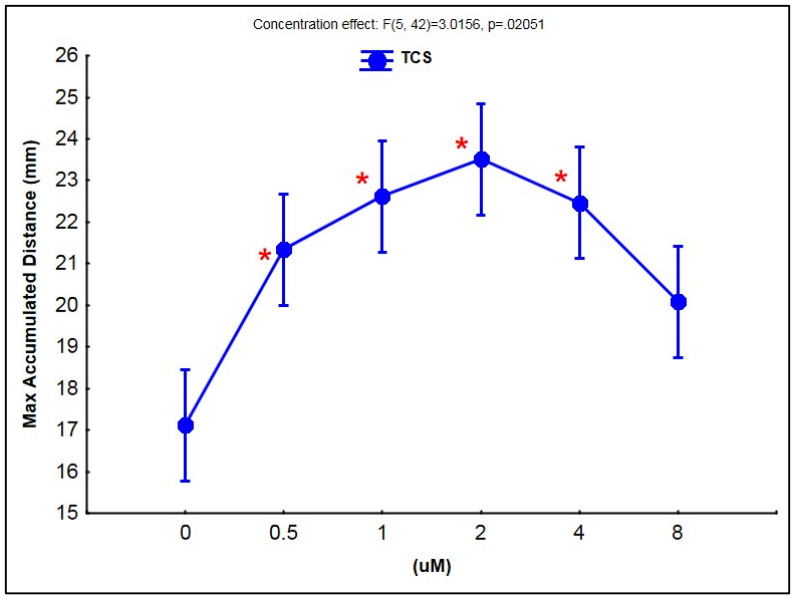

Figure 5: Effects of TCS on maximum accumulated distance in *D. pulex.* The LSD test indicated a significant difference between TCS treated animals and controls at the corresponding concentrations (* P<0.05).

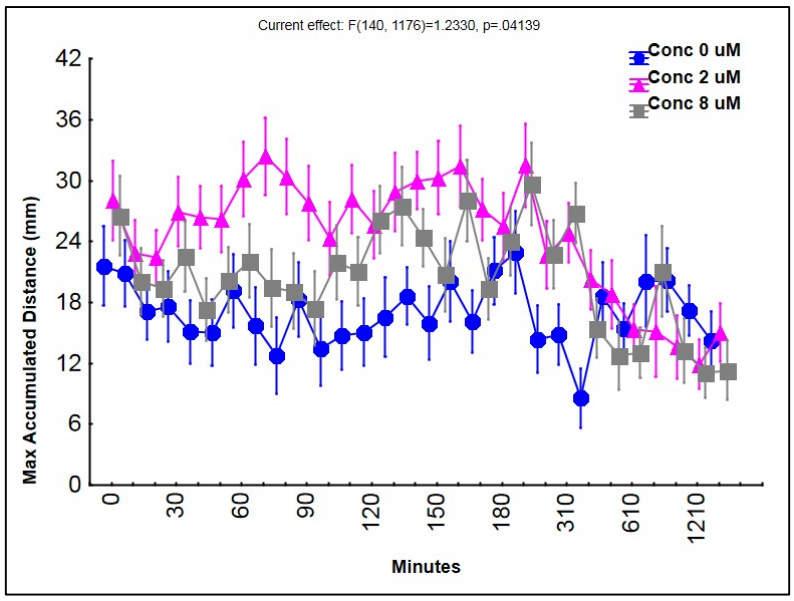

Figure 6: Time-dependent effects of TCS on maximum accumulated distance in *D. pulex*

Angle

The mean angle was significantly changed by TCS in *D. pulex* in a concentration-dependent manner (P<0.05) as shown in Figure 7. The mean angle for concentrations 2

and 4 µM was significantly lower than control (P<0.05, LSD in all cases). The time-dependent effects of concentration on mean angle are shown in Figure 8. The effect of concentration on mean angle was not dependent on time (P>0.50). All the concentrations including control tended to increase in mean angle after 180 minutes. The decrease in mean angle for 0.5, 2, and 4 µM occurred when there was an increase in maximum accumulated distance (see Figure 6).

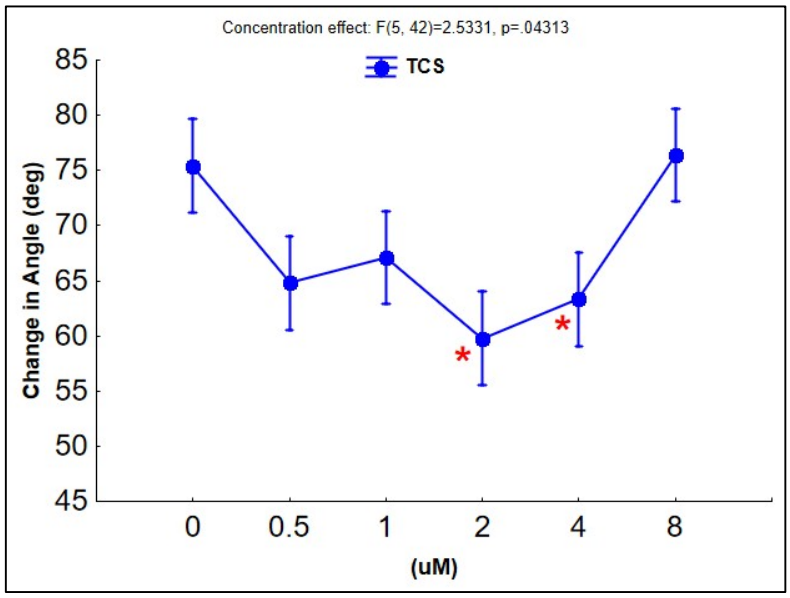

Figure 7: Effects of TCS on mean angle in *D. pulex.* The LSD test indicated a significant difference between TCS treated animals and controls at the corresponding concentrations (* P<0.05).

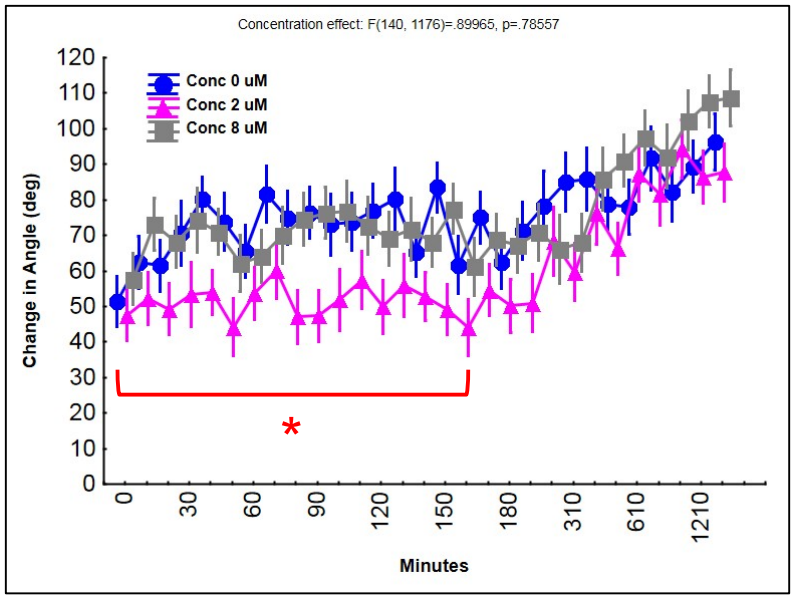

Figure 8: Time-dependent effects of TCS on mean angle in *D. pulex.* There was a significant difference between TCS treated animals and controls at the corresponding time points as indicated by the bracket (Contrast analysis, * P<0.05).

Triclocarban (TCC) (Lower Concentrations)

Distance

TCC elicited a non-significant concentration-dependent effect on maximum accumulated distance (P>0.50) in *D. pulex* as shown in Figure 9. There was not a significant time x concentration effect of TCC on maximum accumulated distance (P>0.50). Figure 10 shows the time-course for the effects of TCC.

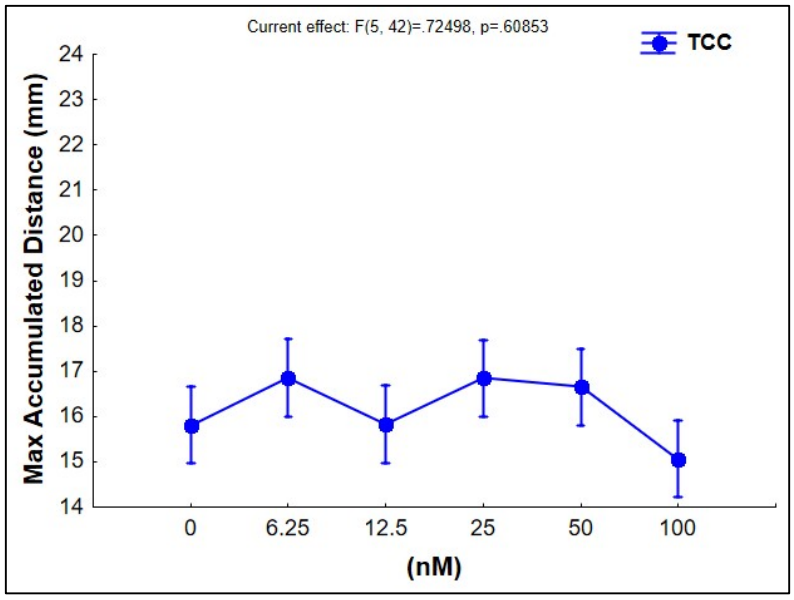

Figure 9: Effects of TCC on maximum accumulated distance in *D. pulex*

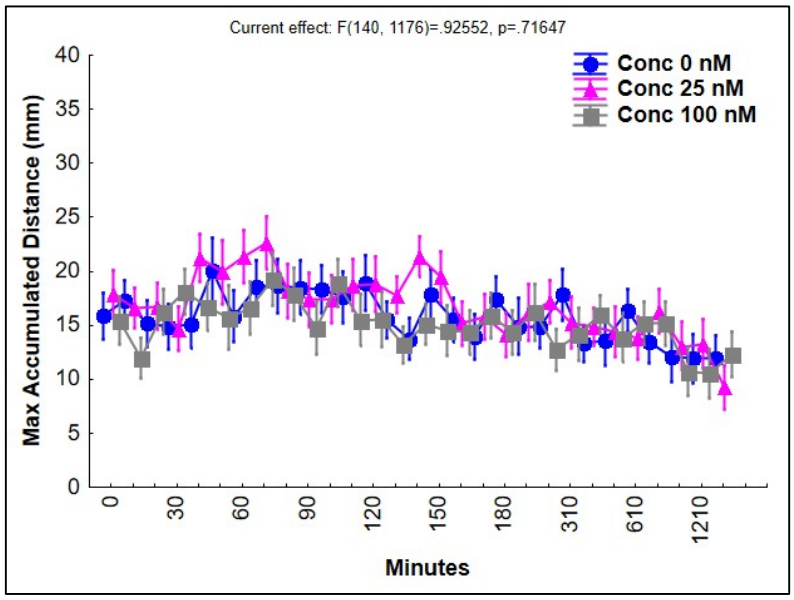

Figure 10: Time-dependent effects of TCC on maximum accumulated distance in *D. pulex*

Angle

As shown in Figure 11, the mean angle in *D. pulex* was not significantly changed by TCC in a concentration-dependent manner (P>0.20). The time-dependent effects of

concentration on mean angle are shown in Figure 12. There was a non-significant trend towards an effect of concentration on mean angle significance (P ~ 0.0507).

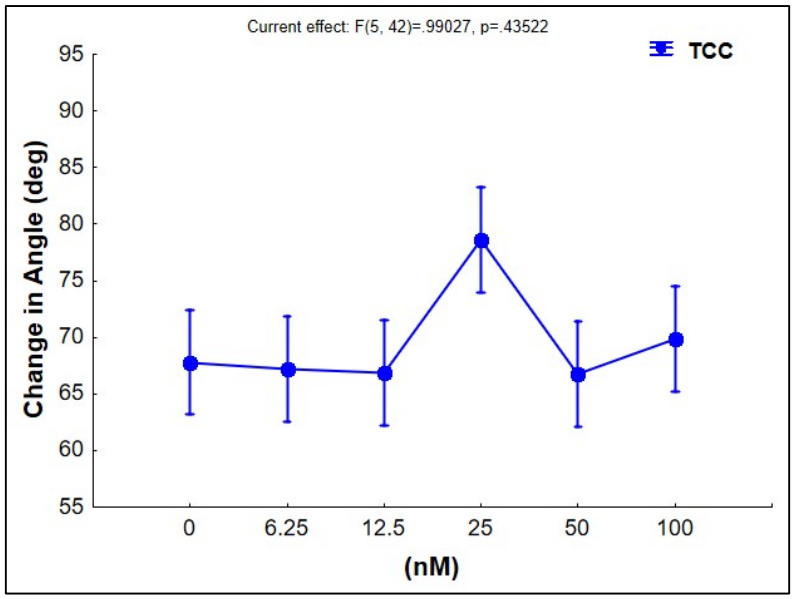

Figure 11:Effects of TCC on mean angle in *D. pulex*

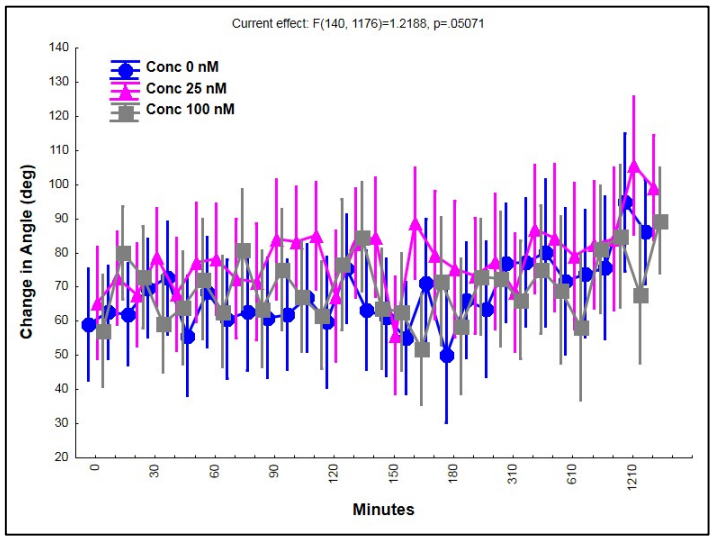

Figure 12: Time-dependent effects of TCC on mean angle in *D. pulex*

Triclocarban (TCC) (Higher Concentrations)

Distance

TCC elicited a non-significant concentration-dependent effect on maximum accumulated distance (P>0.50) in *D. pulex* as shown in Figure 13. There was a non-

significant trend towards time x concentration effect of TCC on maximum accumulated distance (P ~ 0.059). Figure 14 shows the time-course for the effects of TCC.

