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Contaminants Of Emerging Concern: Effects Of Known Neuroactive Agents, Antibiotics, And Chemically Uncharacterized Photodegredates On Behavior And Physiology Of Daphnia Pulex

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CONTAMINANTS OF EMERGING CONCERN: EFFECTS OF KNOWN NEUROACTIVE AGENTS, ANTIBIOTICS, AND CHEMICALLY UNCHARACTERIZED PHOTODEGREDATES ON BEHAVIOR AND PHYSIOLOGY OF *DAPHNIA PULEX*

by

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DEDICATION

I would like to dedicate this work to my parents, Vijay Matta and Priya Matta without whom I would not be where I am right now. They have always provided me with unconditional love and support with no expectations. I would also like to dedicate it to my best friend and brother, Prashant Matta and my young at heart grandmother, Kaushalya Matta. Last but not the least Akshay Jadhav

In loving memory of my grandfather, Sridhar Matta……

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Chapter 1: INTRODUCTION

"Earth provides enough to satisfy every man's needs, but not every man's greed." — Mahatma Gandhi

The level of impact of human activity on the environment has led to the idea of labeling the current epoch as "Anthropocene" (Zalasiewicz et al., 2010; Dirzo et al., 2014; Lewis et al., 2015; Corlett, 2015). Anthropogenic climate change and the dramatic decline in biodiversity are two of the leading reasons for considering the naming of an epoch according to the impact of a single species (Zalasiewicz et al., 2010; Dirzo et al., 2014; Lewis et al., 2015; Corlett, 2015). Intimately related to this impact of human activity on climate and the diversity of life is the alteration of the chemical composition of the biosphere that includes contamination beyond the greenhouse gases. The level of chemical contamination of the environment found now varies greatly from terrestrial superfund sites to the micro-contamination of aquatic ecosystems (Daughton and Ternes, 1999; Kolpin et al., 2002; Ela et al., 2011, Hutchinson et al., 2013).

The chemical contaminants found in the environment are not homogeneously distributed, and their dispersion is affected by their physical and chemical properties. These chemical contaminants can be found in air, soil (including sediment) and water. They may be dissolved in water or chemically or physically attached to soil particles or sediment. They can be found in small spaces between the soil particles, ground water, and aquifers.

Although there is significant chemical contamination affecting environmental health, the recognition of the magnitude of the problem is lagging well behind efforts to directly improve human health through biomedical research. We depend on the environment to provide us with fresh water, food, shelter and clothing, and chemical contamination can disrupt vital ecological services (Lake et al., 2012; Noyes et al., 2009). Sustaining human health requires a healthy environmental too.

There is an urgent need to understand the impact of environment contamination so that appropriate measures can be taken to preserve environmental health and human health. Population growth, industrialization and urbanization have depleted natural resources and lead to extensive air, soil and water contamination. Environmental health is intimately associated with human health and the impact of anthropogenic contamination on both environmental health and human health is poorly understood. The range of visible human impact on the natural environment is quite extensive, and the terrestrial impact from fossil fuel use, deforestation, mining, the spread of invasive species, and extensive loss of wildlife habitat can be readily observed. What is often less easily observed is the impact of anthropogenic contamination of the aquatic environment

Some of the main sources of chemical and microbial contaminates in aquatic systems include runoff from urban, industrial, and agricultural areas, mining operations, and raw untreated sewage and effluent from wastewater treatment plants (Kolpin et al., 2002; Fong et al., 2007; Kummerer, 2009; Mkandawire, 2013, Yager et al., 2014) . The use of pesticides, such as insecticides and herbicides, and antibiotics in the industrialized agricultural system and in the urban areas of the United States is extensive, and a large percentage of these chemicals end up in aquatic systems where they can affect non-target species. Additionally, many of these compounds or may pass through water treatment processes as parent compounds or transformation products (e.g. Ternes et al., 2004) and can threaten the health of humans as well as natural systems.

The diversity of chemicals now contaminating surface and ground water is vast and is rapidly increasing (Kolpin et al., 2002; Lapworth et al., 2012). Chemicals of concern include pharmaceuticals, hormones, pesticides, detergents, polycyclic aromatic hydrocarbons (PAH),

plasticizers, fire retardants, and many others (Kolpin et al.,2002). The original parent compounds that are released into the environment or into wastewater infrastructure can be further transformed through anthropogenic or natural processes into known or unknown transformation products (Kummerer, 2009; Sirtori et al., 2012; Kock-Schulmeyer et al., 2013). Even though we are still in the process of determining the extent to which known compounds have entered the environment as contaminants, there is much less known about the nature of transformation products, their impact and fate.

An understanding of the extent of chemical contamination of the environment has been greatly enhanced in recent years by the development of technology with the sensitivity to detect chemicals at concentrations much lower than previously possible. As advanced analytical methods, such as liquid chromatography-mass spectroscopy (LC-MS), made it possible for scientists and engineers to measure chemicals in the environment at very low concentrations, the extensive micro-contamination of air, soil and water by the chemicals we use has become more apparent. Chemical contaminants found in surface- and ground-water have been termed *contaminants of emerging concern* or CEC's. The U.S. Geological Survey Toxic Substances Hydrology Program defines CECs as follows:

"Contaminants of Emerging Concern" 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". (http://toxics.usgs.gov/regional/emc/).

The USGS carried out a seminal study using five newly developed analytical methods to measure the concentrations of 95 organic wastewater contaminants (OWCs) in water samples from 139 streams across 30 states in United States during 1999 and 2000, and the OCWs were found in approximately 80% of the sampled streams. 82 of 95 compounds were detected and they were associated with residential, agricultural and industrial settings (Kolpin et al., 2002). CEC's have been found in the environment worldwide and are generally found in the range of µg L^{-1} or ng L^{-1} (Kolpin et al., 2002; Kummerer, 2009; Hogenboom et al., 2009), but the amount of CEC's detected varies because of many factors such as geographical area (e.g., urban versus rural) population density, and regulatory policies.

There are a number of ways that the CEC's are released into the environment. These chemical contaminants are always subjected to dynamic changes in the environment which are associated with the principles of source, loading and fate. The main sources are industrial, agricultural and residential. Due to ubiquitous distribution in surface water and ground water, hydrologists use some of these chemicals as tracers for the impact of anthropogenic activity on water systems (e.g., caffeine, sweeteners). Although CEC's are often found in the ground and surface water in very low concentrations, they are being continuously loaded and can be found in a biologically active or inactive form. The chemicals may undergo many changes before, during or after entering the environment that result in changes in the solubility, polarity, toxicity and other properties of compound. These transformation products can be created by biotic processes in the environment, e.g., by bacteria or fungi, or by abiotic processes, such as hydrolysis or photo-oxidation (Kummerer 2009).

Even though these chemicals are present in low concentrations in the environment they can have adverse effects on organisms in the ecosystem. They have been shown to affect the normal development, life cycle, and behavior of a number of non-target aquatic organisms. There is evidence of endocrine disruption due to contaminant exposure in the environment. Natural and synthetic estrogen hormones in surface water have been associated with morphological changes in fish characterized as gonadal intersex, and the changed morphology is also associated with increased expression of vitellogenin (Debrow et al., 1998; Routledge et al., 1998). High concentrations of vitellogenin was found in the plasma of male white perched in the Lower Great Lakes region that were observed to be gonadal intersex, suggesting significant exposure to endocrine disruptors (Kavanagh et al., 2004). Pharmaceuticals in surface waters are also suspected of being capable of endocrine disruption. Antidepressants such as fluoxetine (Prozac), which can bioaccumulate, appear to be potential endocrine disruptors in fish (Mennigen et al. 2011). Fluoxetine has also been shown to stimulate reproduction in invertebrates (Flaherty and Dodson, 2005). Clofibric acid, a cholesterol lowering pharmaceutical, increases the proportion of male off spring in *Daphnia magna* (Flaherty and Dodson, 2005). Demasculinization and complete feminization has been observed in male African clawed frogs exposed to a very potent and commonly used herbicide, atrazine (Hayes et al., 2010), and this feminizing effect has been consistently found among other vertebrate classes (Hayes et al., 2011). Prenatal exposure to atrazine, an endocrine disruptor, has caused malformations of the male genitals such as hypospadias, cryptorchidism and small penis size in humans. (Agopian et al., 2012).

Although the evidence of endocrine disruption occurring within the environment due to anthropogenic contamination is on the rise, evidence of probable behavioral effects due to contaminant exposure is also now being reported. Oxazepam has been shown to alter the

behavior of fish at environmentally relevant concentrations (Brodin et al., 2013). Of particular relevance to humans, the prenatal exposure to chlorpyrifos, an organophosphate insecticide and emerging contaminant, has been associated with changes in brain morphology and neurological deficits (Rauh et al. 2012).

Since there is significant evidence of contaminants of emerging concerns having an impact on environmental as well as human health, improved methods for detecting biological effects are greatly needed. The waterflea, *Daphnia*, is an organism that has been commonly used for water quality testing and is considered a model organism for biomedical research (http://www.nih.gov/science/models/daphnia).. Most often the biological endpoint measured is immobility, an indirect measure of concentration-dependent lethality expressed as an EC_{50} value. While this approach has great utility, it likely underestimates the potential impacts of contaminants because sub-lethal effects are not evaluated. Recently Zein et al. (2014, 2015) have developed an optical tracking technique to evaluate sub-lethal effects of individual contaminants, contaminant combinations, or complex mixtures on *Daphnid* behavior.

Daphnia pulex is a freshwater zooplankton that is a very important in freshwater ecosystems. These animals are found in freshwater ecosystems throughout the world including the Great Lakes watershed in the USA (Carpenter 1987). *Daphnia* are considered to be a keystone species. If a keystone species is lost, the whole aquatic ecosystem is altered. (Flathery and Dodson 2005).

Daphnia feed on phytoplankton such as algae and also bacteria and protozoans. They are in turn a source of food for larger invertebrates and vertebrates in the food web such as hydra, salamanders, fish etc. (Tessier et al., 2000). Therefore, changes in life history of *Daphnia* can bring about an imbalance in the aquatic ecosystem and hence eventually affect freshwater

ecosystems upon which the human population depends, and this is one of the reasons why the EPA has developed standardized bioassays to assess the effects of water contaminants on *Daphnia*. In fact, a recent study has reported such changes in North American aquatic ecosystems due to the loss of *Daphnia* (Jeziorski et al., 2015).

Daphnia are very sensitive to changes in the environment such as availability of food, predation, water chemistry, oxygen level, temperature changes, etc. *Daphnia* also have developed specialized mechanisms to adapt to changes in the environment.. They undergo morphological changes and form helmet and teeth-like structures in the presence of kairomones, chemical hormones released by predators. In addition, *Daphnia* are capable of changing their physiology in response to stress. For example, low oxygen triggers production of Hb and gives a reddish color to the haemolymph and an up-regulation of anaerobic metabolism. Daphnia also respond to environment stressors by switching from asexual to sexual reproduction. (e.g., see Altshuler et al., 2011; Colbourne et al.,2011for review). These adaptations to environmental change are made possible through epigenetic regulatory mechanisms (Coors et al., 2004). Therefore, Daphnia are ideal for evaluating water quality and the toxicity of contaminants of emerging concern, since they are very sensitive to environmental stressors.

The standardized bioassays developed by the EPA take advantage of *Daphnid* sensitivity to environmental changes to evaluate water quality. They are designed to carry out traditional LC_{50} tests, where the focus is on estimating the lethal concentration that kills 50% or more animals. However, significant effects such as altered motor function, developmental changes, changes in life processes such as reproduction and growth can be observed at levels below the LC_{50} concentrations. (Zein et al., 2014; Dodson and Hanazato 1995).