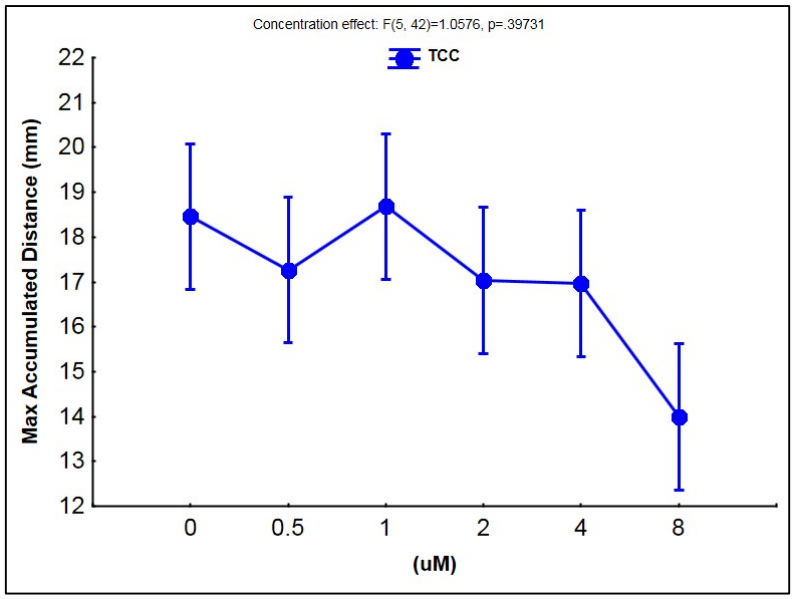

Figure 13: Effects of TCC on maximum accumulated distance in D. pulex

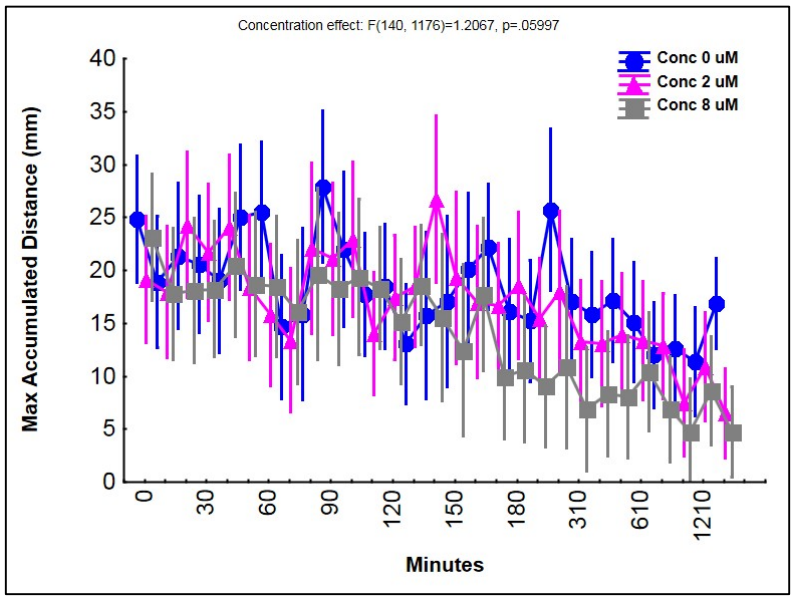

Figure 14: Time-dependent effects of TCC on maximum accumulated distance in D. pulex

Angle

As shown in Figure 15, the mean angle in *D. pulex* was not significantly changed by TCC when only considering concentration (P>0.50). The time-dependent effects of concentration on mean angle are shown in

Figure 16. When examined over time there was a significant increase in mean angle for the 8 μM concentration after 3 hours (P<0.05).

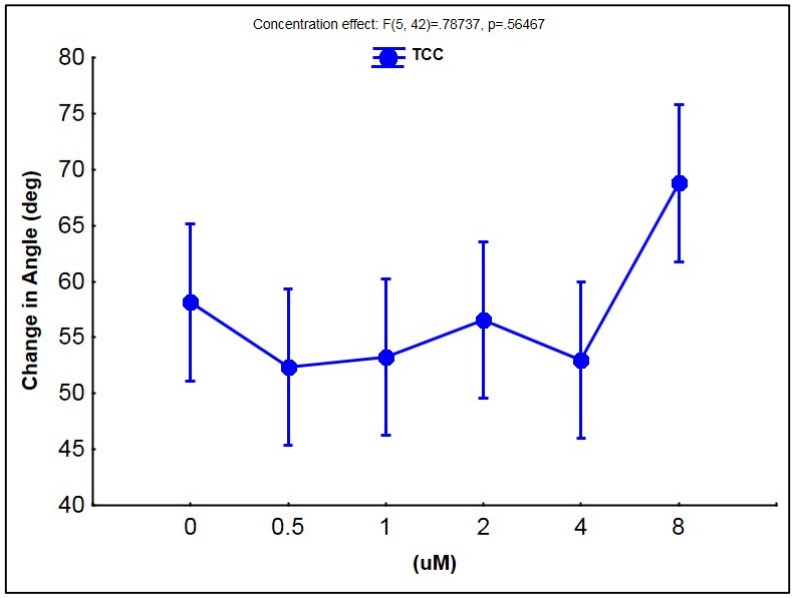

Figure 15: Effects of TCC on mean angle in D. pulex

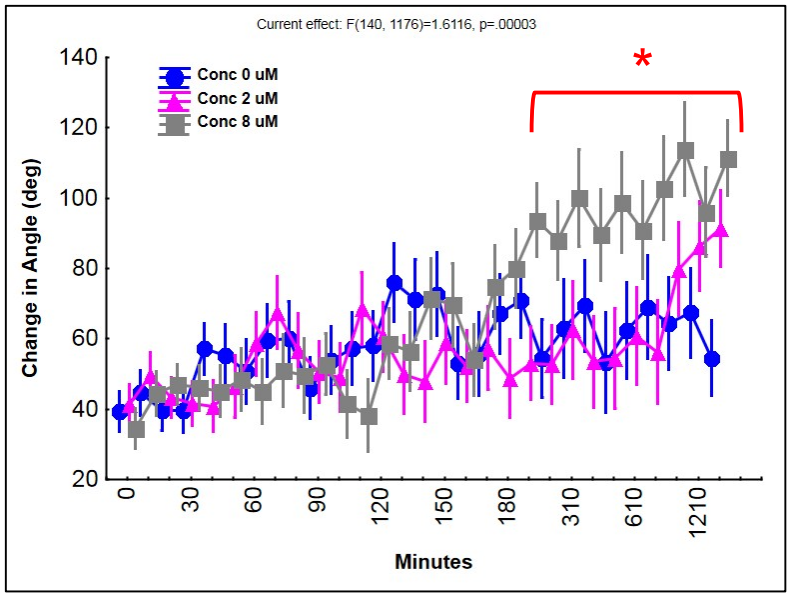

Figure 16: Time-dependent effects of TCC on mean angle in D. pulex. There was a significant difference between 8 µM TCC treated animals and controls at the corresponding time points as indicated by the bracket (Contrast analysis, * P<0.05).

Metformin (Lower Concentrations)

Distance

Metformin elicited a non-significant concentration-dependent effect in *D. pulex* on maximum accumulated distance (P>0.50) as shown in Figure 17. Figure 18 shows there was not a significant time x concentration effect of metformin on maximum accumulated distance (P>0.50).

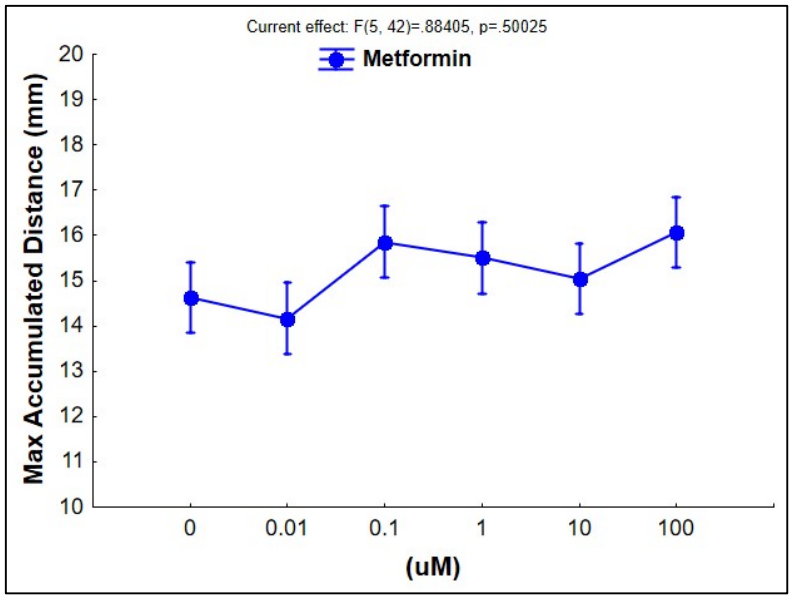

Figure 17: Effects of metformin on maximum accumulated distance in *D. pulex*

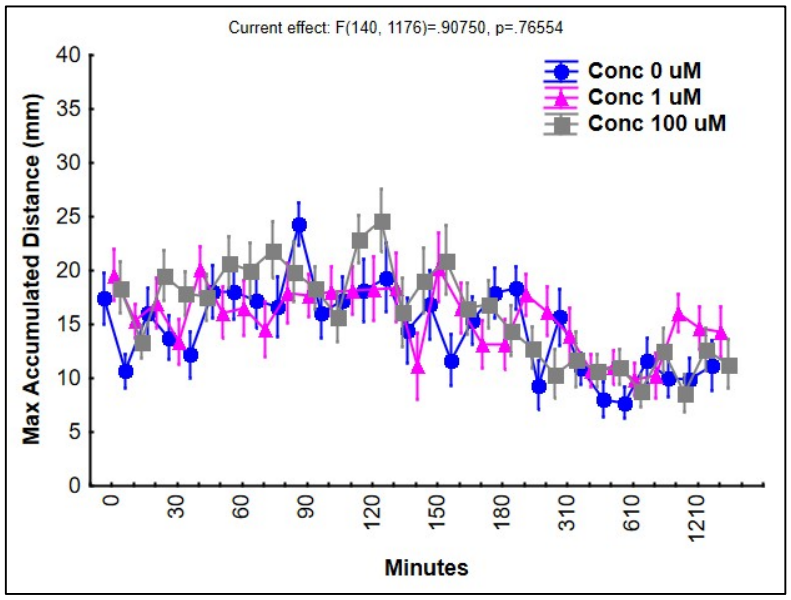

Figure 18: Time-dependent effects of metformin on maximum accumulated distance in *D. pulex*

Angle

As shown in Figure 19, the mean angle in *D. pulex* was not significantly changed by metformin in a concentration-dependent manner (P>0.50). The time-dependent effects

of concentration on mean angle are shown in Figure 20. The effect of concentration on mean angle was not dependent on time (P>0.20).

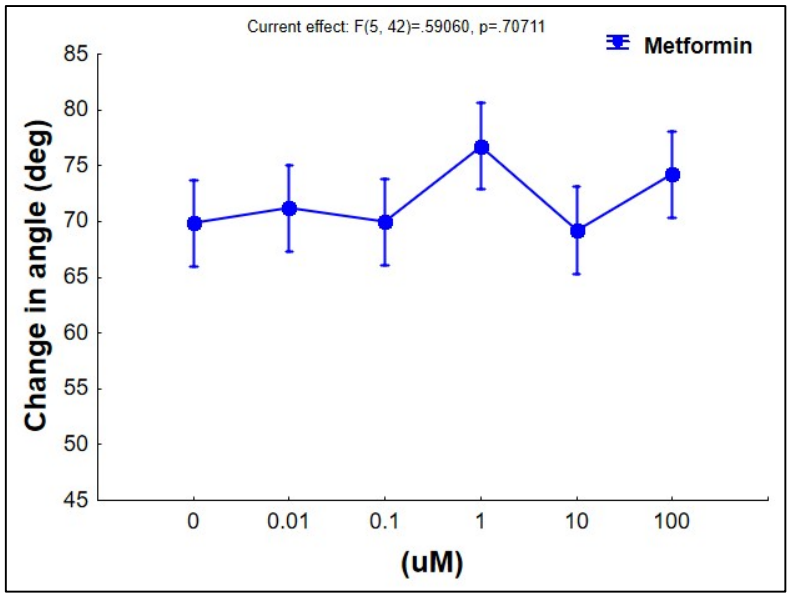

Figure 19: Effects of metformin on mean angle in *D. pulex*

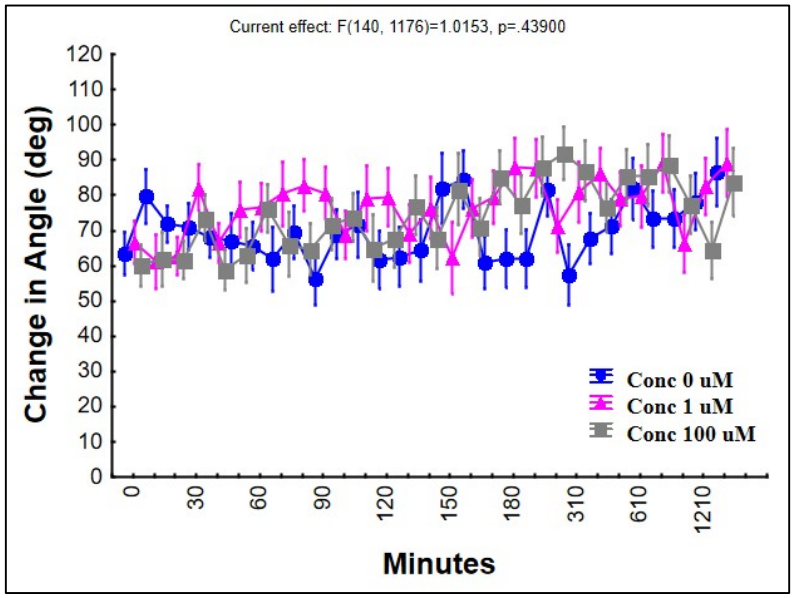

Figure 20: Time-dependent effects of metformin on mean angle in *D. pulex*

Metformin (Higher Concentrations)

Distance

Metformin elicited a non-significant concentration-dependent effect in *D. pulex* on maximum accumulated distance (P>0.20) as shown in Figure 21. Figure 22 shows there was not a significant time x concentration effect of metformin on maximum accumulated distance (P>0.50).

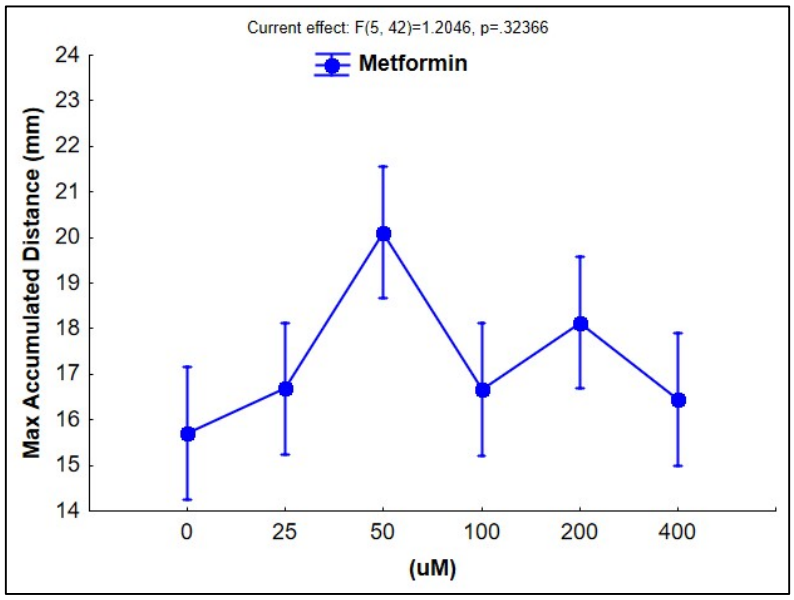

Figure 21: Effects of metformin on maximum accumulated distance in *D. pulex*

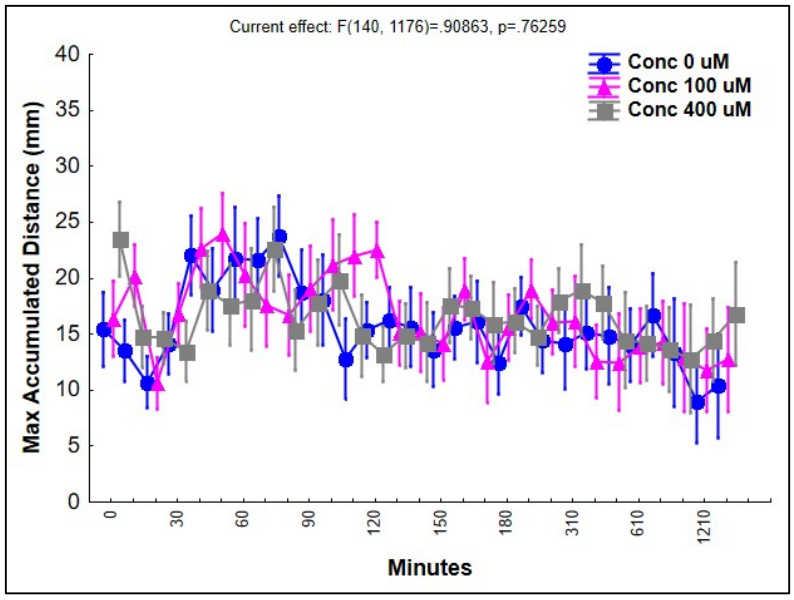

Figure 22: Time-dependent effects of metformin on maximum accumulated distance in *D. pulex*

Angle

As shown in Figure 23, the mean angle in *D. pulex* was not significantly changed by metformin in a concentration-dependent manner (P>0.20). The time-dependent effects of concentration on mean angle are shown in Figure 24. The effect of concentration on mean angle was not significant (P>0.50).

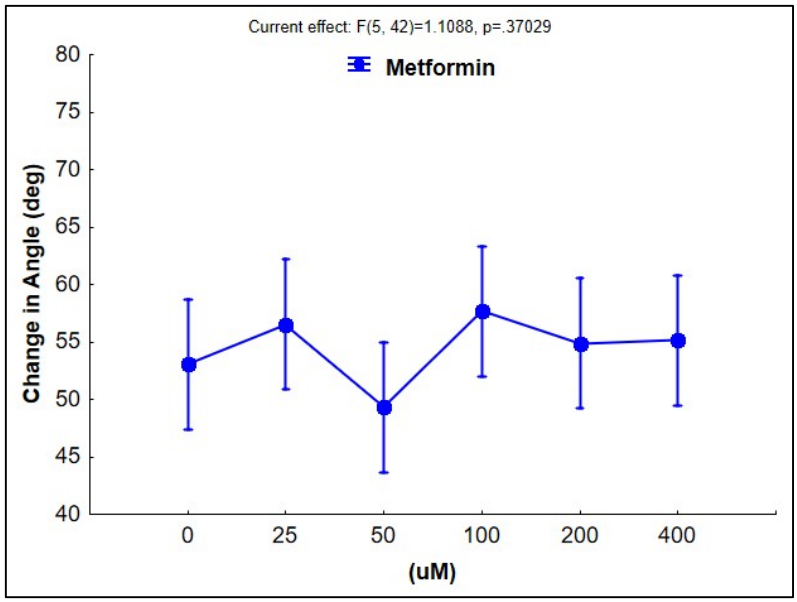

Figure 23: Effects of metformin on mean angle in *D. pulex*

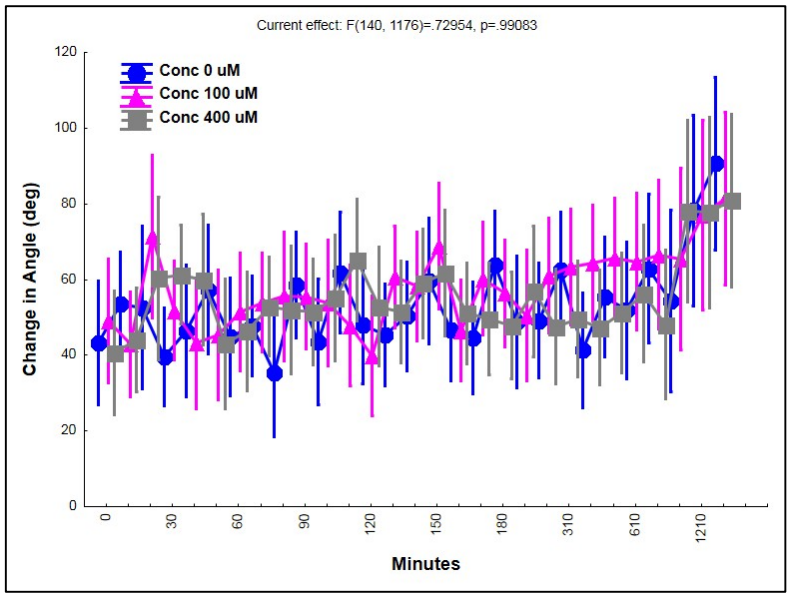

Figure 24: Time-dependent effects of metformin on mean angle in *D. pulex*

Estrone

Distance

Figure 25 shows a non-significant concentration-dependent effect on maximum accumulated distance (P>0.10) in *D. pulex* induced by estrone. There was not a

significant time x concentration effect of estrone on maximum accumulated distance (P>0.50) (See Figure 26).

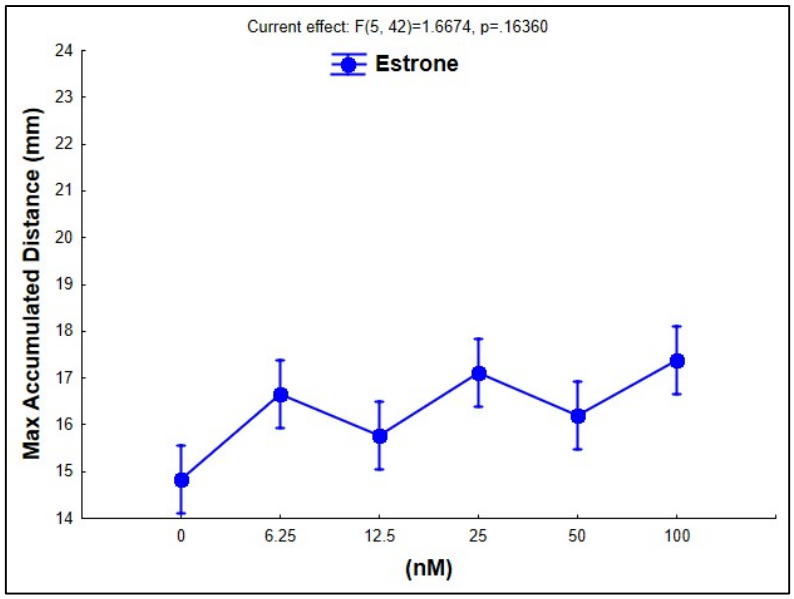

Figure 25: Effects of estrone on maximum accumulated distance in *D. pulex*

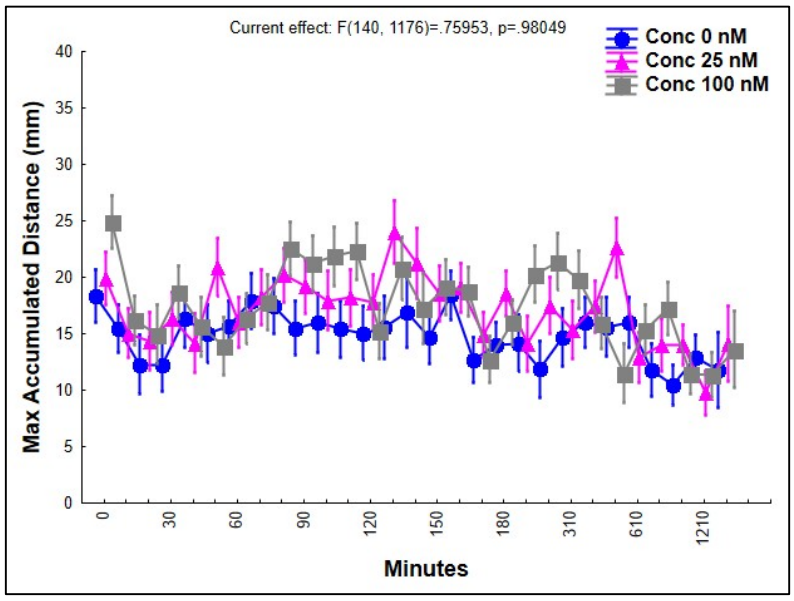

Figure 26: Time-dependent effects of estrone on maximum accumulated distance in *D. pulex*

Angle

The mean angle in *D. pulex* was not significantly changed by estrone (P>0.20) as shown in Figure 27. The time-dependent effects of concentration on mean angle are shown in Figure 28. The effect of concentration on mean angle was not significant (P>0.50).