Regulatory risk assessment generally focuses on single chemicals in a controlled environment whereas organisms are often exposed to complex mixtures (Pavlaki et al., 2011). Since chemical contaminants can interact and have additive, synergistic, or antagonistic effects (Altshuler et al., 2011; Zein et al., 2015) on biological systems there is a need for new assessment methods that can evaluate sub-lethal effects at low concentrations. Since aquatic organisms can be continually exposed to a number of water contaminants simultaneously, assay systems that can evaluate the large number of contaminants as single chemicals, combinations of specific chemicals, or poorly characterized complex mixtures are needed to assess the risk.

This study focuses on utilizing novel *Daphnid* optical bioassays to examine the toxicity of selected contaminants of emerging concern. The chemicals selected for this study are found in surface water. Chemicals that are known neuroactive agents were selected. The insecticide, diazinon, and the antidepressant, fluoxetine are known to have effects on specific neurotransmitter systems. Since *Daphnia* are known to have the target neurotransmitter systems for diazinon (cholinergic system) and fluoxetine (serotonergic system), but would be considered to be "non-target" organisms, it was of interest to characterize the behavioral effects in *Daphnia* resulting from exposure. Triclosan and triclocarban are antibiotics that are used in antibacterial soaps and a number of other products, and these chemicals and their transformation products resulting form photodegredation are commonly found in surface water. Therefore, it was of interest to evaluate the behavioral effects of chemicals that do not have any known biological targets within *Daphnia*.

Two different optical tracking assays were used to evaluate changes in *Daphnid* swimming behavior and *Daphnid* cardio-respiratory function as a result of exposure to diazinon, fluoxetine, triclosan, triclocarban and photodegredates of the two antibiotics.

Diazinon

Diazinon is an organophosphate insecticide. It is an acetylcholinesterase (AChE) inhibitor. AChE breaks down the acetylcholine (ACh) into choline and acetate. Inhibition of AChE results in excessive accumulation of ACh in the synapse and results in toxicity. Diazinon has been used in the United States since 1956. It was used for agricultural as well as household purposes. In 2002, it was outlawed for indoor uses and in 2004 for outdoor uses, such as gardening. It has since been used for agricultural purposes only. Diazinon products are available as dust, granules, liquid concentrate, seed dressings and cattle ear tags (EPA 2012). It can be found in the environment as a result of run-off from farming areas at concentrations of approximately 0.35ug/L (Koplin et al., 2002). It has been shown that there is a significant reduction in reproduction, survival rate, intrinsic rate of natural increase, mean carapace length observed in *Daphnia magna* exposed to diazinon (Sanchez et al., 2000)

Physostigmine

Physostigmine is a reversible acetylcholinesterase inhibitor. It has the same mode of action as the organophosphate insecticides, but does not covalently bind to AChE. It is used to treat glaucoma, Alzheimer's disease and has recently been used for treatment of orthostatic hypotension. To the best of our knowledge, physostigmine has not been identified as a contaminant of emerging concern. However, physostimine has been used extensively in pharmacological studies and serves as a good reference compound that shares the mode of action of many acetylcholinesterase insecticides (Zein et al., 2014).

Fluoxetine

Fluoxetine is an antidepressant frequently observed in surface waters downstream municipal wastewater treatment plant discharges. Fluoxetine is a serotonin reuptake inhibitor

and is classified as a selective serotonin reuptake inhibitor (SSRI). It is an anti-depressant that is highly prescribed under the brand name 'Prozac' (NDC Health, 1999). Fluoxetine is also prescribed to treat compulsive behavior as well as eating and personality disorders (Brooks et al., 2003, Kolpin et al., 2002). It is found in the concentration of approximately 0.012 – 1.4 µg/L in the surface water. Chronic exposure to fluoxetine has shown to significantly increase the number of offspring produced by an individual *Daphnid mother* in her lifetime (Flaherty 2005).

Triclosan and Triclocarban

These two antibiotics are biologically active compounds and are very soluble in water with low biodegradability, hence there is a risk of bioaccumulation in aquatic organisms (Wollenberger et al., 2000). Triclosan (TCS) and Triclocarban (TCC) are polychlorinated aromatic compounds which kill micro-organisms rapidly via a non-specific action (Halden 2014) Although there is literature on TCS and TCC, little is known about or their fate in the environment (Halden and Paull 2005) .

TCS is used as an antiseptic agent in many medical products, shampoos, deodorants, medicinal skin creams, dental products such as mouthwash and toothpaste and is most prevalent in soaps (0.10 – 1.00%) (Singer et al., 2002). The incorporation of TCS into a variety of products has resulted in widespread discharge into the environment from in wastewater treatment plants and into surface waters (Singer et al., 2002). According to the USGS survey carried out in 2002, triclosan has been found in the concentration of approximately 2.3 µg/L (Kolpin et al., 2002). At a relatively low exposure concentration (1 - 16µg/L) *Daphnia magna* are found to increase their rate of reproduction and in size (i.e. body length), whereas the opposite occurs when exposed to higher concentrations (64-128µg/L). In addition, the total number of times

molting occurred per animal was found to decrease when exposed to TCS (Peng et al., 2013). Like TCS, TCC is an antimicrobial agent that is also found in personal care and medical products. About 75% of liquid soaps and 29% of bar soaps manufactured in the United States contain TCC (Perencevich et al., 2001).

This thesis addresses two hypotheses:

(1) The anticholinesterase inhibitor and insecticide, diazinon, has significant effects on swimming behavior and cardiorespiratory function at environmentally relevant concentrations. A corollary to this hypothesis is that other anticholinesterase inhibitors have similar behavioral and cardiorespiratory effects. Physostigmine, which is an anticholinesterase that is well characterized in the literature will serve as a prototypical compound to compare to diazinon.

(2) The broad spectrum antibiotics, triclosan and triclocarban and their photo-degradative products have significant effects on swimming behavior and survival at environmentally relevant concentrations.

Chapter 2: MATERIALS AND METHODS

BEHAVIORAL ASSAY

To evaluate the sub-lethal effects of select emerging contaminants, optical tracking of *Daphnia pulex* was performed according to the method described by Zein et al. (2014).

Animals

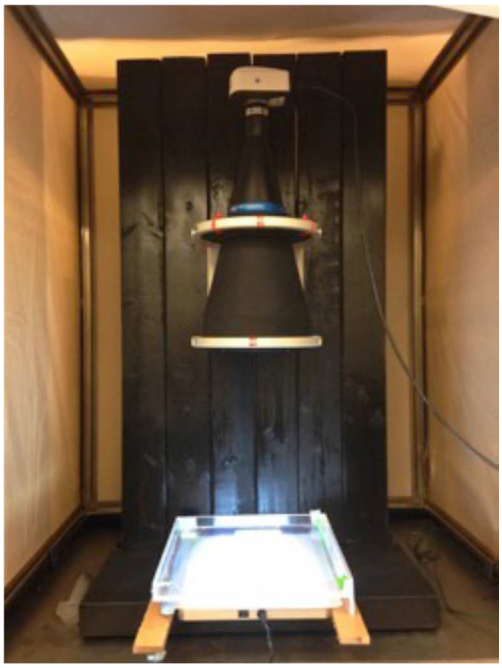
D. pulex utilized in the bioassay were collected from a pond at the Michigan State University Kellogg Biological Station in 2008. *Daphnia pulex* were cultured and maintained in 4L glass jars in an incubator at 21±0.5ºC. The incubator was illuminated to create a 16/8 hour light/dark cycles. The animals were fed 3 times a week with a 50/50 algae mixture of *Ankistrodesmus falcatus* and *Chlamydomonas reinhardii.* The animals were maintained in COMBO culture medium, which can support the growth of zooplankton (Kilham et al., 1998). The composition of COMBO is described below under Drugs, Chemicals and Solutions

24-Well Plate Setup

Daphnia were removed from the culture and passed through a mesh screen to select daphnia of uniform size (>1.4 mm) for the experiments. The selected daphnia were placed randomly in the 24-well plate (Corning Costar, Sigma-Aldrich). A single *daphnia* was placed into each well using an eyedropper. Each well held 3.5 ml of solution when full. Once the daphnia was placed in the well, the excess COMBO present in the wells was removed and discarded with the help of a narrow tip pipette, so as to prevent the dilution of added chemicals solutions. Solutions of different concentrations of the chemicals to be studied were placed randomly in the 24-well plate. The entire plate setup procedure required approximately 15 minutes.

Optical Tracking

Once the 24-well plate was prepared with the solutions and *Daphnia*, it was placed on a clear Plexiglas table with an LED light plate source underneath (Art Light). An Infinity 2-1M digital monochrome camera (Lumenera Corporation, Ottawa, Ontario) was used with a telecentric lens (Opto Engineering, Houston, TX). The setup used to record the swimming behavior of Daphnia is shown in Figure1A and 1B.



1A 1B

Figure1A: Camera, lens and 24 well plate setup for swimming behavior assay. 1B: 24 well plate with solutions and freely swimming Daphnia

A camera resolution of 1280 by1024 was used to capture the videos in an AVI format. Video files were analyzed using the 2D tracking module in Image Pro Premier 9 software (Media Cybernetics, Rockville, MD).

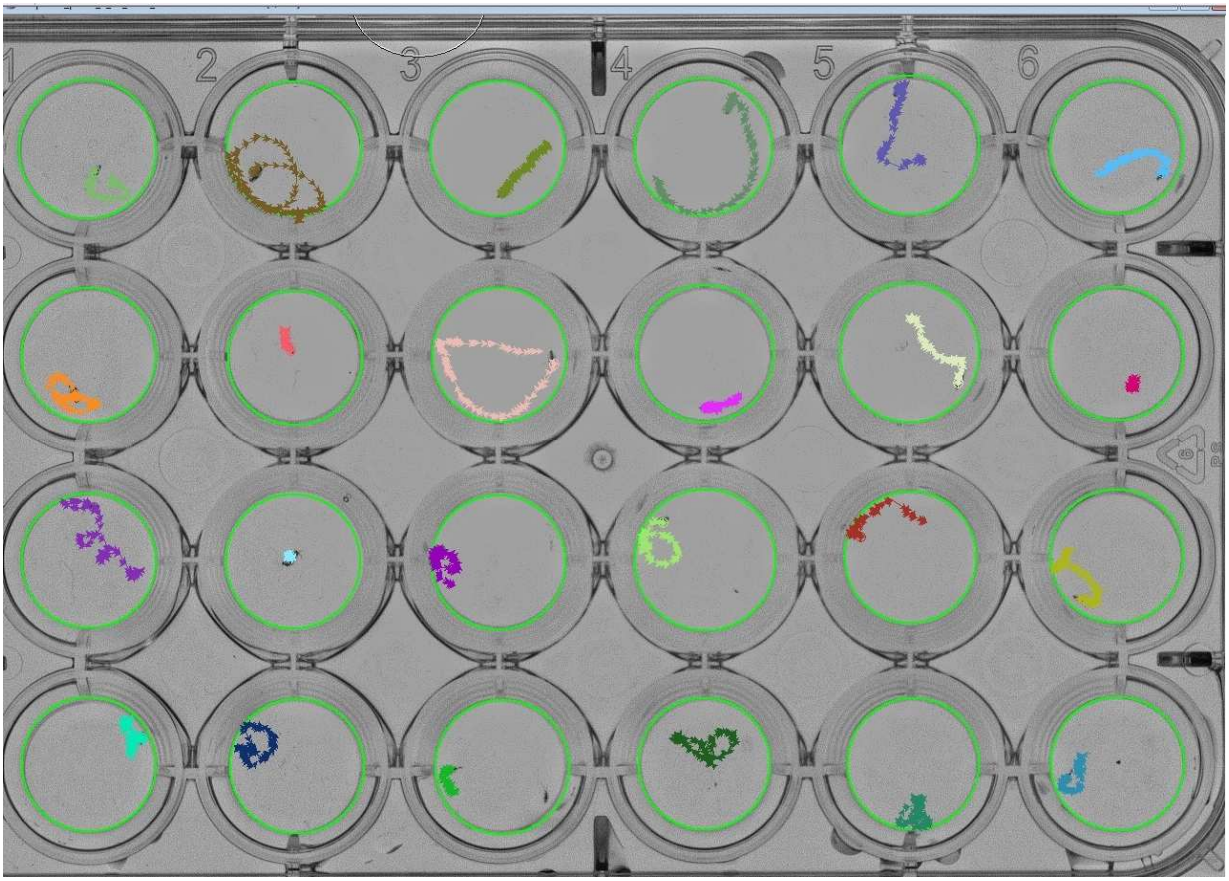


Figure 2

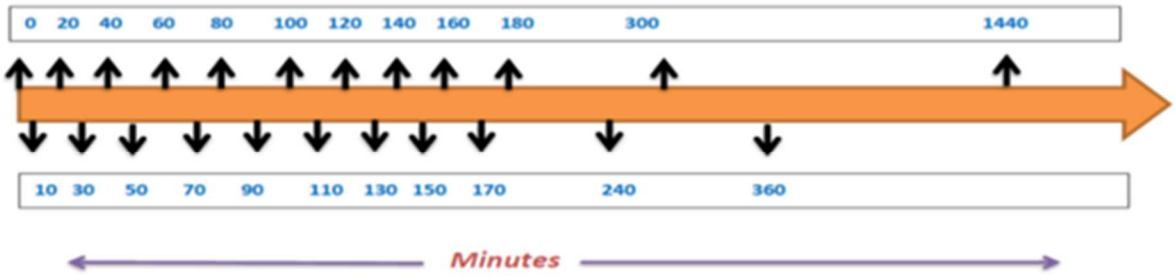
Figure 2: A video file that has been tracked for Daphnid movement. Each well contains one daphnia

Experimental Design

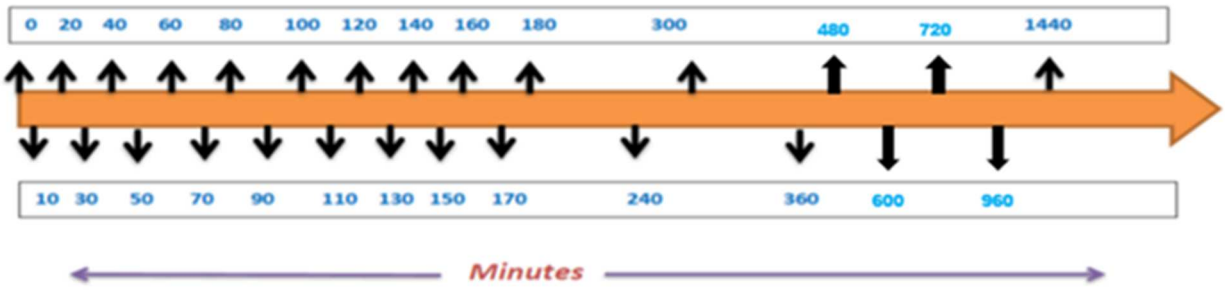
In a typical experiment, 5 second videos were recorded every 10 minutes for 180 minutes, then every hour for the next three hours and at the 24th hour. Therefore at the end of each experiment Daphnia were exposed to each chemical for 24 hours. Every 5 second

recording consisted of a total of 145 frames that were used to track and quantify *Daphnia* movement.

The two parameters used to quantify *Daphnia* swimming movements were maximum accumulated distance and mean angular change. Accumulated distance was measured by the summing the distance covered between two frames over the course of the 5 sec video (145 frames). The angular change was measured by comparing the change in direction of movement over the course of 145 frames. Rigorously, angular change is based on the change in the vector (the direction of movement that occurs between frames) across three frames (see Zein et al., 2014 for more details). The change in angle is reported as the mean angle for the 5 sec video. Fig3A and Fig3B illustrates the timeline used for the experiments



A) known biological targets



B) unknown biological targets

Figure 3A: Timeline for experiments for Contaminants of emerging concern with known biological target. **3B**: Timeline for experiments for Contaminants of emerging concernwith unknown biological targets

Statistical Analysis

Statistica (Version10, Tulsa, OK, USA) was used for statistical analysis. A repeated measures of analysis of variance (ANOVA) with time as the repeated measure was used to evaluate swimming behavior. The dependent variables were Accumulated Distance (mm) and Mean Angle (degrees). The independent variables were parameters (Maximum Accumulated Distance and Mean Angle), chemical, and concentration. A three-way ANOVA was used to evaluate the behavioral results from exposure to TCS and TCC. The dependent variables were accumulated distance and mean angle. The independent variables were chemical (COMBO/TCS/TCC), concentration (low, high) and time (0 to 90 minutes). The least significant difference (LSD) post-hoc test was used for multiple comparisons following ANOVA. In selected cases contrast analysis was also used to compare a series means across groups following ANOVA.

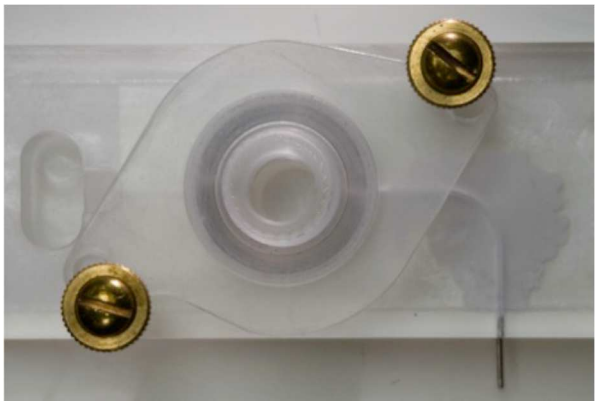
PHYSIOLOGICAL ASSAY

Aquatic Chamber:

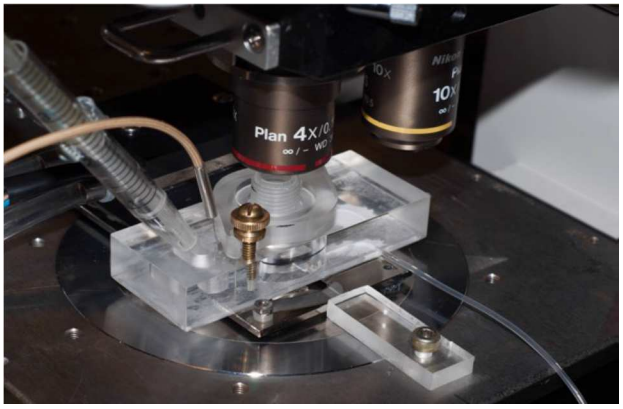
A rectangular 8.0cm x 3.2cm x 1.5cm (LWH) aquatic chamber was custom built from a 1.5cm thick Plexiglas by the College of Engineering machine shop, Wayne State University. A 2cm hole drilled in the middle of the rectangular Plexiglass base and the bottom was sealed with a regular rectangular borosilicate microscope glass slide. Two 23-gauge stainless steel hypodermic tubes were used as an inlet and outlet to the cylindrical chamber for the perfusion of solutions. A small collection well near the outlet was used to collect the solutions exiting the chamber and measure water temperature using a temperature probe (Physitemp Instruments Inc., Clifton, NJ). A Plexiglass top with a threaded cylindrical plunger was used to seal the perfusion chamber. The cylindrical plunger used a round rubber gasket and circular cover glass

to complete the seal. The chamber was placed on the microscope stage on top of a TS-4SPD heating and cooling stage (Physitemp Instruments, Inc.).

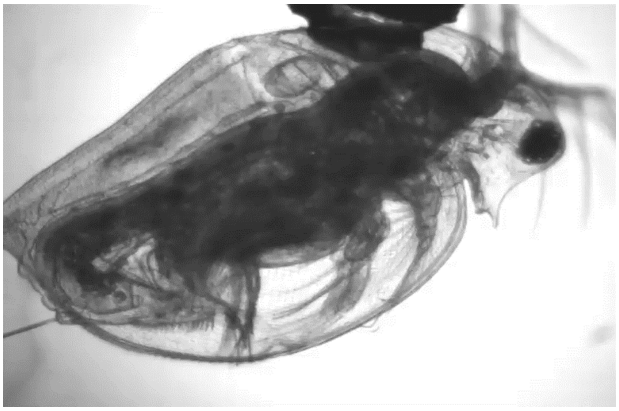
4A



4B



4C



4D

Figure 4A: Side view of the aquatic chamber. **4B:** Top view of the aquatic chamber. **4C:** Aquatic chamber with setup, microscope, temperature probe, inlet and outlet tubes. **4D:** Daphnia on a pin

Animal Preparation:

An adult *Daphnia* was isolated and glued on the tip of 33 gauge stainless steel tubing which was 10 mm in length using <1nL of cyanoacrylate glue. The stainless steel tubing was glued to the dorsal side of the head between anterior of the heart and the posterior to the eye in parallel to the anterior-posterior body axis. The 33 gauge tubing holding the animal was placed into a 26 gauge tube fixed inside the aquatic chamber to place the animal in a consistent viewing position. The chamber was filled with COMBO and then was closed with a top chamber-viewing insert. The animals were able to freely move their swimming antennae and appendages once inside the chamber. Each animal was observed under a 4x Nikon microscope objective. The animal's heart, appendages, swimming antennae, eye movement and gut were clearly visible through the translucent exoskeleton.

Experimental Design:

10 sec video recordings were recorded every 10 minutes using an Infinity 2M-1 monochrome camera (Lumenera Corporation, Ottawa, Ontario). The video file was analyzed for motor activity using the 2D tracking module from Image Pro Premier 9 software (Media Cybernetics, Rockville, MD). Animal movement was analyzed by measuring density-intensity changes within an area of interest (AOI). The oscillating movement of the heart wall or appendages through this defined AOI was used to calculate beat rate per minute.

Optical Tracking

At the beginning of the experiment COMBO was perfused through the chamber for 30 minutes at a rate of 10μL/min at 20ºC. This temperature was close to that of the culture in the incubator. Temperature was then reduced to 15ºC after 30 minutes and perfused with COMBO continued for one hour at the rate of 10μL/min. When drugs or chemicals were delivered to the chamber the pump rate was increased to 250μL/min for approximately 2.5 minutes and then

returned back to 10µL/min. The duration of drug or chemical application depended on the timing of the responses observed and occurred over a period of 1- to 4-hours.

Statistical Analysis

The statistical analysis was carried out using Statistica software (Version10, Tulsa, OK, USA). A repeated measures ANOVA with time as the repeated measure was used to analyze motor activity. The dependent variables were HR and ABR. The independent variables were parameter (HR/ABR) and concentration. In selected cases contrast analysis was used to compare a series of means across groups after ANOVA.

SURVIVAL ASSAY

The survival assay was carried out for a period of 5 days. The *Daphnia* were selected for these experiments using a screen to filter by size and were approximately 1-day old. On exposure Day-0, different concentrations of the solution to be tested were prepared in EPA water (http://water.epa.gov/scitech/methods/cwa/wet/upload/2007_07_10_methods_wet_disk2_atx7-10.pdf). . Daphnia were temporally collected in a watch glass until a total of five were obtained which were then transferred as a group into a 150 ml beaker containing a test solution. There were 4 replicates of each test solution, and therefore a total of 20 animals were used for each test solution. To minimize light exposure, the beakers were placed into an open lab drawer and covered with foil (shiny side out) to ensure darkness because the compounds are photo-sensitive but to also sufficient air-flow. Temperature was monitored by a thermometer kept in the lab drawer. On Day-1, each beaker was checked for number of surviving *Daphnia* and the survival number was noted as well as the room temperature. On day-2 the solutions were changed. Two hours prior to changing the solutions, the *Daphnia* were fed with reconstituted YTC mix (Carolina biological, Burlington, NC,). A few drops of the mix was gently pipetted into each

beaker. Once the *Daphnia* were allowed to feed for 2 hours, they removed from the beaker and placed on a watch glass and the number of surviving *Daphnia* was noted. The beakers were cleaned with a few drops of EPA water to remove any food residue at the bottom of the beaker and refilled with fresh EPA water. Once the *Daphnia* are placed back into the beaker the watch glass was rinsed with methanol followed by EPA water and then wiped down to avoid any cross-contamination. On Day-3 and Day-4, the number of surviving daphnia in each beaker and room temperature was recorded.