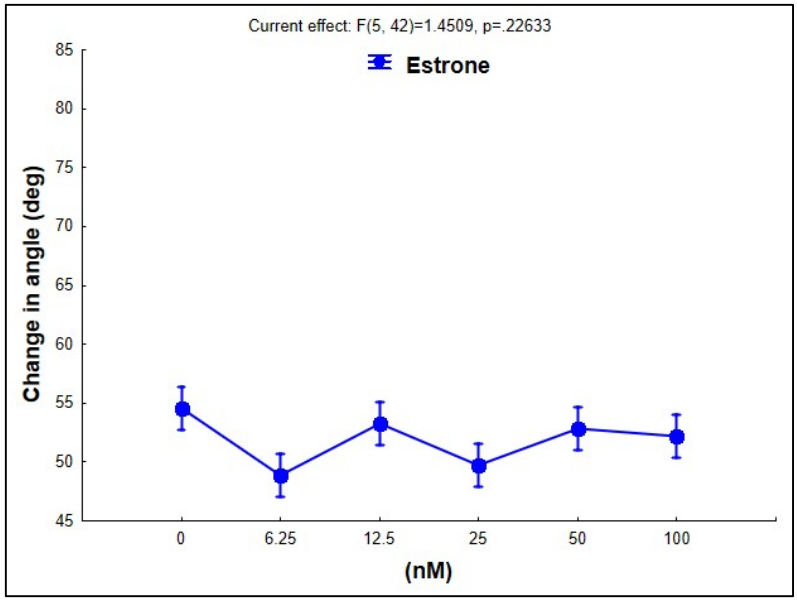

Figure 27: Effects of estrone on mean angle in *D. pulex*

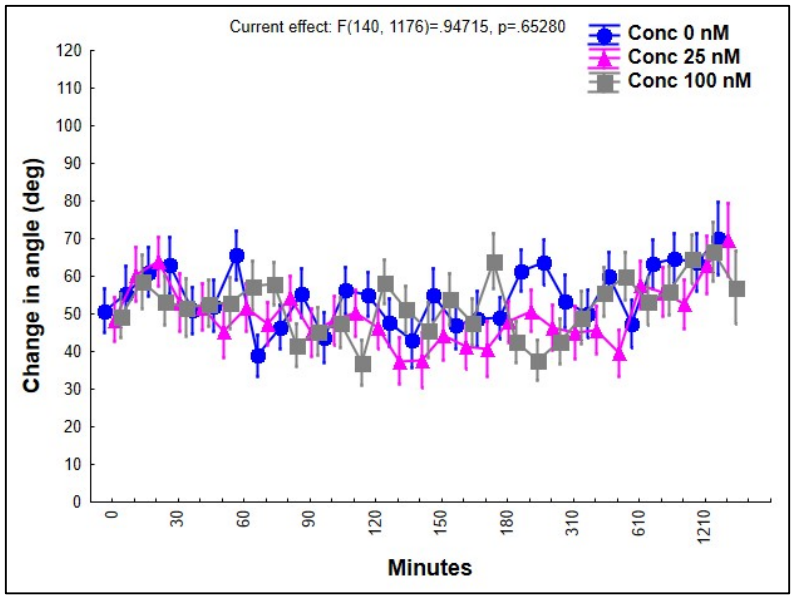

Figure 28: Time-dependent effects of estrone on mean angle in *D. pulex*

Chapter 4: EDCs Effects on the Behavior of *Danio rerio*

Results

Triclosan (TCS)

Distance

Although TCS did not elicit a significant concentration-dependent effect on maximum accumulated distance when time was not included as a factor (concentration effect, P>0.05) in *D. rerio* (Figure 29), there was a significant time x concentration effect of TCS on maximum accumulated distance (P<0.05). Figure 30 shows the time-course for the effects of TCS. The time point when peak stimulation of swimming occurred was at approximately 15 minutes with the 0.25 µM and 2 µM concentration showing the greatest values for maximum accumulated distance.

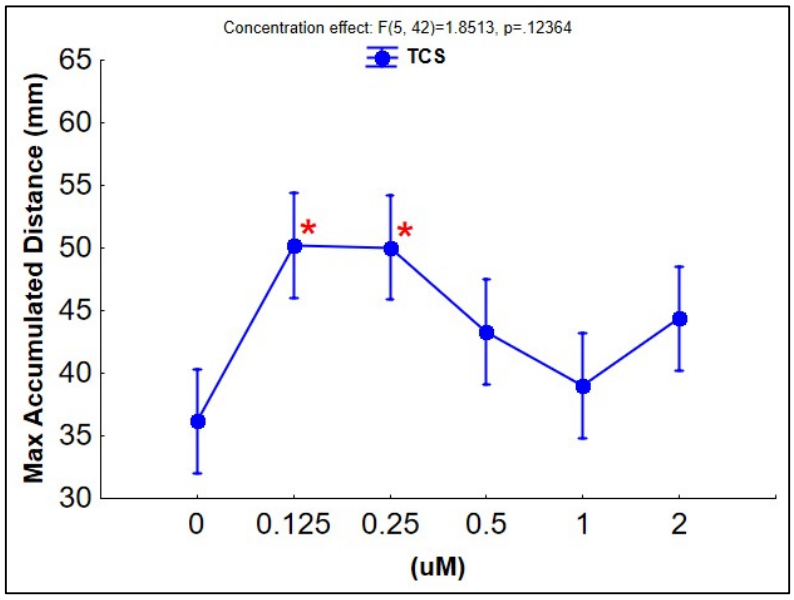

Figure 29: Effects of TCS on maximum accumulated distance *in D. rerio.* The LSD test indicated a significant difference between TCS treated animals and controls at the corresponding concentrations (* P<0.05).

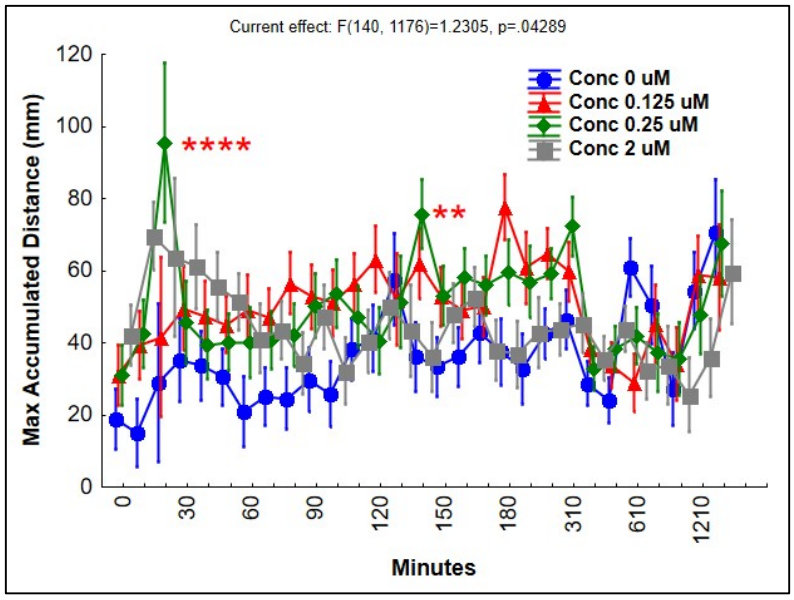

Figure 30: Time-dependent effects of TCS on maximum accumulated distance *in D. rerio.* There was a significant difference between TCS treated animals and controls at the corresponding time points as indicated by the asterisks (Contrast analysis, ** P<0.01, **** P<0.001).

Angle

The mean angle was not significantly changed by TCS in *D. rerio* as shown in Figure 31. The time-dependent effects of concentration on mean angle are shown in Figure 32. The effect of concentration on mean angle was not dependent on time (P>0.50). The lower values of mean angle for concentrations 0.125 μM and 0.5 μM relative to control are consistent with the elevated maximal accumulated distance observed in Figure 26 and a stimulatory effect on swimming behavior. All the concentrations including control tended to increase in mean angle after 310 minutes.

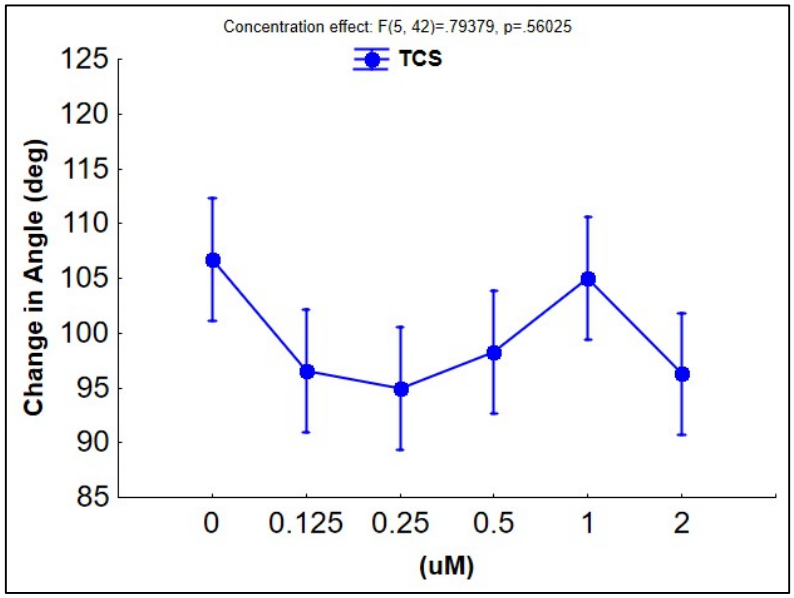

Figure 31: Effects of TCS on mean angle *in D. rerio*

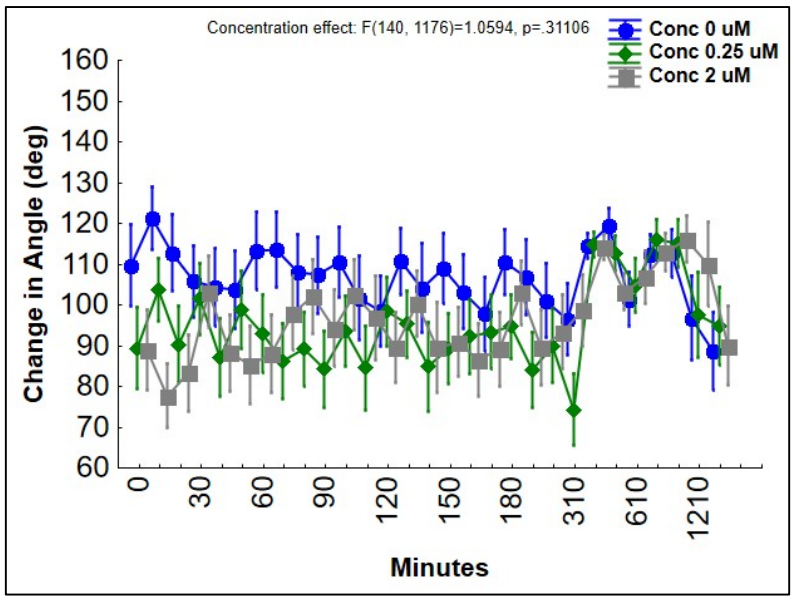

Figure 32: Time-dependent effects of TCS on mean angle *in D. rerio*

Triclocarban (TCC) (Higher Concentrations) (0.5 µM – 8 µM)

In general, the µM concentrations of TCC used for *D. pulex* (0.5 µM – 8 µM, described above) were lethal to *D. rerio* (as shown in Table 1). TCC in the nM

concentration range did not elicit significant behavioral changes (see below for detailed concentrations).

Table 1: Triclocarban (TCC) lethality in Zebrafish

	0	0.5 µM	1 µM	2 µM	4 µM	8 µM
Alive	4	3	1	1	1	2
Dead	0	1	3	3	3	2

TCC (Lower Concentrations) (6.25 nM – 100 nM)

Distance

TCC elicited a non-significant concentration-dependent effect on maximum accumulated distance (P>0.50) in *D. rerio* as shown in Figure 33. There was not a significant time x concentration effect of TCC on maximum accumulated distance (P>0.50). Figure 34 shows the time-course for selected concentrations of TCC.

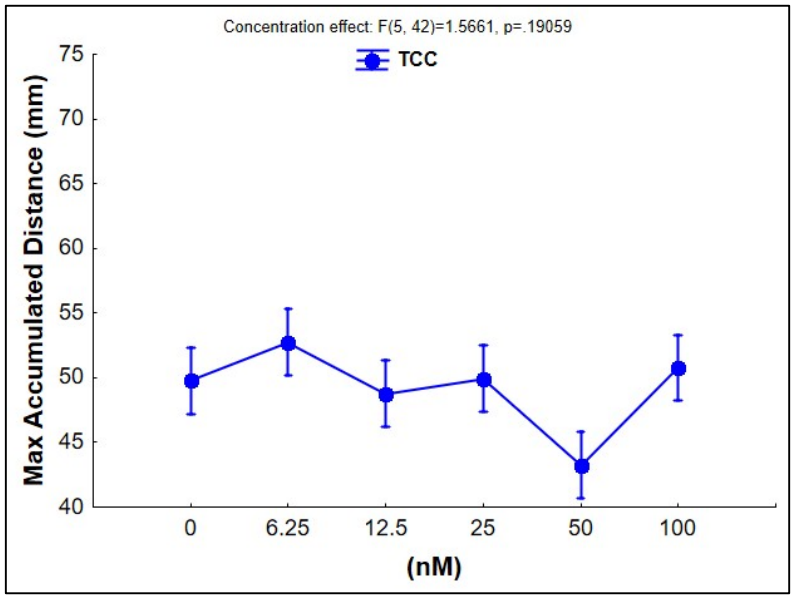

Figure 33: Effects of TCC on maximum accumulated distance *in D. rerio*

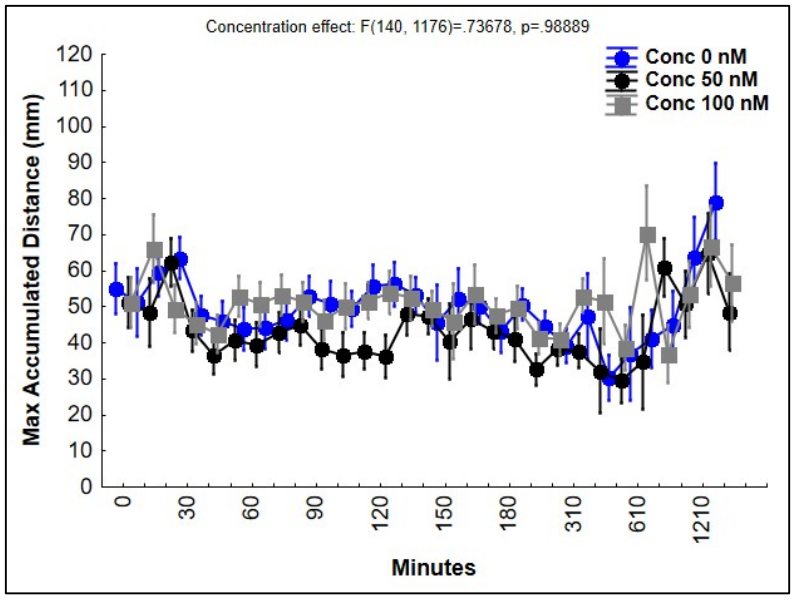

Figure 34: Time-dependent effects of TCC on maximum accumulated distance *in D. rerio*

Angle

As shown in Figure 35, the mean angle in *D. rerio* was not significantly changed by TCC in a concentration-dependent manner (P>0.20). There was a non-significant effect of concentration on mean angle of *D. rerio* over time (P>0.50, Figure 36). However, the mean angle was increased after 3 hours due to inactivity of *D. rerio*.

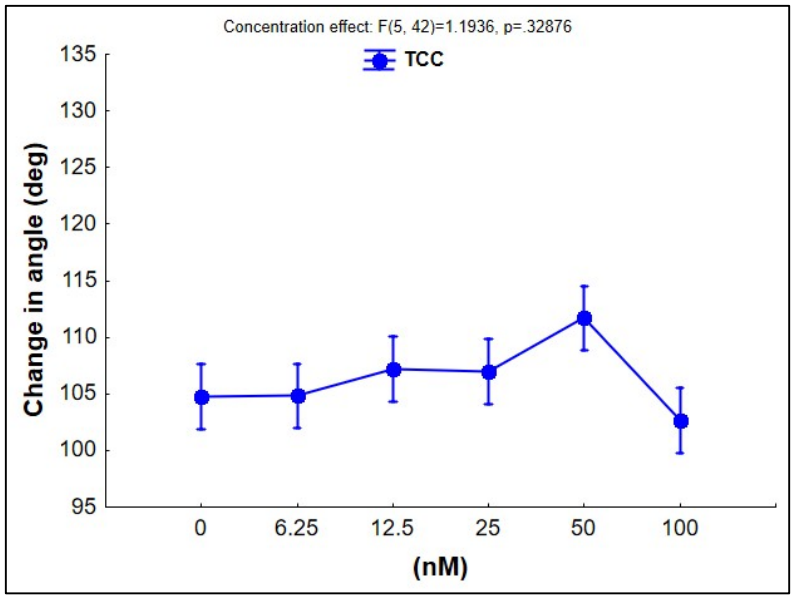

Figure 35: Effects of TCC on mean angle *in D. rerio*

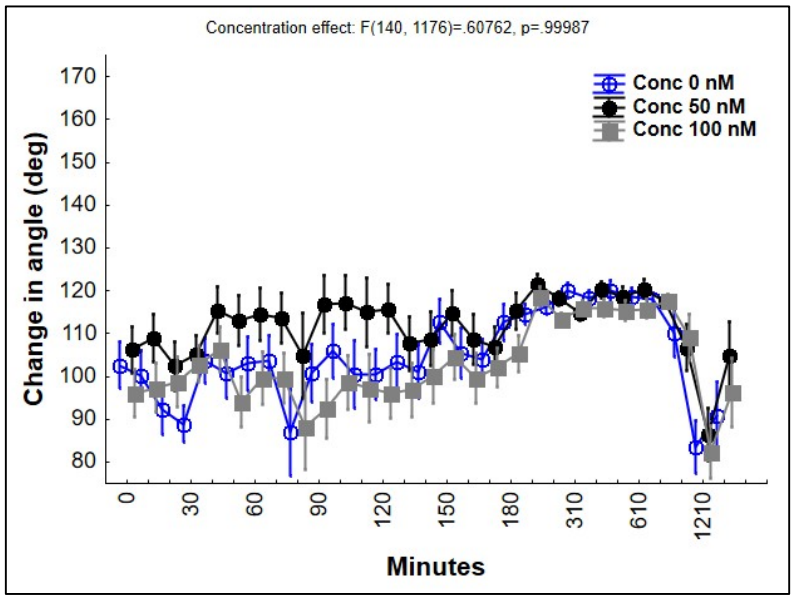

Figure 36: Time-dependent effects of TCC on mean angle *in D. rerio*

Metformin

Distance

There was a non-significant trend for metformin to elicit a concentration-dependent effect in *D. rerio* on maximum accumulated distance (P ~ 0.072) as shown in Figure 37. There was a decrease in maximum accumulated distance at the 0.01, 1, 10 and 100 µM

concentration. Figure 38 shows selected concentrations of metformin. There was not a significant time x concentration interaction effect of metformin on maximum accumulated distance (P>0.50). The 0.01 and 1 μM concentration have lower values for maximum accumulated distance earlier in the time course.

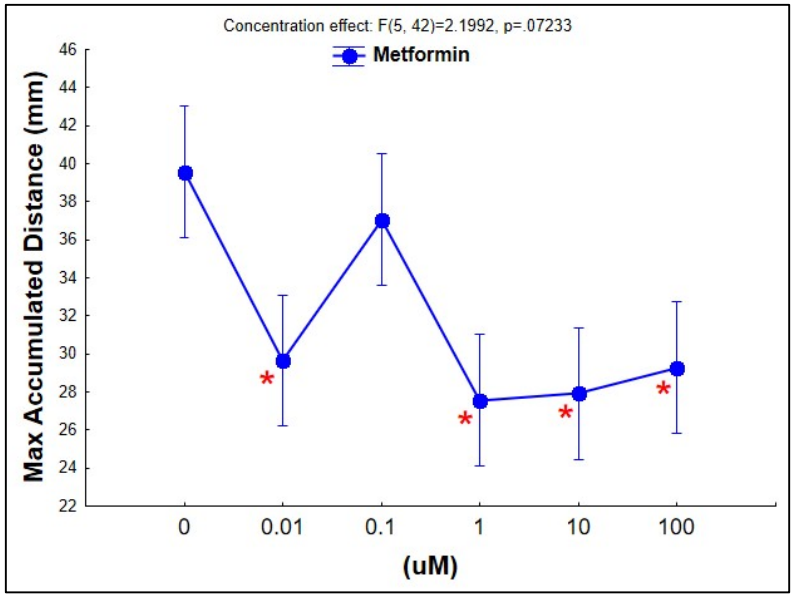

Figure 37: Effects of metformin on maximum accumulated distance *in D. rerio.* The LSD test indicated a significant difference between metformin treated animals and controls at the corresponding concentrations (* P<0.05).

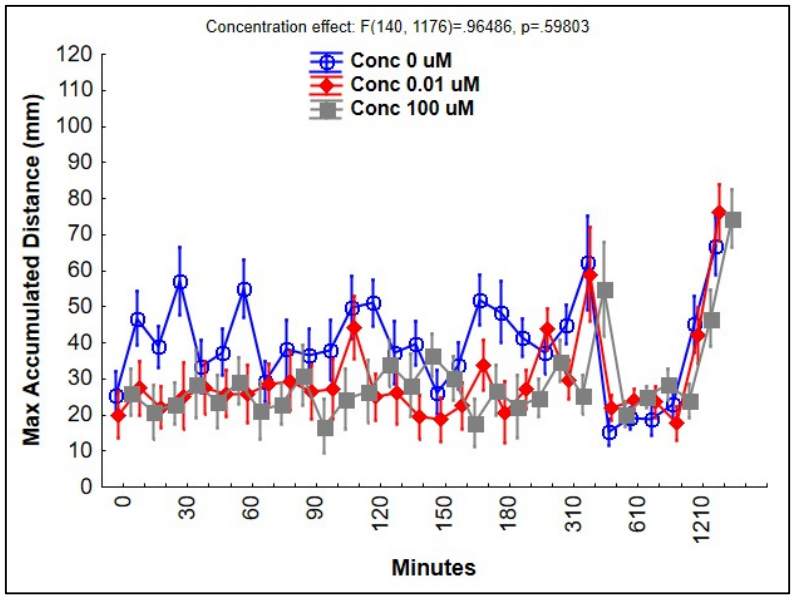

Figure 38: Time-dependent effects of metformin on maximum accumulated distance *in D. rerio*

Angle

As shown in Figure 39, the mean angle in *D. rerio* was increased significantly by metformin in a concentration-dependent manner ($P<0.05$). The time-dependent effects of concentration on mean angle are shown in Figure 40. The effect of concentration on mean angle was not dependent on time ($P>0.20$). However, it is clear that there is an increase in mean angle relative to control early in the time course, consistent with the results depicted in Figure 35.

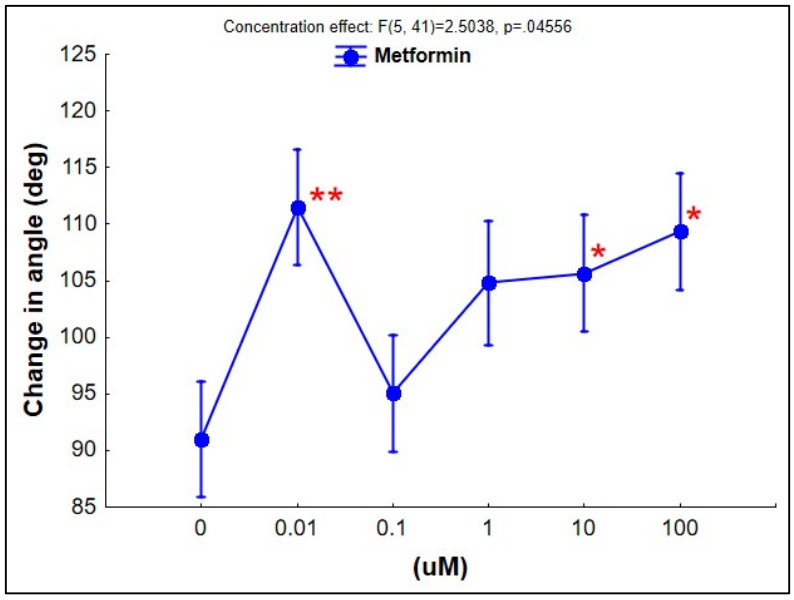

Figure 39: Effects of metformin on mean angle *in D. rerio.* The LSD test indicated a significant difference between metformin treated animals and controls at the corresponding concentrations (* P<0.05, ** P<0.01).

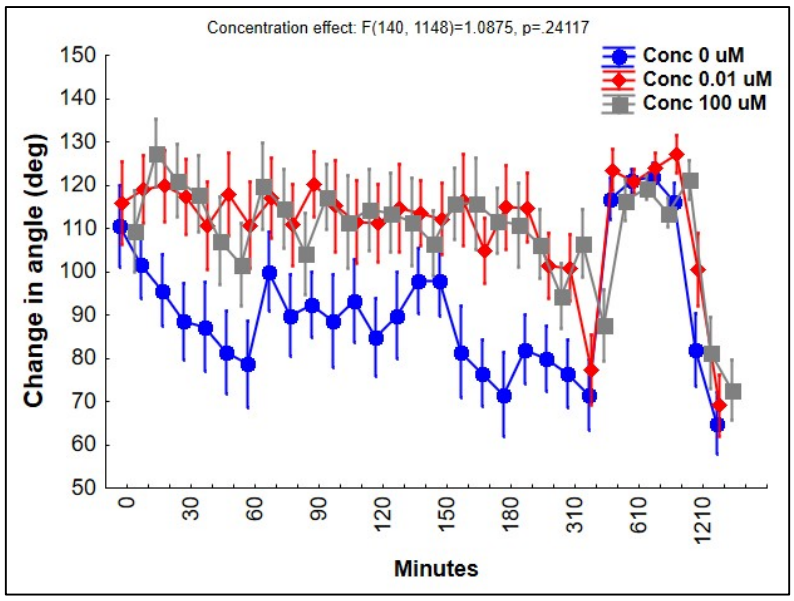

Figure 40: Time-dependent effects of metformin on mean angle *in D. rerio*

Estrone

Distance

Figure 41 shows a non-significant trend for concentration-dependent effect on maximum accumulated distance (P ~ 0.061) in *D. rerio* induced by estrone. The maximum accumulated distance for the 50 nM and 100 nM concentrations was higher than control. Although there was not a significant time x concentration interaction effect of estrone on maximum accumulated distance (P>0.50, see Figure 42), the 50 and 100 nM concentrations exhibit an increased maximum accumulated distance relative to control. This is consistent with behavioral stimulation during the early portion of the time course.

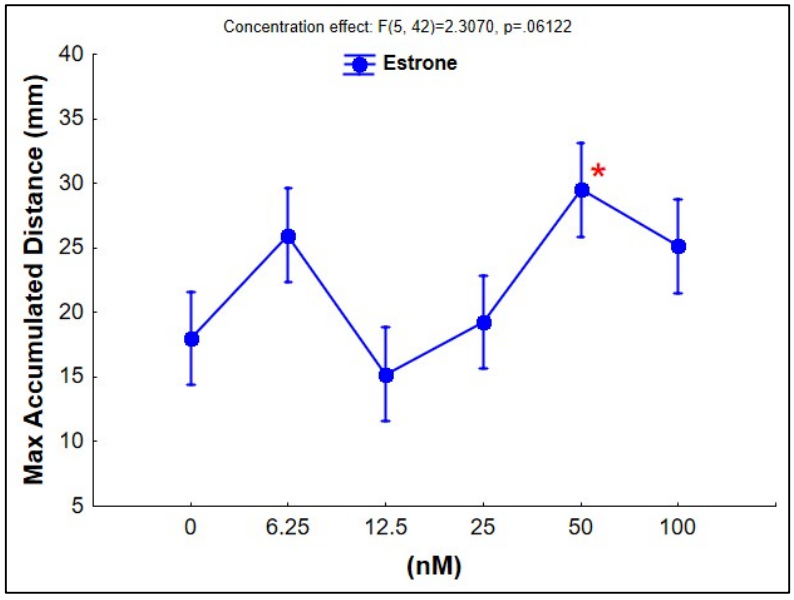

Figure 41: Effects of estrone on maximum accumulated distance *in D. rerio.* The LSD test indicated a significant difference between estrone treated animals and controls at the corresponding concentration (* P<0.05).