Drugs, Chemicals, and Solutions

Stock solutions of each chemical as well as the dilutions were made on the same day as the experiments performed. All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The drugs, physostigmine (10mM stock solution) and fluoxetine(10mM stock solution), used in this study were directly dissolved in COMBO except for diazinon. A 10 mM diazinon stock solution was made in acetone and then test solutions were made in COMBO using a serial dilution technique. The highest concentration of diazinon used contained 0.002% acetone. All of the control solutions in diazinon experiments also contained 0.002% acetone to nullify if there is any effect of acetone . Physostigmine and fluoxetine were stored at room temperature. Diazinon was stored in a cool and dark place. The stock solution for parent compounds and photolyzed triclosan and triclocarban (10mM) were made in methanol and the dilutions were prepared in EPA moderately hard water. Parent triclosan and triclocarban were placed in a foil packet in amber colored bottles at room temperature, whereas the photolyzed compounds were covered with foil and kept in a cool and dark place.

Chapter 3: Contaminants with known targets

RESULTS

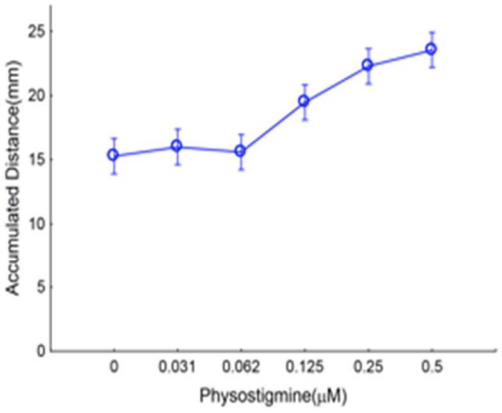
Physostigmine

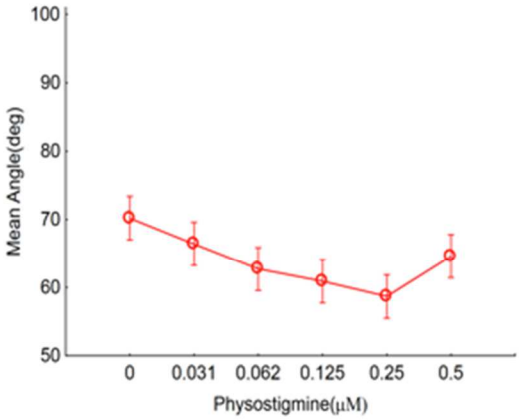
Behavioral analysis

Figure 5A illustrates the effects of physostigmine on accumulated distance. A significant stimulatory effect of physostigmine on swimming behavior was observed as a concentration-dependent increase in accumulated distance ($F_{5,108}$ = 6.96, P<0.001), with the accumulated distance for the 0.125, 0.25 and 0.5µM concentrations significantly greater than the 0µM control (LSD test, P<0.05 for all cases). The effect of physostigmine on mean angle is illustrated in Figure 5B. A non-significant decrease in mean angle was also elicited by physostigmine, with the lowest mean value observed at a concentration of 0.25µM.

The time-course for the effects of physostigmine on accumulated distance is illustrated in Figure 6A. Physostigmine elicited a significant concentration-dependent effect over the time-course on accumulated distance (concentration x time interaction, $F_{115, 2484}$ = 1.99, P<0.001). Contrast analysis for the 60 to 240 minute time interval indicated that the accumulated distance for the 0.125, 0.25 and 0.5µM concentrations were significantly greater than the 0µM control (P<0.05 in all cases). The highest concentration, 0.5µM, eventually caused a significant depression in accumulated distance relative to control at the 6- and 24-hour time-points (LSD test, P<0.05 in both cases). Visual observations of the video recordings at 6-and 24-hours indicated that 14 out of 19 and 16 out of 19 of the animals were immobilized by the 0.5µM concentration respectively. Figure 6B, C, and D separately illustrate the effects of each of the concentrations on accumulated distance relative to control

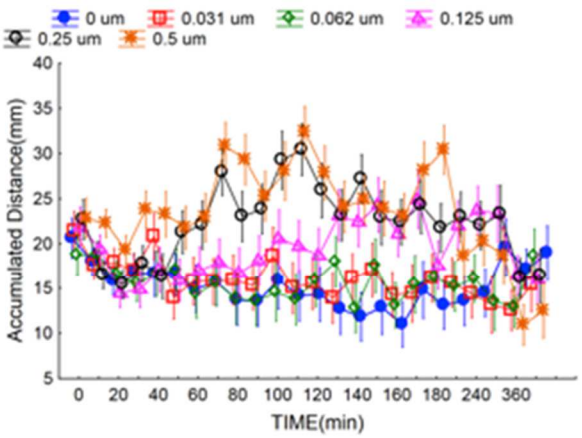
The time-course for the effects of physostigmine on mean angle is illustrated in Figure 7A. Physostigmine elicited a significant concentration-dependent decrease in mean angle over time (concentration x time interaction, $F_{115, 2482} = 1.43$, $P<0.005$). The 0.125, 0.25 and 0.5µM concentrations were significantly lower than the 0µM control for the 60 to 240 minute time interval (contrast analysis, $P<0.05$ in all cases). The highest concentration, 0.5µM elicited a significant increase in mean angle at the 6- and 24-hour time-points (LSD test, $P<0.05$ in both cases) Figure 7B, C, and D separately illustrate the effects of each of the concentrations on mean angle relative to control



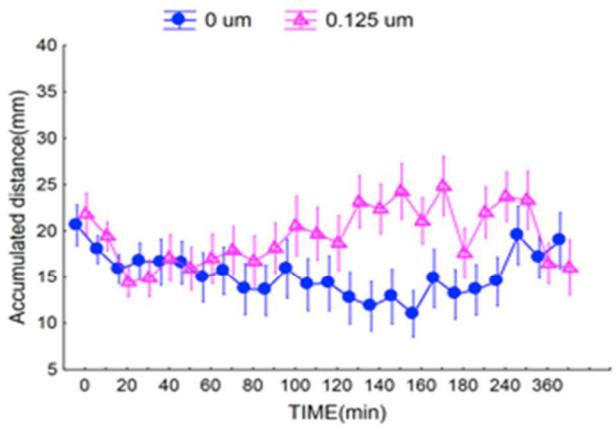


5A 5B

Figure 5: Behavioral response of *Daphnia pulex* to physostigmine. Error bars are the standard error. **5A**: Effects of physostigmine on accumulated distance. **5B**: Effects of physostigmine on mean angle.



6A



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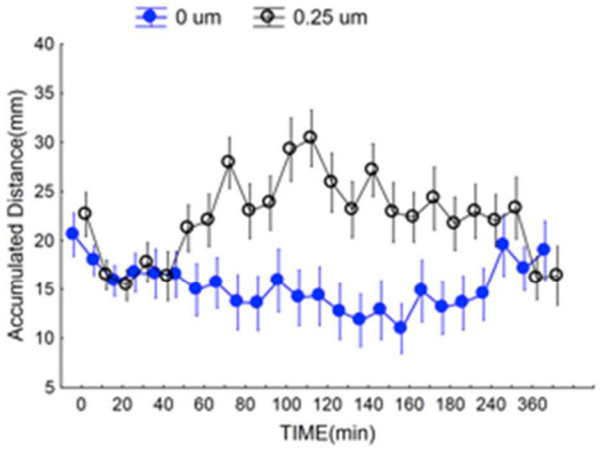
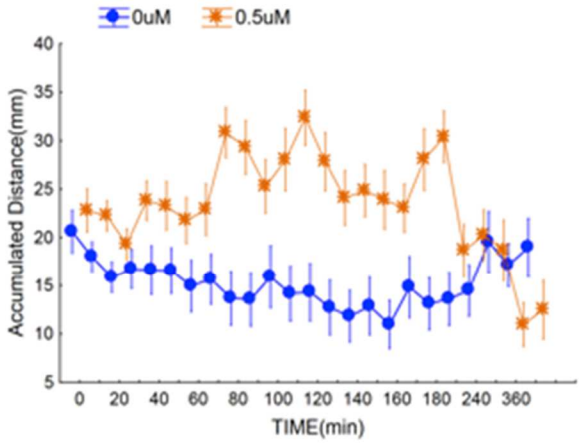
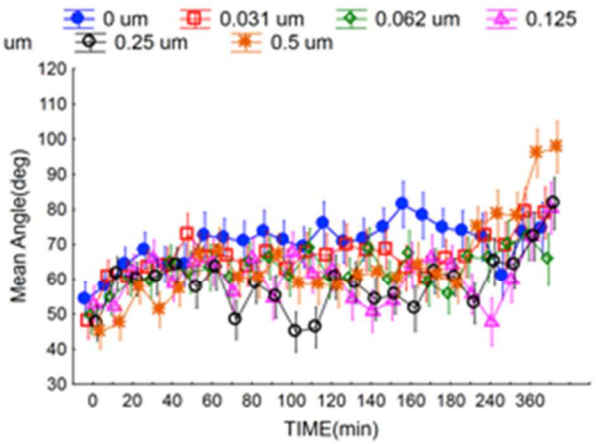


6C

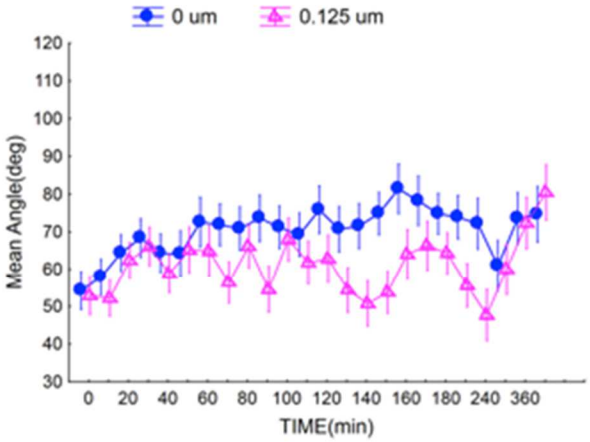


6D

Figure 6A: Time-dependent effects of physostigmine on accumulated distance. Error bars are the standard error. **6B**: Effect of 0.125 µM on accumulated distance with respect to control. **6C:** Effect of 0.25µM on accumulated distance with respect to control. **6D**: Effect of 0.5µM on accumulated distance with respect to control



7A



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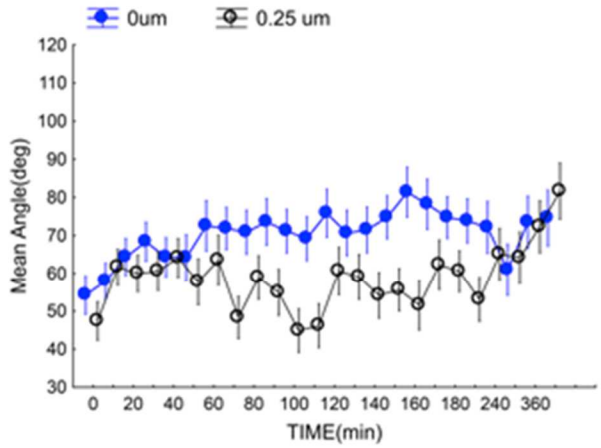
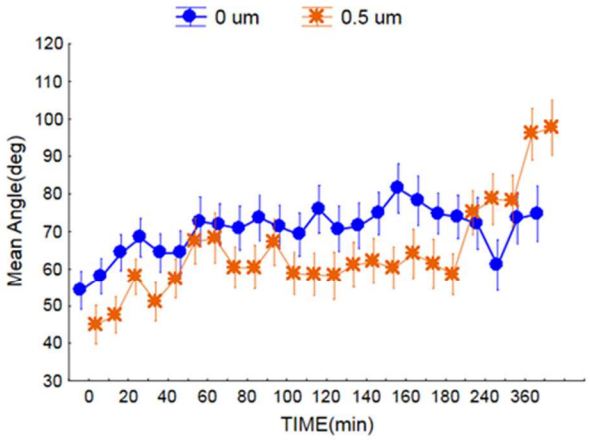