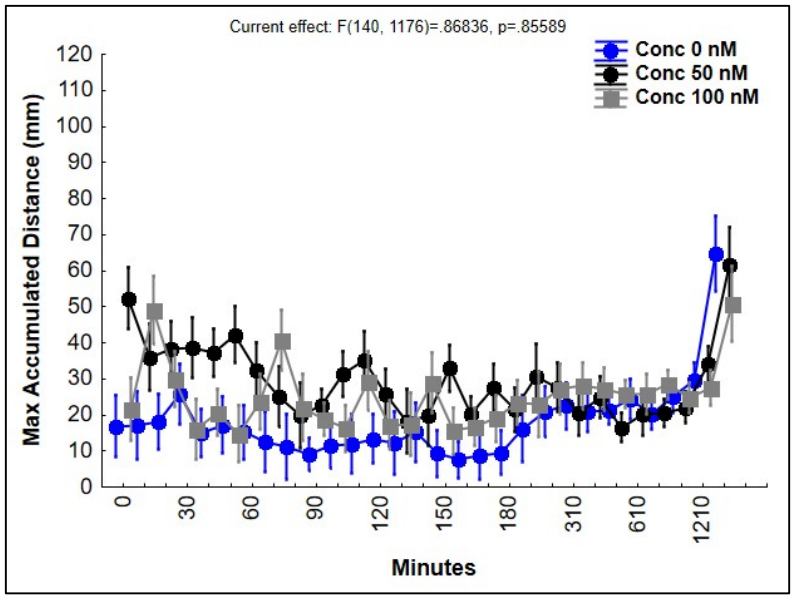

Figure 42: Time-dependent effects of estrone on maximum accumulated distance *in D. rerio*

Angle

The mean angle in *D. rerio* was significantly changed by estrone ($P<0.05$) as shown in Figure 43. The time-dependent effects of for 50 and 100 nM concentrations on mean angle are shown in Figure 44. The effect of concentration on mean angle over time was not significant ($P>0.50$). However, the mean angle values for 6.25, 50 and 100 nM estrone are lower than controls, and this is consistent with behavioral stimulation earlier in the time course (see Figure 40).

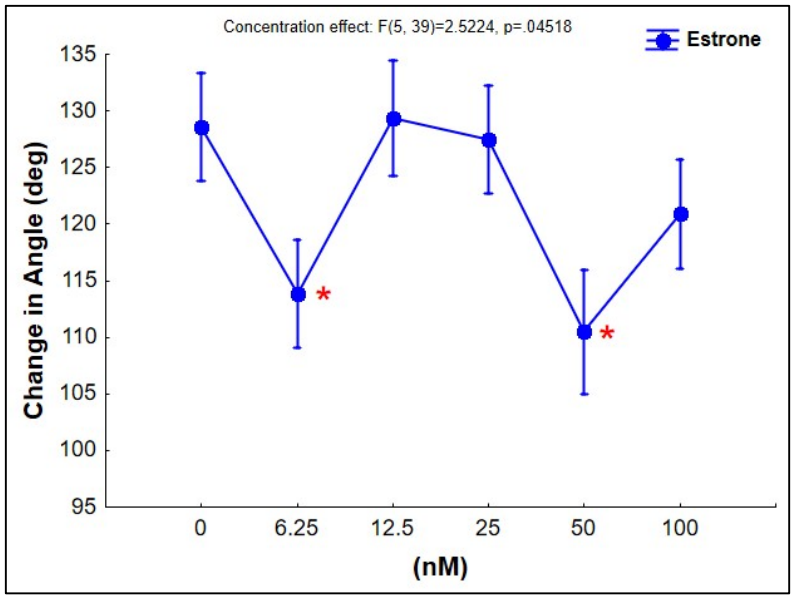

Figure 43: Effects of estrone on mean angle *in D. rerio.* The LSD test indicated a significant difference between estrone treated animals and controls at the corresponding concentrations (* P<0.05).

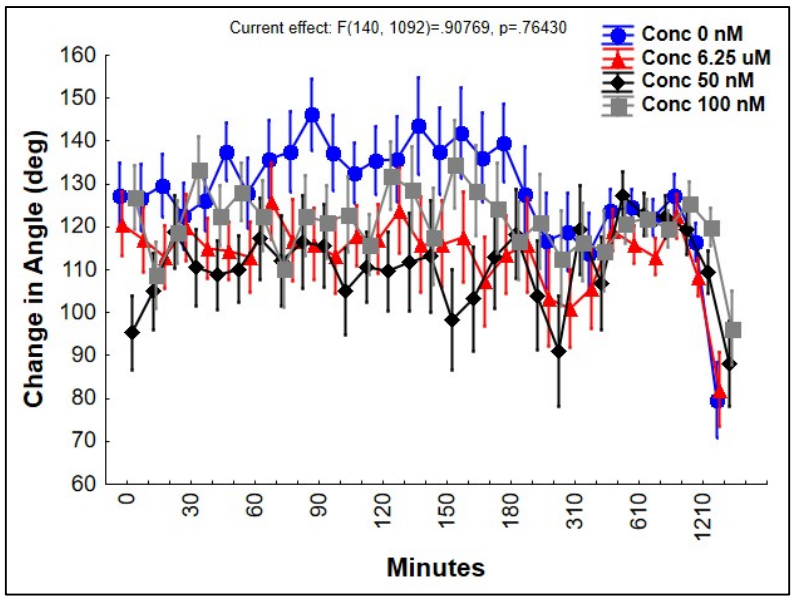

Figure 44: Time-dependent effects of estrone on mean angle *in D. rerio*

Comparison of behavioral studies between *Danio rerio* and *Daphnia pulex*:

TCS significantly stimulated *D. pulex* swimming behavior (+/-). However, the effect on *D. rerio* was a significant increase in maximum accumulated distance without a corresponding decrease in mean angle (+/0). The nM concentration range of TCC did not affect *D. rerio* or *D. pulex*. However, the µM concentration of TCC was lethal to *D.rerio*, but not *D. pulex*. TCC in the µM range did significantly increase mean angle of *D. pulex* with a trend to decrease maximum accumulated distance. There was a non-significant trend for metformin to decrease maximum accumulated distance and increase mean angle in *D. rerio* swimming behavior, but it did not affect *D. pulex* behavior. Similarly, estrone affected the behavior of *D. rerio* with significant effects on mean angle, but did not significantly affect *D. pulex* behavior.

Table 2: Summary of behavioral studies

EDC	***D. rerio*** **(concentration)**	***D. pulex*** **(concentration)**
TCS	+/0 (0.5μM-8μM)	+/- (0.5μM-8μM)
TCC	0/0 (6.25nM-100nM)	0/+? (6.25nM-100nM)
	Fatalities all concentrations: 1 Plate (0.5μM-8μM)	-?/+ (0.5μM-8μM)
Metformin	- ?/+ (0.01-100 μM)	0/0 (0.01-100 μM)
		0/0 (25 μM-400 μM)
Estrone	+ ?/- (6.25nM-100nM)	0/0 (6.25nM-100nM)

Maximum Accumulated Distance / Mean Change in Angle

(+): Increase activity

(-): Decrease activity

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

Chapter 5: Discussion & Conclusion

Summary of major findings

These behavioral studies of swimming behavior following exposure to potential EDCs provide the essential information to: (1) assess behavioral responsiveness over a specific concentration range, (2) determine the nature of the behavioral change (stimulation, inhibition, or alteration of turning behavior), (3) evaluate the concentration-dependent 24-hr survival rate, (4) provide a comparison of differences in response across species, and (5) provide a comparison of differences in response across chemicals. The results obtained will be critical for subsequent phases of the EDC project, which include the examination of morphological effects of specific chemical concentrations during development, followed by an assessment of these specific chemical concentrations on gene expression. Furthermore, the characterization of behavioral changes due to EDC exposure helps identify potential neurotoxic actions of the EDCs, which can be further explored, and will provide a context that is essential for evaluation of alterations in gene expression. For example, the similarity in EDC altered gene pathways associated with specific endocrine disruptive mechanisms (e.g., estrogenicity) may be more easily discerned when the comparisons among chemicals include EDCs that exhibit contrasting behavioral responses.

TCS clearly stimulated swimming behavior in *D. pulex*, with a significant increase in maximum accumulated distance and a corresponding decrease in turning (decrease in mean angle). However, in zebrafish TCS only significantly increased maximum accumulated distance. Therefore, TCS appeared to be stimulatory in both species, but

there were some qualitative differences in the behavior observed, with no effect on turning behavior in zebrafish.

TCS and TCC are both antimicrobials and fungicides with similar, dioxin-like structures, that have two benzene rings carrying multiple chlorines (Halden, 2014). However, the findings strongly suggest that there are significant differences in behavioral sensitivity and toxicity to exposure across these two species. In zebrafish, the lower concentration range (6.25nM - 100nM) of TCC did not elicit any significant behavioral effects, while the higher concentration range (0.5 μM - 8μM) was lethal to approximately 50% of fish (12 out 24). On the other hand, in *D. pulex*, both the lower and higher concentration ranges of TCC identified an alteration in turning behavior exhibited as an increase in turning in the absence of increased maximum accumulated distance, and this pattern achieved significance at the higher concentrations. It is possible that a toxic threshold may have been reached at the higher concentrations that might become more severe (e.g., affecting survival) if the duration of exposure were increased beyond 24 hours.

Metformin exposure caused a significant increase in turning behavior and a trend towards a decrease in maximum accumulated distance in the zebrafish, but *D. pulex*, behavior was not significantly affected even when the *D. pulex* was challenged at higher concentrations than the zebrafish. This result strongly suggests that the zebrafish are more sensitive to metformin than are *D. pulex*.

Estrone significantly inhibited the turning behavior in the zebrafish, but there was not a corresponding significant increase in maximal accumulated distance. There was no significant effect on behavior in *D. pulex* over the same concentration range. This

strongly suggests that there are significant species-dependent differences in sensitivity to the effects of this steroid hormone on behavior. This finding is consistent with the known differences between the endocrine systems of vertebrates and crustaceans like *D. pulex* (LeBlanc, 2007).

TCS

This study demonstrated that TCS causes significant stimulation of *D. rerio* swimming behavior at concentrations of (0.5μM - 8μM), observed mainly as a significant increase in maximum accumulated distance. Saley et al. (2016) showed that TCS can bioaccumulate and cause cardiac toxicity in zebrafish. When 8 to 120 hours post fertilization (hpf) zebrafish were exposed to concentrations of TCS ranging from 0.4 to 400 μg/L (0.001 to 1.38 μM), significant cardiac changes were observed. Pericardial edema occurred following exposure to 40 μg/L (0.14 μM) of TCS. Moreover, cardiac output was significantly reduced in embryonic zebrafish after exposure to 400 μg/L of TCS. A significant regurgitation effect in embryos was noted after exposure to as low as 0.4 μg/L of TCS. Therefore, acute exposure of developing fish to TCS elicited serious cardiac toxicity (Saley, Hess, Miller, Howard, & King-Heiden, 2016). Oliveira et al. (2009) found that TCS exposure in zebrafish larvae affected the levels of three different biomarkers: cholinesterase, lactic dehydrogenase, and glutathione S-transferase activity at 0.25mg/l or 0.86 uM) (Oliveira, Domingues, Grisolia, & Soares, 2009). The effect of TCS on cholinesterase suggests that a cholinergic mechanism is worth examining for a role in the stimulation of swimming behavior since cholinergic system modulates the swimming activity of both zebrafish (Eddins, Cerutti, Williams, Linney, & Levin, 2010) and *D. pulex* (Zein et al., 2014).

The fact that TCS was shown to bioaccumulate (Orvos, Versteeg, Inauen, Capdevielle, Rothenstein, & Cunningham, 2002) means that acute exposure at nominal concentrations may underestimate the toxicity following more chronic exposure. Some of these effects of TCS on development observed by Saley et al. (2016) may be the result of endocrine disruption, and the observation that TCS can bioaccumulate (Orvos et al., 2002) increases the potential for endocrine disruption. Ishibashi et al. (2004) has shown that TCS exposure in male medaka fish at 20 or 100 ug/Kg can cause the induction of vitellogenin (Ishibashi, Matsumura, Hirano, Matsuoka, Shiratsuchi, Ishibashi, Takao, & Arizono, 2004). Raut and Angus (2010) have also shown that TCS exposure can cause the induction of vitellogenin and decrease sperm production in male mosquito fish. Vitellogenin is a biomarker for endocrine disruption in the male fish since it is predominantly produced by females as an egg yolk precursor protein. The reduction in sperm production is consistent with an estrogenic or anti-androgenic effect of TCS (Raut & Angus, 2010).

In the present study, TCS (0.5μM - 8μM) exhibited the clearest examples of swimming behavior stimulation. In 2013, Peng et al. examined reproduction and growth effects on *Daphnia magna* following TCS exposure and found significant effects in a lower range of concentrations. They performed a chronic study that lasted 21 days where they exposed *D. magna* to 1-16 μg/L (0.003 μM – 0.06 μM) of TCS. They found a significant increase in the total number of neonates per female and increased body length. Higher concentrations of TCS 64–128 μg/L (0.22 μM – 0.44 μM), caused a significant decrease in neonates and decrease body length. Moreover, the total number of molting events per adult was decreased compared to controls at TCS concentrations of 16 ($p<0.05$), 64

($p<0.05$), and 128 µg/L (Peng, Luo, Nie, Liao, Yang, & Ying, 2013). It is possible that the studies by Peng et al. (2013) may be the result of TCS acting as an endocrine disrupter in *Daphnia,* with an outcome of a reduced number of offspring per female.

TCC

The lower concentrations of TCC used in the present study did not elicit any significant behavioral effects in zebrafish, while higher concentrations of TCC caused significant lethality. However, Wang et al. (2016) showed that TCC exposure can cause significant reproductive alterations in zebrafish. Exposure of sexually mature zebrafish (3–4 months old) to TCC resulted in reproductive impairment. After 21 days of exposure to 5 µg/L (0.016 µM) TCC, the growth of follicles was significantly affected in females, and retarded spermatogenesis and a low sperm count was observed in males (Wang, Du, Gao, Zhang, & Giesy, 2016). The effect of TCC on spermatogenesis and sperm count suggests that TCC has endocrine disrupting effects in zebrafish. In the present study TCC did not produce any significant effects on the swimming behavior of *D. pulex.* To the best of our knowledge this is the first report examining the behavioral effects of TCC.