7C



7D

Figure 7A: Time-dependent effects of physostigmine on mean angle. Error bars are the standard error. **7B**: Effect of 0.125 µM on mean angle with respect to control. **7C:** Effect of 0.25µM on mean angle with respect to control. **7D**: Effect of 0.5µM on mean angle with respect to control

Physiological Analysis

Physostigmine elicited a significant concentration- and time-dependent effect on HR and ABR, which is depicted in Figure 8 (Concentration x Time x Parameter effect, P<0.001). The 0.5 µM concentration did not elicit any significant effects (contrast analysis, P>0.40 for both parameters), but the 1 µM and 2 µM concentrations completely suppressed ABR after 60 min of exposure (contrast analysis, p<0.001 in both cases). There were small, but non-significant decreases in HR for the 1 µM and 2 µM concentrations (contrast analysis, P>0.10 in both cases). It should be noted that the reduction in ABR observed most often exhibited an irregular pattern, with intermittent pauses in the movement of the appendages followed by resumption of activity at a lower level until activity ceased. A partial recovery from the effects of exposure to high concentrations of physostigmine was observed following perfusion with drug-free COMBO solution. Recovery was monitored for *D. pulex* and on average it took approximately 60 min for ABR to reach 50% of its ABR reading prior to drug application (n=10).

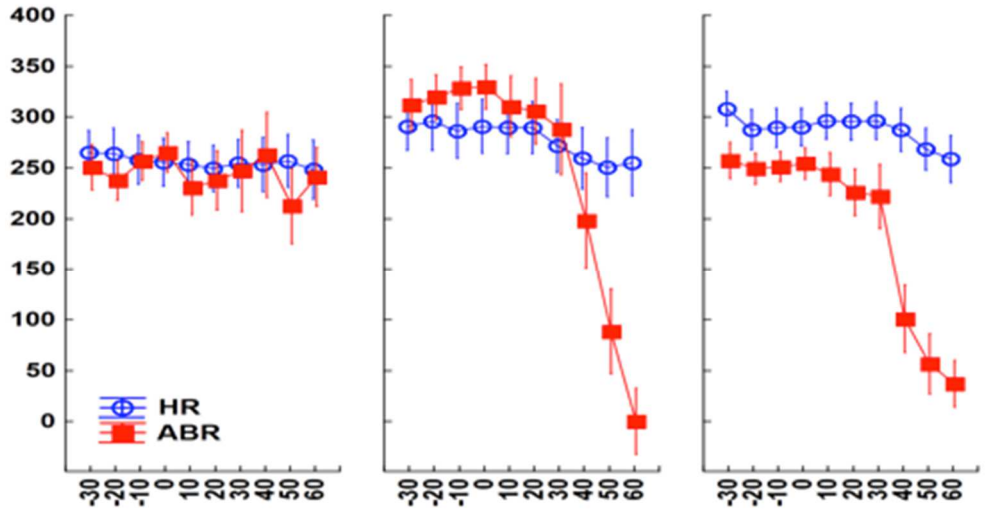


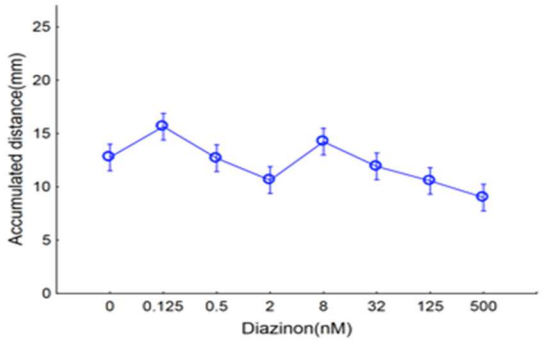
Figure 8: Effects of physostigmine on HR and ABR of *Daphnia pulex*: Concentrations: 0.5, 1 and 2µM. time points -30 to 0 show animals in COMBO. Time points 0 to 60 show the period the animal was in contact with the drug, physostigmine.

Diazinon

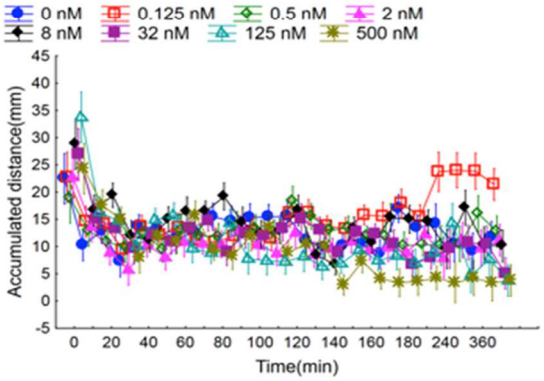
Behavioral analysis

Figure 9A illustrates the effects of diazinon on accumulated distance. A significant effect of diazinon on swimming behavior was observed as concentration-dependent changes in accumulated distance ($F_{7, 64}$= 2.93, P<0.01). The accumulated distance for the 500 and 125 nM concentrations were significantly lower than the 0nM control (LSD test, P<0.05 in both cases). The 0.125nM concentration stimulated swimming behavior and the accumulated distance was significantly higher than the 0nM control (LSD test, P< 0.05). The time-course for the effects of diazinon on accumulated distance is shown in Figure 9B. There was a significant time x concentration effect ($F_{161,1472}$ = 1.78, P<0.001) and the effects of diazinon on accumulated distance increased progressively over time, with the effects from the lower concentrations taking a little longer to develop than the higher concentrations. An analysis of the concentration-dependent effects of diazinon which focused on the time points after 120 minutes is shown in Figure 9C. When this later period in the time-course, was examined (after 120 minutes) significant effects of diazinon on accumulated distance was found ($F_{7, 64}$ = 2.93, P < 0.001), and there was a decrease in the size of the standard errors associated with the mean values. This reduction in standard error reflected the more pronounced effects of diazinon in the later part of the time-course. Contrast analysis, examining the period from 120 minutes to 24-hours indicated that the 0.125 nM concentration significantly elevated the accumulated distance relative to control (P<0.05), while the 500 and 125 nM concentrations significantly decreased accumulated distance (P<0.05). Figure 10D, E and F separately illustrate the effects of each of the 0.125nM, 125nM, and 500nM concentrations on accumulated distance relative to control

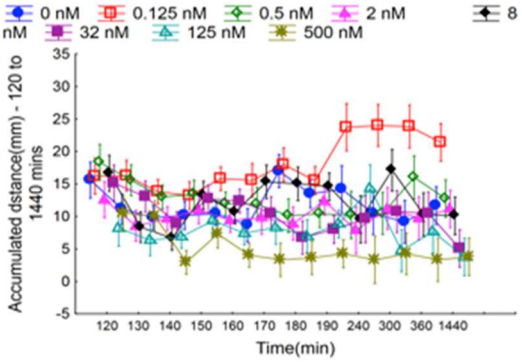
The effect of diazinon on mean angle is illustrated in Figure 11A. A significant concentration-dependent increase in mean angle was elicited by diazinon ($F_{7, 64} = 4.90$, $P<0.001$) with the lowest mean value observed at a concentration of 0.125nM and the highest mean value observed at a concentration of 500nM. Figure 11B shows the significant time- and concentration-dependent effects of diazinon on mean angle ($F_{161, 1472} = 2.48$, $P<0.001$). When the later time-course after 120 minutes was examined a significant time- and concentration-dependent effect was observed with a corresponding decrease in the magnitude of the standard errors associated with the mean values (Figure 11C, $F_{7, 64} = 10.84$, $P< 0.001$) When individual animal recordings were evaluated at the 24hr time point, immobilization was observed in the following proportions for the various concentrations (immobilized/total): 0(0/9), 0.125 (0/9), 0.5 (4/9), 2 (3/9), 8 (1/9), 32 (9/9), 125 (9/9), and 500 nM (9/9). Figure 12D, E and F separately illustrate the effects of each of the concentrations on accumulated distance relative to control



9A

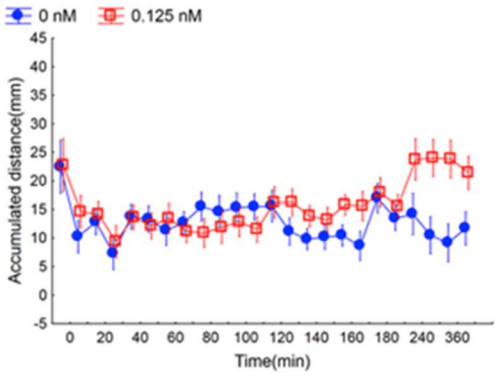


9B

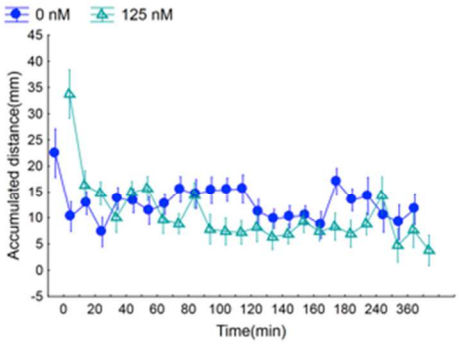


9C

Figure9: Behavioral response of *Daphnia pulex* to diazinon. Error bars are the standard error. **9A:** Effects of diazinon on Accumulated distance. **9B:** Time-dependent effects of diazinon on accumulated distance **9C**: Time-dependent effects of diazinon on accumulated distance from from 120 to 1440 minutes



10A



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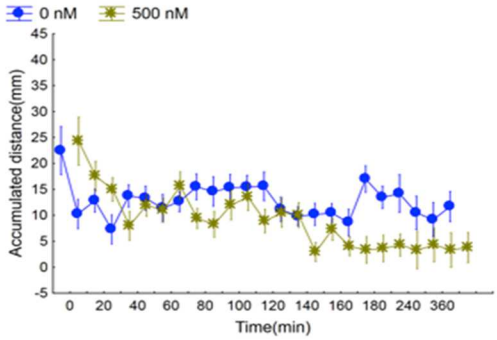
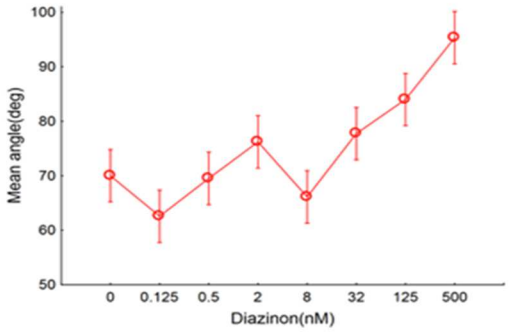
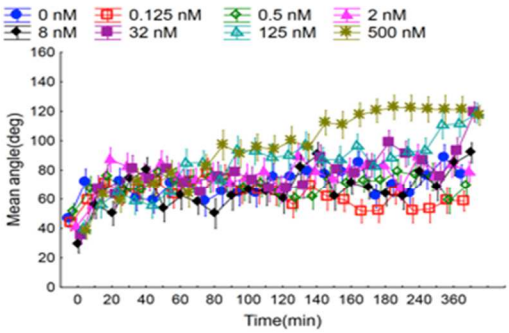