Metformin

In a recent study by Banerjee et al. (2015), embryonic zebrafish were used as an *in vivo* model to complement *in vitro* studies focused on the molecular network underlying metformin action during Neural Crest (NC) cells formation. They exposed 2- and 1000-cell stage embryonic zebrafish cells to metformin concentrations from 10 to 50 mM. All of the metformin concentrations inhibited induction, migration, and differentiation of NC cells except for the 50mM concentration, which exhibited lower survival rate (1%-3%). Abnormal NC development causes many diseases including, fronto-nasal dysplasia,

DiGeorge syndrome and others. This study demonstrated the neurotoxic effects of metformin in zebrafish (Banerjee, Dutta, & Pal, 2016).

Since metformin is incompletely metabolized in humans, it can be found in WWTP effluents in Wisconsin, U.S., at concentrations ranging from 1 to 47 µg/L (0.006 µM – 0.285 µM). Niemuth et al. (2015), exposed adult fathead minnows (*Pimephales promelas*) to a similar concentration of metformin for 4 weeks. VTG, which is produced in the liver of females, was expressed at a significantly higher level in the metformin-treated males when compared with controls. This study concluded that there were significant endocrine disrupting effects of metformin in *Pimephales promelas.* The results of the current thesis show that zebrafish (*Danio rerio)* behavior can be significantly affected by metformin, too. To the best of our knowledge, there is no published data on the effects of metformin exposure on daphnia yet. There were no significant behavioral effects of meformin on *D. pulex* in this study, again demonstrating a species-dependent effect on behavior, with zebrafish being more sensitive than *D. pulex.*

Estrone

There are many reports in the literature demonstrating reproductive disruption upon exposure to estrogenic contaminants (Lee et al., 2013); (Preciados et al., 2016); (Xin, Susiarjo, & Bartolomei, 2015)). These effects include induction of VTG, decreased fertility, and feminization of male fish (Söffker & Tyler, 2012). Most of the literature agrees with this study's findings, that estrone causes behavioral changes in zebrafish in concentrations as low as 6.25 nano-molar concentration. In 2013, (Notch & Mayer) conducted a study of estrogen effects on embryonic zebrafish by exposing them to 100 ng/L of 17α-ethinylestradiol (EE2), 100 ng/L of estrone (E1), or a combination of EE2 and

E1 and sampled at 12, 24, 48, 72 and 96-hour post fertilization (hpf). At 24 hpf to 96 hpf, there was a statistically significant increase of plasma vitellogenin-1 (VTG1) mRNA in embryonic zebrafish that were exposed to both compounds. At 72 hpf, embryos exposed to only EE2 showed a significant increase in VTG1. Another example, is where Japanese medaka were exposed to estrogenic hormones in nanogram per liter concentrations and the exposure was associated with intersex morphology (i.e., testis–ova) in these fish (Metcalfe et al., 2001). Although estrone has been shown to bioaccumulate in *Daphnia* (Mezcua et al., 2004), evidence for estrogen signaling in Crustacea has not been reported (LeBlanc, 2007), and this is consistent with the lack of significant behavioral effects of estrone on daphnia at relatively high concentrations used in this study.

Conclusions

This study completes the behavioral component of the EDC project, with a focus on selected PPCPs suspected or known to be endocrine disrupting chemicals. EDCs are known to have multiple properties capable of activating multiple concentration-dependent mechanisms and can achieve varied effects on the endocrine system or other systems (e.g., see (Kenakin, 2009); (Oliveira et al., 2009). The need to screen for multiple chemical properties that can elicit multiple biological responses is necessary for the development of assay systems that can detect EDC activity (e.g., estrogenicity) and differentiate the identity of the responsible chemicals, a concentration-dependent fingerprint of possible biological activities. With the large number of EDCs found in aquatic systems, identification of the principle chemical entities responsible for endocrine disruption can be a challenge and a biological tool in the form of a multi-tiered assay system would be an asset complimenting analytical chemistry.

The series of experiments examining the effects of four chemicals, TCS, TCC, metformin, and estrone on the swimming behavior of two different aquatic species, zebrafish (vertebrate) and *D. pulex* (invertebrate), has demonstrated significant concentration-dependent differences in responses across the series of chemicals, between species for a given chemical (metformin, estrone), and similarities in response to a chemical by both species (TCS). In the next phase of the EDC project, morphological effects resulting from exposure to these two chemicals will be examined. This effort will be mostly focused on zebrafish because of the estrogen-based steroid hormone system associated with female vertebrates, but there will also be evaluation of selected chemicals such as TCS 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.

Appendix A

Stock Solution Calculations

Triclosan (TCS)

Stock solution of 50mM

$\text{Molecular weight of TCS} = 289.54 \text{ g/mol}$

$$50 \text{ mM} = \frac{16.8\text{mg}}{0.289\text{mg/ml}} \times \frac{1}{\text{volume (ml)}}$$

Volume= 1.16 ml of acetone

Concentrations studied:

[0, 0.5, 1.0, 2.0, 4.0, 8.0 µM] – *Daphnia pulex*

[0, 0.125, 0.25, 0.50, 1.0, 2.0 µM] – *Danio rerio*

Triclocarban (TCC)

Stock solution of 50mM

$\text{Molecular weight of TCC} = 315.58 \text{ g/mol}$

$$50 \text{ mM} = \frac{4 \text{ mg}}{0.315 \text{ mg/ml}} \times \frac{1}{\text{Volume (ml)}}$$

Volume= 0.25 ml of acetone

Concentrations studied:

[0, 6.25, 12.5, 25, 50, 100 nM] - *Daphnia pulex*, *Danio rerio*

[0, 0.5, 1.0, 2.0, 4.0, 8.0 µM] - *Daphnia pulex*, lethal in 50% of *Danio rerio*

Metformin

Stock solution of 10 mM

$\text{Molecular weight of metformin} = 165.62 \text{ g/mol}$

$$10 \text{ mM} = \frac{7 \text{ mg}}{0.165 \text{ mg/ml}} \times \frac{1}{\text{Volume (ml)}}$$

Volume= 4.24 ml of nano water

Concentrations studied:

[0, 0.01, 0.1, 1.0, 10, 100 µM] - *Daphnia pulex*, *Danio rerio*

[0, 25, 50, 100, 200, 400 µM] - *Daphnia pulex*

Estrone

Stock solution of 1mM

$$\text{Molecular weight of estrone} = 270.37\ \text{g/mol}$$

$$1\ \text{mM} = \frac{2\ \text{mg}}{0.270\ \text{mg/ml}} \times \frac{1}{\text{Volume (ml)}}$$

Volume= 7.40 ml of nano water

Concentrations studied:

[0, 6.25, 12.5, 25, 50, 100 nM] *Daphnia pulex*, *Danio rerio*

Appendix B

Daphnia pulex

Effect	TCS: ANOVA with repeated measures (Time) Dependent variable: Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	46048717	1	46048717	1112.530	0.000000
Conc code	624101	5	124820	3.016	0.020510
Error	1738422	42	41391		
TIME	1293378	28	46192	8.988	0.000000
TIME*Conc code	887110	140	6336	1.233	0.041395
Error	6043616	1176	5139		

Effect	TCS: ANOVA with repeated measures (Time) Dependent variable: Mean angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	6399461	1	6399461	1549.107	0.000000
Conc code	52322	5	10464	2.533	0.043134
Error	173505	42	4131		
TIME	169860	28	6066	16.869	0.000000
TIME*Conc code	45294	140	324	0.900	0.785570
Error	422909	1176	360		

Effect	TCC: ANOVA with repeated measures (Time) Dependent variable: Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	405522.9	1	405522.9	658.7844	0.000000
conc code	3255.2	5	651.0	1.0576	0.397312
Error	25853.6	42	615.6		
TIME	20492.6	28	731.9	11.8550	0.000000
TIME*conc code	10429.2	140	74.5	1.2067	0.059969
Error	72601.6	1176	61.7		

Effect	TCC: ANOVA with repeated measures (Time) Dependent variable: Mean angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	4524135	1	4524135	398.4440	0.000000
conc code	44701	5	8940	0.7874	0.564671
Error	476889	42	11355		
TIME	188422	28	6729	12.2469	0.000000
TIME*conc code	123978	140	886	1.6116	0.000026
Error	646183	1176	549		

Effect	Metformin (2 folds diff. high conc): ANOVA with repeated measures (Time) Dependent variable: Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	416162.0	1	416162.0	854.5284	0.000000
conc code	2933.3	5	586.7	1.2046	0.323656
Error	20454.3	42	487.0		
TIME	7982.8	28	285.1	3.3468	0.000000
TIME*conc code	10836.3	140	77.4	0.9086	0.762589
Error	100177.7	1176	85.2		

Effect	Metformin (2 folds diff. high conc): ANOVA with repeated measures (Time) Dependent variable: Mean angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	4124331	1	4124331	2264.622	0.000000
conc code	10096	5	2019	1.109	0.370288
Error	76490	42	1821		
TIME	92796	28	3314	6.393	0.000000
TIME*conc code	52950	140	378	0.730	0.990828
Error	609670	1176	518		

Effect	Metformin (10 folds diff. low conc): ANOVA with repeated measures (Time) Dependent variable: Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	322010.2	1	322010.2	2264.924	0.000000
conc code	628.4	5	125.7	0.884	0.500251
Error	5971.2	42	142.2		
TIME	10968.3	28	391.7	9.856	0.000000
TIME*conc code	5049.6	140	36.1	0.908	0.765539
Error	46739.8	1176	39.7		

Effect	Metformin (10 folds diff. low conc): ANOVA with repeated measures (Time) Dependent variable: Mean angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	7187988	1	7187988	2061.173	0.000000
conc code	10298	5	2060	0.591	0.707108
Error	146468	42	3487		
TIME	35383	28	1264	3.245	0.000000
TIME*conc code	55353	140	395	1.015	0.439001
Error	457955	1176	389		

Effect	Estrone: ANOVA with repeated measures (Time) Dependent variable: Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	370629.9	1	370629.9	3050.113	0.000000
Conc Code	1013.1	5	202.6	1.667	0.163602
Error	5103.6	42	121.5		
TIME	6573.2	28	234.8	5.261	0.000000
TIME*Conc Code	4745.0	140	33.9	0.760	0.980488
Error	52476.5	1176	44.6		

Effect	Estrone: ANOVA with repeated measures (Time) Dependent variable: Mean angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	3748245	1	3748245	4865.227	0.000000
Conc Code	5589	5	1118	1.451	0.226325
Error	32357	42	770		
TIME	37197	28	1328	3.971	0.000000
TIME*Conc Code	44356	140	317	0.947	0.652804
Error	393381	1176	335		

Danio rerio

Effect	TCS: ANOVA with repeated measures (Time) Dependent variable: Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	2675807	1	2675807	661.0771	0.000000
conc code	37467	5	7493	1.8513	0.123643
Error	170001	42	4048		
TIME	82848	28	2959	4.2910	0.000000
TIME*conc code	118794	140	849	1.2305	0.042887
Error	810919	1176	690		

Effect	TCS: ANOVA with repeated measures (Time) Dependent variable: Mean angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	13816782	1	13816782	1909.333	0.000000
conc code	28721	5	5744	0.794	0.560252
Error	303931	42	7236		
TIME	65458	28	2338	6.507	0.000000
TIME*conc code	53286	140	381	1.059	0.311061
Error	422499	1176	359		

Effect	TCC: ANOVA with repeated measures (Time) Dependent variable: Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	3369575	1	3369575	2211.766	0.000000
conc code	11930	5	2386	1.566	0.190593
Error	63986	42	1523		
TIME	52124	28	1862	4.639	0.000000
TIME*conc code	41389	140	296	0.737	0.988893
Error	471878	1176	401		

Effect	TCC: ANOVA with repeated measures (Time) Dependent variable: Mean angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	15742759	1	15742759	8360.695	0.000000
conc code	11238	5	2248	1.194	0.328756
Error	79084	42	1883		
TIME	86951	28	3105	17.194	0.000000
TIME*conc code	15364	140	110	0.608	0.999872
Error	212397	1176	181		

Effect	Metformin: ANOVA with repeated measures (Time) Dependent variable: Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	1410717	1	1410717	507.8838	0.000000
conc code	30543	5	6109	2.1992	0.072333
Error	116661	42	2778		
TIME	141250	28	5045	15.6792	0.000000
TIME*conc code	43461	140	310	0.9649	0.598026
Error	378367	1176	322		

Effect	Metformin: ANOVA with repeated measures (Time) Dependent variable: Mean angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	14392383	1	14392383	2361.553	0.000000
conc code	76298	5	15260	2.504	0.045559
Error	249873	41	6094		
TIME	198846	28	7102	19.686	0.000000
TIME*conc code	54923	140	392	1.088	0.241166
Error	414126	1148	361		

Effect	Estrone: ANOVA with repeated measures (Time) Dependent variable: Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	684530.5	1	684530.5	225.0142	0.000000
conc code	35090.7	5	7018.1	2.3070	0.061220
Error	127771.0	42	3042.2		
TIME	69009.5	28	2464.6	7.8185	0.000000
TIME*conc code	38322.3	140	273.7	0.8684	0.855889
Error	370709.3	1176	315.2		

Effect	Estrone: ANOVA with repeated measures (Time) Dependent variable: Mean angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	19116241	1	19116241	3614.690	0.000000
conc code	66699	5	13340	2.522	0.045180
Error	206251	39	5288		
TIME	78788	28	2814	7.164	0.000000
TIME*conc code	49910	140	356	0.908	0.764303
Error	428887	1092	393		

References

Agopian, A. J., Lupo, P. J., Canfield, M. A., & Langlois, P. H. (2013). Case-control study of maternal residential atrazine exposure and male genital malformations. *Am J Med Genet A, 161A*(5), 977-982. doi:10.1002/ajmg.a.35815

Baker, T. R., Peterson, R. E., & Heideman, W. (2013). Early dioxin exposure causes toxic effects in adult zebrafish. *Toxicol Sci, 135*(1), 241-250. doi:10.1093/toxsci/kft144

Balmford, A., & Bond, W. (2005). Trends in the state of nature and their implications for human well-being. *Ecol Lett, 8*(11), 1218-1234. doi:10.1111/j.1461-0248.2005.00814.x

Banerjee, P., Dutta, S., & Pal, R. (2016). Dysregulation of Wnt-Signaling and a Candidate Set of miRNAs Underlie the Effect of Metformin on Neural Crest Cell Development. *Stem Cells, 34*(2), 334-345.