10C

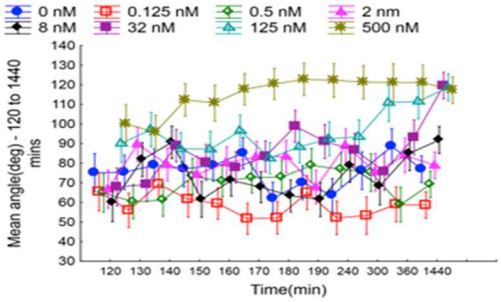
Figure 10: Effect of different concentrations of Diazinon on accumulated distance with respect to control. **10A**: Effect of 0.125nM on accumulated distance with respect to control. **10B**: Effect of 125 nM on accumulated distance with respect to control. **10C**: Effect of 500nM on accumulated distance with respect to control



11A

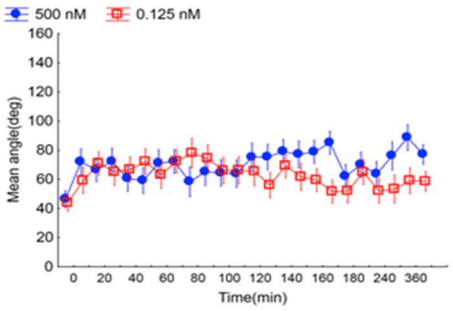


11B

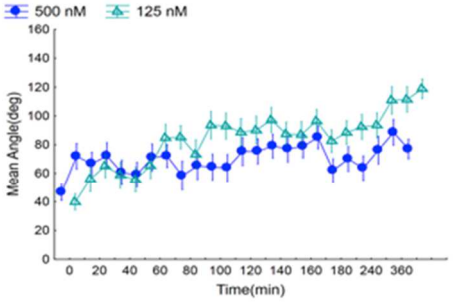


11C

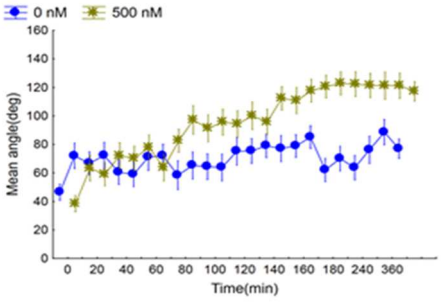
Figure11: Behavioral response of *Daphnia pulex* to diazinon. Error bars are the standard error. **11A:** Effects of diazinon on mean angle. **11B:** Time-dependent effects of diazinon on Mean angle **11C:** Time-dependent effects of diazinon on Mean angle from 120 to 1440 minutes



12A



12B



12C

Figure 12: Effect of different concentrations of diazinon on mean angle with respect to control. **12A**: Effect of 0.125nM on mean angle with respect to control. **12B**: Effect of 125nM on mean angle with respect to control. **12C**: Effect of 500nM on mean angle with respect to control

Physiological Analysis

The physiological effects of diazinon (0.5 – 4.0µM) on heart rate (HR) and appendage beat rate (ABR) are depicted in Figure 13. For all of the diazinon responses analyzed, a minimum of 3 hours of responses were recorded. In selected cases a longer time-course was recorded. Diazinon elicited a significant concentration- and time-dependent effect on HR and ABR (Concentration x Time Interaction, $F_{42,126}$ =2.65, P<0.001) over the 3 hour period of exposure. The physiological effect of diazinon also differed significantly between the HR and ABR parameters (Concentration x Time x Parameter Interaction, $F_{42,126}$ =3.62, P<0.001). An LSD test indicated that HR was not significantly affected by diazinon within the 3 hour exposure period. Diazinon elicited a time- and concentration-dependent inhibition of ABR. Within the 3-hour exposure period ABR was significantly inhibited during only one time period, 140 minutes, following 0.5µM diazinon exposure (P<0.05). However, the ABR was completely inhibited after 3 hours in 2 out of 3 cases. 1 µM diazinon significantly inhibited ABR at time periods greater than 140 minutes (P<0.05 in all cases). 2µM diazinon significantly inhibited all time periods greater than 90 minutes (P<0.05 in all cases).

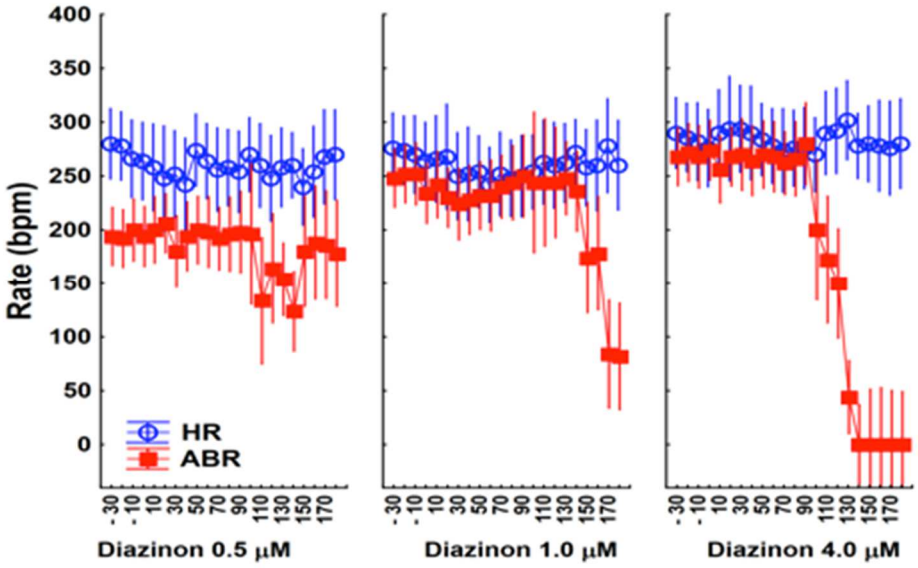


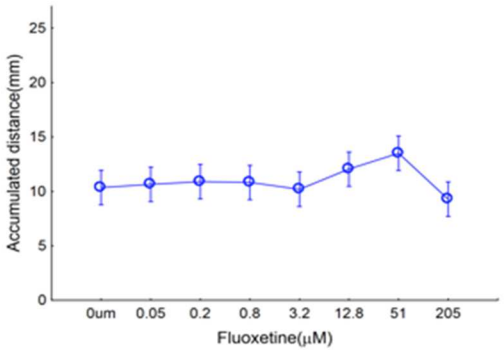
Figure 13: Effects of diazinon on HR and ABR of Daphnia pulex: Concentrations: 0.5, 1 and 4µM

Fluoxetine

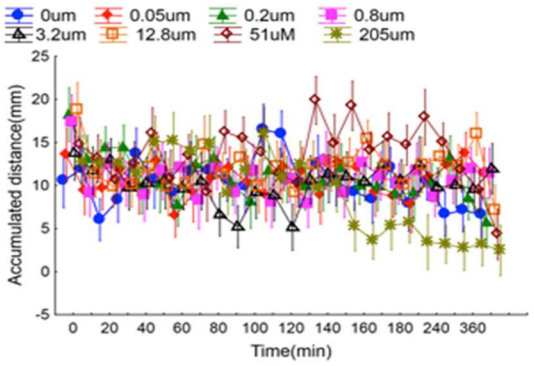
Figure 14A illustrates the effects

of fluoxetine on accumulated distance. The concentration-effect of fluoxetine on accumulated distance was not significant ($F_{7, 64}$= 0.66, P>0.50). The time-course for the effects of fluoxetine on accumulated distance is shown in Figure 14B. There was a significant time x concentration effect ($F_{161, 1472}$ = 1.42, P<0.001) on accumulated distance. An analysis of the concentration-dependent effects of fluoxetine which focused on the time points after 120 minutes is shown in Figure 14C. A significant effect of fluoxetine on accumulated distance was also found for this later period in the time-course ($F_{77, 704}$ = 1.33, P < 0.01). Contrast analysis, examining the period from 120 minutes to 24-hours indicated that the 51μM concentration significantly elevated the accumulated distance relative to control (P<0.05), while the 205μM concentration significantly decreased accumulated distance (P<0.05). Figure 15A and B separately illustrate the effects of each of the concentrations on accumulated distance relative to control.

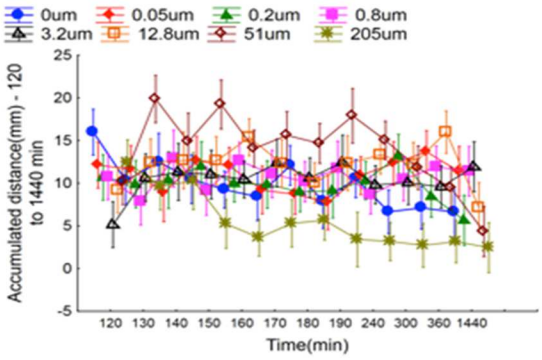
The effect of fluoxetine on mean angle is illustrated in Figure 16A. The concentration effect of fluoxetine on mean angle was not significant ($F_{7, 64}$ = 1.80, P>0.50). Figure 16B shows the significant time- and concentration-dependent effects of fluoxetine on mean angle (F= 1.85, P<0.001). An analysis of the concentration-dependent effects of fluoxetine which focused on the time points after 120 minutes is shown in Figure 16C. When the later time-course after 120 minutes was examined a significant time- and concentration-dependent effect was also observed ($F_{77, 704}$ = 1.57, P< 0.05). When individual animal recordings were evaluated at the 24hr time point, immobilization was observed in the following proportions for the various concentrations (immobilized/total): 0 (1/9), 0.05 (1/9), 0.2 (1/9), 0.8 (1/9), 3.2 (2/9), 12.8 (2/9), 51(9/9) and 205(9/9). Figure 17A and B illustrate the effects of each of the concentrations on mean angle relative to control separately.



14A

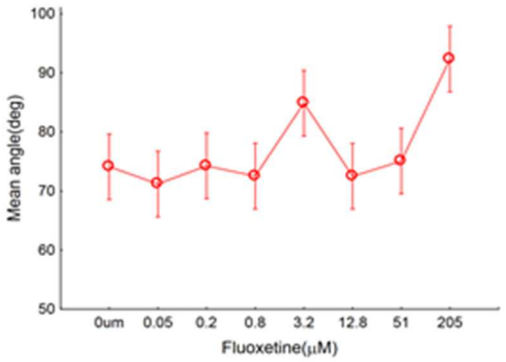


14B

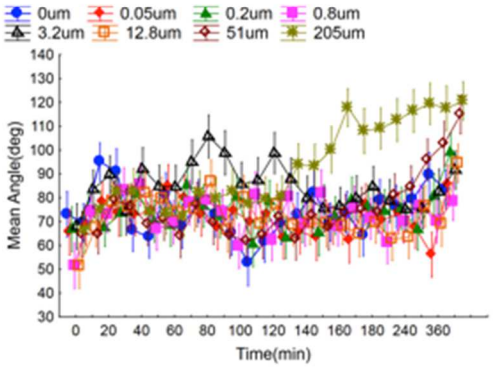


14C

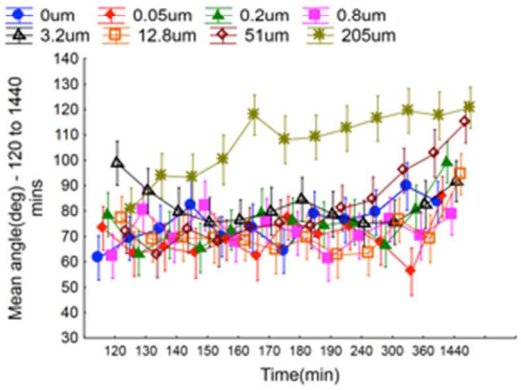
Figure14: Behavioral response of *Daphnia pulex* to fluoxetine. Error bars are the standard error. **14A:** Effects of fluoxetine on Accumulated distance. **14B:** Time-dependent effects of fluoxetine on accumulated distance **14C**: Time-dependent effects of fluoxetine on accumulated distance from 120 to 1440 minutes



15A

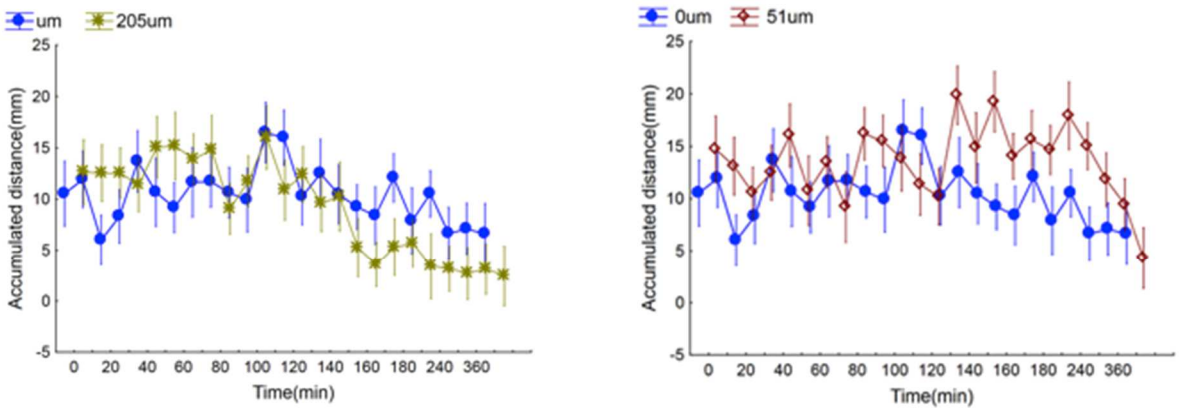


15B



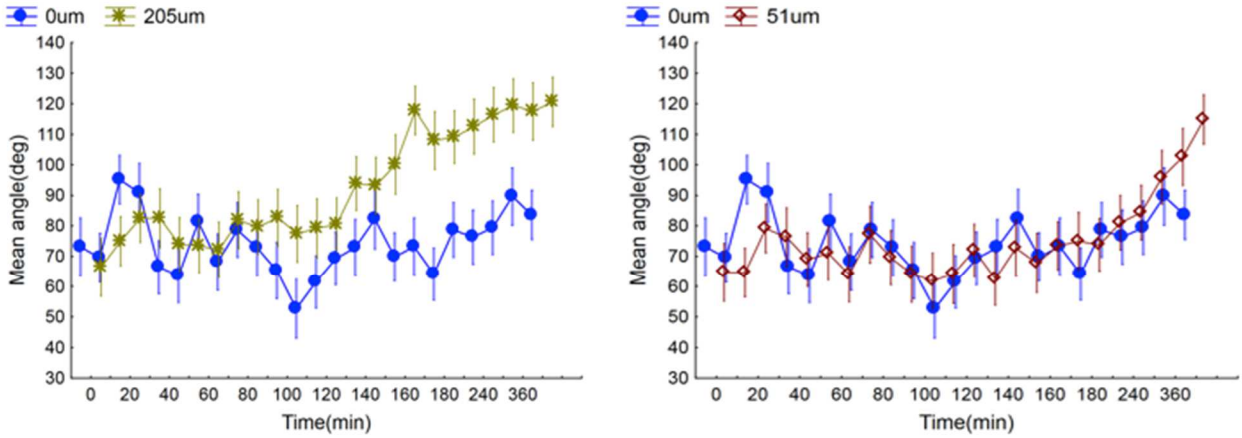
15C

Figure15: Behavioral response of *Daphnia pulex* to fluoxetine. Error bars are the standard error. **15A:** Effects of fluoxetine on mean angle. **15B:** Time-dependent effects of fluoxetine on Mean angle **15C:** Time-dependent effects of fluoxetine on Mean angle from 120 to 1440 minutes



16A 16B

Figure 16: Effect of different concentrations of fluoxetine on accumulated distance with respect to control. **16A**: Effect of 205µM on accumulated distance with respect to control. **16B**: Effect of 51µM on accumulated distance with respect to control



17A 17B

Figure 17: Effect of different concentrations of fluoxetine on mean angle with respect to control. **17A**: Effect of 205µM on mean angle with respect to control. **17B**: Effect of 51µM on mean angle with respect to control

DISCUSSION

Physostigmine

Daphnia pulex are known to have a cholinergic neurotransmitter system, including homologs for the synthetic enzyme, choline acetyltransferase, the vesicular ACh transporter, the degradative enzyme acetylcholinesterase, and nicotinic and muscarinic receptors (McCoole et al., 2012a). In the present study the acetylcholinesterase (AChE) inhibitor, physostigmine, elicited a significant and relatively rapid concentration-dependent increase in maximum accumulated swimming distance over the 24 hour time-course. The onset time for the increase in distance was also concentration-dependent, with higher concentrations, 0.25 – 0.5 μM, producing significant stimulation of swimming earlier in the time-course (less than one hour) than the lower 0.125 μM concentration (greater than one hour). As has been shown in a previous study by Zein et al. (2014) examining a shorter 90 minute time-course, the increase in swimming distance was associated with a significant decrease in mean angle over the first three to four hours for these same concentrations (0.125 – 0.5 μM), which is indicative of a decrease in turning behavior. However, by the sixth hour of the time-course the higher, 0.5 μM physostigmine concentration, elicited a significant increase in mean angle that was associated with animals becoming immobilized due to exposure. To the best of our knowledge, this study and the previous paper by our lab, Zein et al. (2014), represent the only behavioral studies examining the effects of physostimine on *Daphnia pulex*.

Physostigmine (0.5 to 2 μM) also elicited a significant concentration-dependent inhibition of appendage beat rate (ABR), but not heart rate (HR). The onset for ABR inhibition was approximately 30 to 40 minutes at the 1 and 2 μM concentration (Hannan et al., in preparation). The lack of effects of physostigmine on heart rate differs from the inhibitory effect on *Daphnia magna* heart rate described by Baylor (1942). However, the experimental setup used by Baylor differs significantly, and Baylor does not show these physostigmine results, concluding that these were “toxic” effects. To the best of our knowledge this is the first report of the inhibitory effects of physostigmine on ABR. Inhibition of ABR would be expected to affect both feeding and respiratory function.

Diazinon

The AChE inhibitor and emerging contaminant, diazinon, elicited concentration-dependent behavioral effects on maximum accumulated distance and mean angle qualitatively similar to physostigmine. Diazinon appeared to be more potent than physostigmine since stimulation of swimming behavior, seen as an increase in maximum accumulated distance, was detected at the lowest concentration of 0.125 nM. The stimulatory effects of diazinon were observed with a much more rapid onset relative to physostigmine at the 125 nM concentration (i.e., the first, time-0, reading for diazinon versus greater than one hour for physostigmine). A corresponding decrease in mean angle was also seen for 125 nM diazinon. It should be noted that the stimulatory response to the lowest 0.125 nM concentration of diazinon occurred particularly late in the time-course, after approximately four hours of exposure. However, the increase in mean angle over the diazinon concentration range studied tended to predominate for most of the higher diazinon concentrations, indicating an increase in turning behavior eventually that eventually resulted in 100% immobility at 24 hours for the highest concentrations (32-500 nM). The increase in mean angle and increase in immobilized animals over this extended concentration range and longer 24-hour time course is consistent with our previous reports examining responses to higher concentrations of diazinon (e.g., 1 and 2 μM) over a shorter 90-minute period (Zein et al., 2015).

To the best of our knowledge this study and the previous paper by Zein et al. (2015) represent the only behavioral studies examining the effects of diazinon on *Daphnia pulex*. The 0.125 nM diazinon concentration is lower than the LC_{50} value of 0.9μg/L (2.9 nM) reported by Fernandez et al. (1994) for *Daphnia magna*. For Daphnia LC_{50} assays immobility rather than death is typically used as the end point, and it is assumed that immobility was the end point used by Fernandez et al. (1994) in their 24-hour study even though their methods describe the end point as mortality. In the present study, immobilization was not observed after 24 hours of exposure at 0.125 nM.

This diazinon concentration is environmentally relevant since similar concentrations have been detected in surface waters (e.g., see Kolpin et al., 2002), and the behavioral stimulation observed in the

present study suggests that diazinon might affect behavior in the real world habitat. For example, alterations in behavior may affect survival by making daphnia more prone to predation. Higher concentrations of diazinon have been found to increase the rate of predation of *Daphnia pulex* by *Hydra littoralis* under controlled experimental conditions (Zein et al., in preparation). Since contaminants of emerging concern are generally found as complex mixtures, the presence of bioactive concentrations of diazinon in the aquatic environment may exhibit increased toxicity due to additive or synergistic interactions with other contaminants. For example, Zein et al. (2015) reported a potential synergistic interaction between diazinon and the detergent metabolite, 4-nonylphenol, which is also commonly found in surface waters.

Diazinon elicited physiological responses qualitatively similar to physostigmine, eliciting an inhibition of ABR at the two higher concentrations studied, 1 and 4 □M, without affecting HR. However, the onset of the response was much later in the time-course for the effect on ABR. The onset of the ABR response for diazinon was more than two hours after the initiation of exposure compared to 30 to 40 minutes for similar physostigmine concentrations.

Comparison of physostigmine and diazinon

While the qualitative effects of the two AChE inhibitors, physostigmine and diazinon, were very similar, the quantitative aspects in terms of concentrations eliciting a certain level of response and onset times in particular were quite different. Although the mode of action is very similar for these two agents, the mechanism of acetylcholinesterase inhibition is different. Physostigmine is a carbamate inhibitor, which elicits a reversible inhibition of enzyme activity. The inhibitory effect is reversed by hydrolysis of the carbamate group from AChE (Taylor, 2011). Diazinon is an organophosphate inhibitor which is converted to an active metabolite, diazoxon, that creates a long-lasting covalent bond to AChE (Kretschmann et al., 2011). Although, these differences in action may contribute to the differing response profiles between these two agents, the difference in octanol/water coefficients may play a larger role. Diazinon was shown to be capable of eliciting a stimulation of swimming behavior that was recorded at the “0” time-point, which suggests that diazonon is rapidly generated. However, the time to onset of

response varied widely among the various responses measured, with a particularly long onset for inhibition of ABR even though the diazinon concentrations required to elicit these responses were much higher. The octanol-water partition coefficient for physostigmine is only 1.17 (Greig et al., 2000), where as that for diazinon is 3.81 (Nemeth-Konda et al., 2002). The difference in octanol-water partition coefficients suggests that differences in partitioning of these two agents between lipid and water phases may influence the rate of onset of the responses. This result in turn suggests that at least some portion the AChE pool associated with the behavioral response (e.g., increase in maximum accumulated swimming distance) and physiological response (ABR) may differ in anatomical location or have different enzyme inhibition thresholds.

An additional potential factor is the selectivity of physostigmine and diazinon as enzyme inhibitors. AChE inhibitors are often capable of inhibiting multiple esterases. For example, diazinon and physostigmine can inhibit both acetylcholinesterase and butyrylcholinesterase (Musilek et al., 2011; Taylor, 2011), and organophosphates are known to also affect neuropathy target esterase (NTE, Richardson et al., 2013). Although the genome of *Daphnia pulex* has been sequenced (e.g., see Coulbourne et al., 2011), and various esterases have been identified (McCoole, 2012a), the biological impact of inhibiting different esterases, particularly acetylcholinesterase, is still an ongoing field of inquiry in *Daphnia*.

Since animals like *Daphnia* live in the aquatic environment for their entire life cycle, exposure to chemical contaminants can occur over an entire life span. Contaminants like diazinon are also known to bioaccumulate in *Daphnia*, and this can lead to greater toxicity (Kretschmann et al. 2011; Nyman et al., 2014). Fernandez et al. (1994) found that exposure of *Daphnia magna* to lower concentrations of diazinon for 5 hours significantly inhibited filtration (µl/animal/hour) and ingestion (cells/animal/hour) at concentrations of 0.47 µg/l (~ 1.5 nM) and 0.60 µg/l (~ 2nM), respectively. These findings taken together suggest that exposure to very low concentrations of diazinon for a sufficiently long enough time period can impair feeding in Daphnia, and therefore may impact the survival and fitness of this keystone species.