Bradley, P. M., Journey, C. A., Button, D. T., Carlisle, D. M., Clark, J. M., Mahler, B. J., . . . VanMetre, P. C. (2016). Metformin and Other Pharmaceuticals Widespread in Wadeable Streams of the Southeastern United States. *Environmental Science & Technology Letters, 3*(6), 243-249. doi:10.1021/acs.estlett.6b00170

Eddins, D., Cerutti, D., Williams, P., Linney, E., & Levin, E. D. (2010). Zebrafish provide a sensitive model of persisting neurobehavioral effects of developmental chlorpyrifos exposure: comparison with nicotine and pilocarpine effects and relationship to dopamine deficits. *Neurotoxicol Teratol, 32*(1), 99-108. doi:10.1016/j.ntt.2009.02.005

Grassi, M., Rizzo, L., & Farina, A. (2013). Endocrine disruptors compounds, pharmaceuticals and personal care products in urban wastewater: implications for

agricultural reuse and their removal by adsorption process. *Environ Sci Pollut Res Int, 20*(6), 3616-3628. doi:10.1007/s11356-013-1636-7

Green, R. E., Newton, I., Shultz, S., Cunningham, A. A., Gilbert, M., Pain, D. J., & Prakash, V. (2004). Diclofenac poisoning as a cause of vulture population declines across the Indian subcontinent. *Journal of Applied Ecology, 41*, 793-800.

Halden, R. U. (2014). On the need and speed of regulating triclosan and triclocarban in the United States. *Environ Sci Technol, 48*(7), 3603-3611. doi:10.1021/es500495p

Ishibashi, H., Matsumura, N., Hirano, M., Matsuoka, M., Shiratsuchi, H., Ishibashi, Y., . . . Arizono, K. (2004). Effects of triclosan on the early life stages and reproduction of medaka Oryzias latipes and induction of hepatic vitellogenin. *Aquatic Toxicology, 67*(2), 167-179.

Kenakin, T. (2009). Quantifying Biological Activity in Chemical Terms: A Pharmacology Primer To Describe Drug Effect. *Chemical Biology, 4*(4), 249-260.

Key Issues: Toxic Chemicals - High Risk Issue. (2017). Retrieved from http://www.gao.gov/key_issues/toxic_chemicals/issue_summary

Kilham, S. S., Kreeger, D. A., Lynn, S. G., Goulden, C. E., & Herrera, L. (1998). COMBO: A defined freshwater culture medium for algae and zooplankton. *Hydrobiologia, 377*, 147-159.

Kolpin, D. W. (2017). Contaminants of Emerging Concern in the Environment Investigation. Retrieved from https://toxics.usgs.gov/investigations/cec/index.php

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., & Buxton, H. T. (2002). Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999−2000: A National

Reconnaissance. *ENVIRONMENTAL SCIENCE & TECHNOLOGY, 36*(6), 1202-1211. doi:10.1021/es011055j

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., & Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. *Environmental Science & Technology, 36*(6), 1202-1211. doi:Doi 10.1021/Es011055j

Kummerer, K. (2009a). Antibiotics in the aquatic environment - A review - Part I. *Chemosphere, 75*(4), 417-434. doi:Doi 10.1016/J.Chemosphere.2008.11.086

Kummerer, K. (2009b). The presence of pharmaceuticals in the environment due to human use - present knowledge and future challenges. *Journal of Environmental Management, 90*(8), 2354-2366. doi:Doi 10.1016/J.Jenvman.2009.01.023

LeBlanc, G. A. (2007). Crustacean endocrine toxicology: a review. *Ecotoxicology, 16*(1), 61-81. doi:10.1007/s10646-006-0115-z

Lee, H. R., Jeung, E. B., Cho, M. H., Kim, T. H., Leung, P. C. K., & Choi, K. C. (2013). Molecular mechanism (s) of endocrine-disrupting chemicals and their potent oestrogenicity in diverse cells and tissues that express oestrogen receptors. *Journal of cellular and molecular medicine, 17*(1), 1-11.

Metcalfe, C. D., Metcalfe, T. L., Kiparissis, Y., Koenig, B. G., Khan, C., Hughes, R. J., . . . Potter, T. (2001). Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (Oryzias latipes). *Environmental Toxicology and Chemistry, 20*(2), 297-308.

Mezcua, M., Gómez, M. J., Ferrer, I., Aguera, A., Hernando, M. D., & Fernández-Alba, A. R. (2004). Evidence of 2,7/2,8-dibenzodichloro-p-dioxin as a photodegradation product of triclosan in water and wastewater samples. *Analytica Chimica Acta, 524*(1), 241-247. doi:http://dx.doi.org/10.1016/j.aca.2004.05.050

Niemuth, N. J., Jordan, R., Crago, J., Blanksma, C., Johnson, R., & Klaper, R. D. (2015). Metformin exposure at environmentally relevant concentrations causes potential endocrine disruption in adult male fish. *Environ Toxicol Chem, 34*(2), 291-296. doi:10.1002/etc.2793

Notch, E. G., & Mayer, G. D. (2013). Impact of environmental estrogens on nucleotide excision repair gene expression in embryonic zebrafish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 157*(4), 361-365. doi:http://dx.doi.org/10.1016/j.cbpc.2013.03.004

Oaks, J. L., Gilbert, M., Virani, M. Z., Watson, R. T., Meteyer, C. U., Rideout, B. A., . . . Khan, A. A. (2004). Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature, 427*, 630-633.

Oliveira, R., Domingues, I., Grisolia, C. K., & Soares, A. M. V. M. (2009). Effects of triclosan on zebrafish early-life stages and adults. *Environmental Science and Pollution Research, 16*(6), 679-688. doi:Doi 10.1007/S11356-009-0119-3

Orvos, D. R., Versteeg, D. J., Inauen, J., Capdevielle, M., Rothenstein, A., & Cunningham, V. (2002). Aquatic toxicity of triclosan. *Environmental toxicology and chemistry, 21*(7), 1338-1349.

Peng, Y., Luo, Y., Nie, X. P., Liao, W., Yang, Y. F., & Ying, G. G. (2013). Toxic effects of triclosan on the detoxification system and breeding of Daphnia magna. *Ecotoxicology, 22*(9), 1384-1394. doi:10.1007/s10646-013-1124-3

Preciados, M., Yoo, C., & Roy, D. (2016). Estrogenic Endocrine Disrupting Chemicals Influencing NRF1 Regulated Gene Networks in the Development of Complex Human Brain Diseases. *International Journal of Molecular Sciences, 17*(12). doi:10.3390/ijms17122086

Rauh, V. A., Perera, F. P., Horton, M. K., Whyatt, R. M., Bansal, R., Hao, X., . . . Peterson, B. S. (2012). Brain anomalies in children exposed prenatally to a common organophosphate pesticide. *Proc Natl Acad Sci U S A, 109*(20), 7871-7876. doi:10.1073/pnas.1203396109

Raut, S. A., & Angus, R. A. (2010). Triclosan has endocrine-disrupting effects in male western mosquitofish, Gambusia affinis. *Environmental toxicology and chemistry, 29*(6), 1287-1291.

Rempel, M. A., & Schlenk, D. (2008). Effects of environmental estrogens and antiandrogens on endocrine function, gene regulation, and health in fish. *Int Rev Cell Mol Biol, 267*, 207-252. doi:10.1016/S1937-6448(08)00605-9

Saley, A., Hess, M., Miller, K., Howard, D., & King-Heiden, T. C. (2016). Cardiac Toxicity of Triclosan in Developing Zebrafish. *Zebrafish, 13*(5), 399-404. doi:10.1089/zeb.2016.1257

Shultz, S., Baral, H. S., Charman, S., Cunningham, A. A., Das, D., Ghalsasi, G. R., . . . Prakash, V. (2004). Diclofenac poisoning is widespread in declining vulture

populations across the Indian subcontinent. *Proc Biol Sci, 271 Suppl 6*, S458-460. doi:10.1098/rsbl.2004.0223

Söffker, M., & Tyler, C. R. (2012). Endocrine disrupting chemicals and sexual behaviors in fish–a critical review on effects and possible consequences. *Critical reviews in toxicology, 42*(8), 653-668.

Vandenberg, L. N., Colborn, T., Hayes, T. B., Heindel, J. J., Jacobs, D. R., Jr., Lee, D. H., . . . Myers, J. P. (2012). Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocr Rev, 33*(3), 378-455. doi:10.1210/er.2011-1050

Wang, P., Du, Z., Gao, S., Zhang, X., & Giesy, J. P. (2016). Impairment of reproduction of adult zebrafish (Danio rerio) by binary mixtures of environmentally relevant concentrations of triclocarban and inorganic mercury. *Ecotoxicol Environ Saf, 134P1*, 124-132. doi:10.1016/j.ecoenv.2016.08.026

Xin, F., Susiarjo, M., & Bartolomei, M. S. (2015). Multigenerational and transgenerational effects of endocrine disrupting chemicals: A role for altered epigenetic regulation? *Semin Cell Dev Biol, 43*, 66-75. doi:10.1016/j.semcdb.2015.05.008

Zein, M. A., McElmurry, S. P., Kashian, D. R., Savolainen, P. T., & Pitts, D. K. (2014). Optical bioassay for measuring sublethal toxicity of insecticides in Daphnia pulex. *Environ Toxicol Chem, 33*(1), 144-151. doi:10.1002/etc.2404

Zein, M. A., McElmurry, S. P., Kashian, D. R., Savolainen, P. T., & Pitts, D. K. (2015). Toxic effects of combined stressors on Daphnia pulex: Interactions between diazinon, 4-nonylphenol, and wastewater effluent. *Environmental Toxicology and Chemistry*. doi:10.1002/etc.2908

ABSTRACT

ENDOCRINE-DISRUPTING PROPERTIES OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS (PPCPS): AN EVALUATION USING AQUATIC MODEL ORGANISMS

by:

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Thousands of chemicals have introduced into the environment as a result of human activity since the industrial revolution, and the U.S. Government Accountability Office has estimated that as many as 1,500 new chemical entities are synthesized each year ("Key Issues: Toxic Chemicals - High Risk Issue," 2017). Many of these chemicals are now found in surface water and ground water and can have detrimental effects on environmental health and on human health. This anthropogenic contamination has resulted in the labeling of the diverse array of chemicals found in water, which are not routinely monitored or regulated, as contaminants of emerging concern or CECs. Some of the CECs of greatest concern are those capable of disrupting endocrine system function.

The endocrine system of humans and wildlife is designed to be very sensitive to endogenous signaling molecules we call hormones. Exogenous chemicals that can mimic

or augment the signaling by hormones are capable of disrupting the normal function of endocrine systems, and this subset of CECs has been called endocrine disrupting chemicals or EDCs. EDCs can have a broad range of deleterious impacts on biological function and can affect development and reproduction, and cause cancer. Although EDC effects have been reported in the literature, the number of newly released and existing chemicals in the environment underscores the need for better EDC detection tools. Some of the most commonly observed evidence for endocrine disruption in the environment comes from the observation of feminized male fish or altered sex ratios with fewer males downstream of wastewater effluent outfalls. Evidence strongly suggests that these EDC effects are due to the estrogenic and/or anti-androgenic influence of chemical contaminants in the water. Some of the known or suspected EDCs fall into the category of pharmaceuticals and personal care products (PPCPs).

We proposed that two aquatic model organisms, one invertebrate – *Daphnia pulex* (waterflea), and one vertebrate - *Danio rerio* (zebrafish), can be used to detect the estrogenicity and anti-androgenic effects of known or suspected PPCPs in water. The hypothesis was that known or suspected EDCs have detectable behavioral effects, and that the characterization of these behavioral effects, when combined with developmental and gene expression data, will enable the creation of a mathematical model that can identify chemicals contributing to the estrogenicity or ant-angdrogenic qualities of contaminated water. Furthermore, the study of the impact of EDCs on the behavior of two different aquatic organisms can increase the discriminating power of the behavioral results and broaden the application of these results to the assessment of potential ecological impact.

This study of behavioral effects is one component of a larger EDC project, and it focused on selected PPCPs suspected or known to be endocrine disrupting chemicals: estrone, triclosan (TCS), triclocarban (TCC), and metformin. A novel optical bioassay examined the effects of these four chemicals on the swimming behavior of the two different aquatic species. Significant concentration-dependent differences in responses were found across the series of chemicals and between species for a given chemical (metformin, estrone), and similar responses to one chemical were found for both species (TCS). 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 in the last phase of the project, 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. This new bioassay approach will compliment and expand the power of existing analytical chemistry techniques and enable more efficient evaluation of the complex issues associated with the contamination of aquatic systems by CECs.

Autobiographical Statement

I'm a Doctor of Pharmacy graduated from King Saud University, Saudi Arabia. During my childhood, my interest in pharmacy rose since my dad is a chemistry professor who taught me that everything surrounding us is a unique chemical formula. While continuing my education, my passion increased toward chemistry, mathematics, and biology which lead me to the doctor of pharmacy path.

My motivation to pursue higher education in Pharmaceutical Sciences came from my passion to continuously increasing my pharmaceutical knowledge and strengthen my research skills. My goal is to be a competent scientist contributing to global wellness, educating others entering this field, and providing patients with the benefits of the most up-to-date information in the field.

Dr. David Pitts's knowledge, experience in science and research and his love to keeping the environment safe made me eager to work with him. His lab is a multi-disciplinary environment conjoining pharmacology, toxicology, and environmental studies, adding that to my clinical pharmacy background, made it the ideal place for me to expand my knowledge and research skills.

After going through my Master's degree, my interest in science, environment, and research grew even bigger, which is pushing me towards pursuing a PhD degree.