Fluoxetine

Daphnia are known to have a serotonergic neurotransmitter system. Homologs of the synthetic enzyme, tryptophan hydroxylase, serotonin transporters, and serotonin receptors have been identified in *Daphnia pulex* (McCoole et al., 2012b). Fluoxetine is a selective serotonin reuptake inhibitor and emerging contaminant. Fluoxetine was not found to have significant behavioral effects in this study except at relatively high concentrations. The 51µM concentration elicited a transitory stimulatory effect on swimming behavior expressed as an increase in maximum accumulated distance after two hours of exposure and this lasted for approximately four hours when maximum accumulated distance began to decline. The mean angle was elevated at the 24-hour time point. The 205µM concentration decreased maximum accumulated distance after two hours exposure and this was associated with an increase in mean angle. Both of these fluoxetine concentrations, 51 and 205µM resulted in 100% immobilization of the animals by the 24-hour time point. Given the relatively high concentrations of fluoxetine needed to elicit these behavioral effects, and without experiments conducted in the presence of a serotonin antagonist, it remains possible that the responses may be non-specific and not related to effects on the serotonergic system.

Although the observation of effects of swimming behavior resulting from fluoxetine exposure required high concentrations in the present study, there are a number of studies that have reported effects on *Daphnid* reproduction and life cycle. Compos et al. (2012) reported that exposure to the selective serotonin (5HT) reuptake inhibitors, fluoxetine (~30 nM–230 nM) and fluvoxamine (~7 nM-70 nM), increased *Daphnia magna* juvenile development rate, and increased clutch size and decreased the size of offspring. These effects could be reversed by a 5HT antagonist, indicating that they were likely produced by 5HT receptor stimulation resulting from transporter inhbition. These drugs also elicited changes in oxygen consumption (increased) and carbohydrate levels (decreased). Perry et al. (2008) found that exposure to 9 to 90 nM fluoxetine affected the length of newborn. *Daphnia magna*, with a larger effect on second generation animals relative to first generation. Since the behvioral results observed in the present study occurred at exposure levels that were aproximately an order of magnitude higher than the fluoxetine concentrations used by Compos (2012) and Perry (2008), it is possible that the more biologically

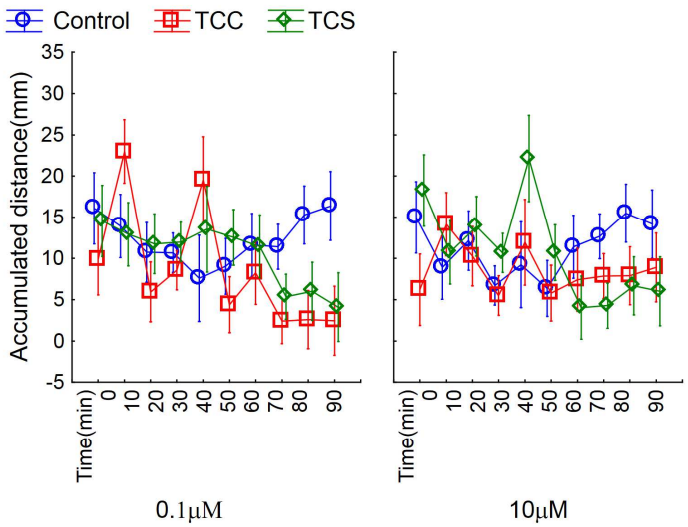
important role of 5HT in Daphnia is related to reproduction and regulation of life cycle rather than modulation of motor activity.

Chapter 4: Emerging Contaminants with unknown targets

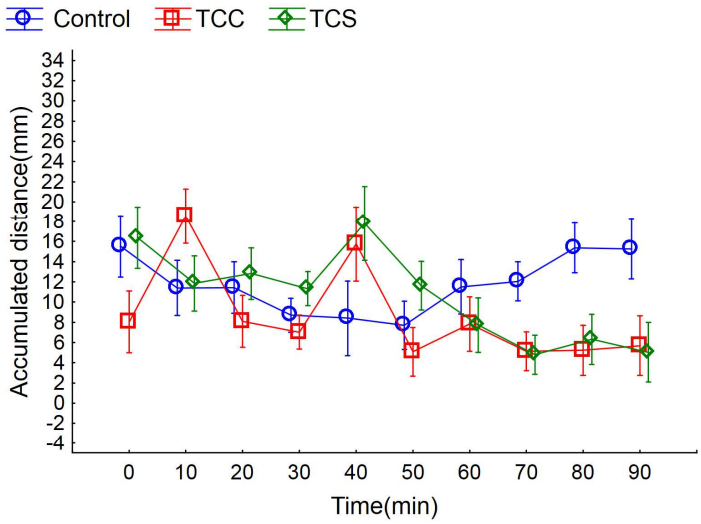
RESULTS

Triclocarban and Triclosan in COMBO

The effects of TCS and TCC exposure on distance are shown in Figure 18A and 18B. Since the concentration effect ($F_{1,18}$=5.09, P>0.50), the concentration x time interaction ($F_{9,162}$=43.01, P>0.50), and the concentration x chemical x time interaction ($F_{18,\ 162}$=32.93, P>0.50) for accumulated distance were not significant, there was no significant difference found between the low and high concentrations (see Figure 18A). However, there was a significant time effect ($F_{9,\ 162}$= 153.43, P< 0.005) and a significant time x chemical interaction ($F_{18,\ 162}$=128.06, P<0.005) indicating that there was a significant chemical effect on accumulated distance that was dependent on time (see Figure 18B). Contrast analysis comparing means at 70 to 90 minutes indicated that the accumulated distance for TCC and TCS were significantly lower than that of controls (P<0.05 in all cases).As shown in Figure 19A and 19B, a similar result was found for mean angle. The concentration effect ($F_{1,\ 18}$ = 0.0, P>0.5), concentration x time interaction ($F_{9,\ 162}$ = 1.60, P>0.1), and concentration x chemical x time interaction ($F_{18,\ 162}$ = 1.10, P>0.2) were not significant. There was a significant time effect ($F_{9,162}$ = 7.39,P<0.001) and time x chemical interaction ($F_{18,192}$ = 3.09,P<0.001), again indicating a significant chemical effect on mean angle (see Figure 19B). Contrast analysis comparing means at 70 to 90 minutes indicated that the mean angle for TCC and TCS were significantly higher than that of controls (P<0.05 in all cases).

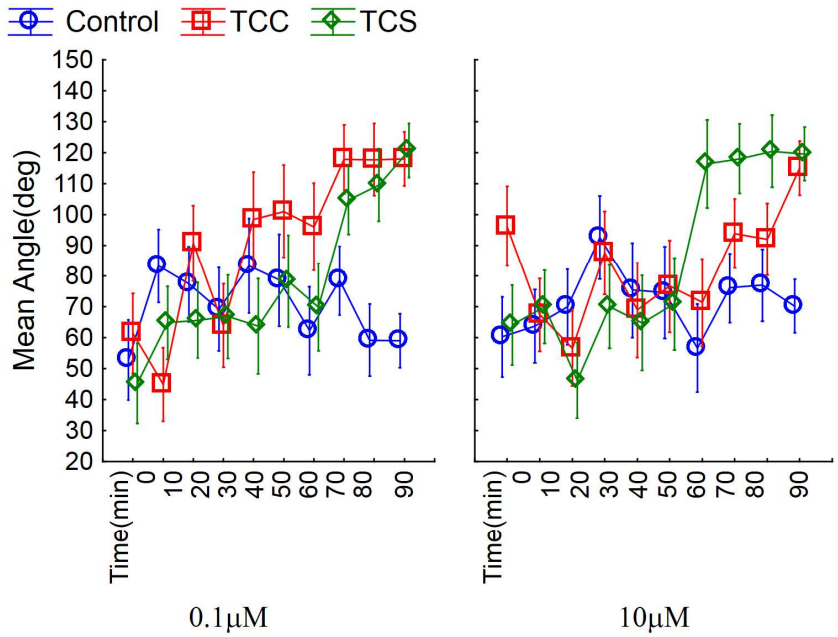


18A

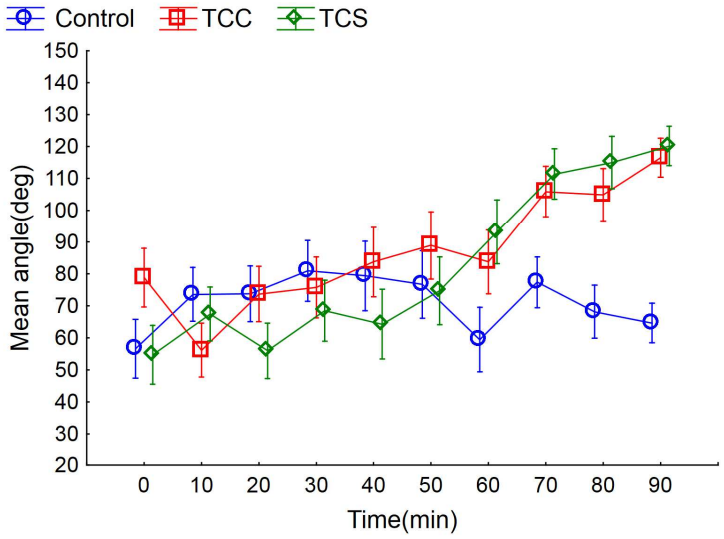


18B

Figure18A: Effect of different concentrations of TCC and TCS over time on accumulated distance. **18B**: Time course for the effects of TCC and TCS on Accumulated distance.



19A



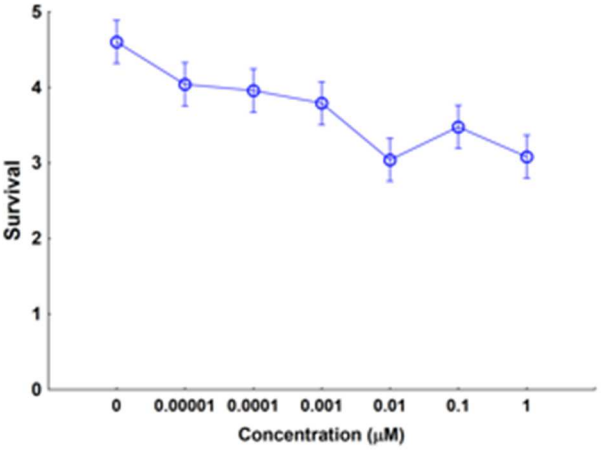
19B

Figure19A: Effect of different concentrations of TCC and TCS over time on mean angle. **19B**: Time course for the effects of TCC and TCS on mean angle.

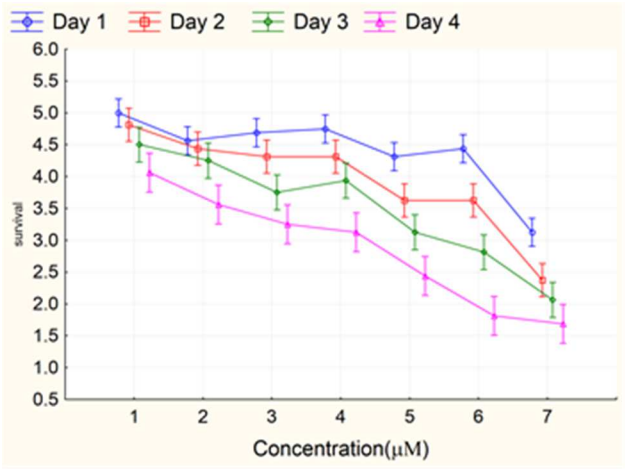
Triclocarban and Triclosan in EPA

Survival Analysis

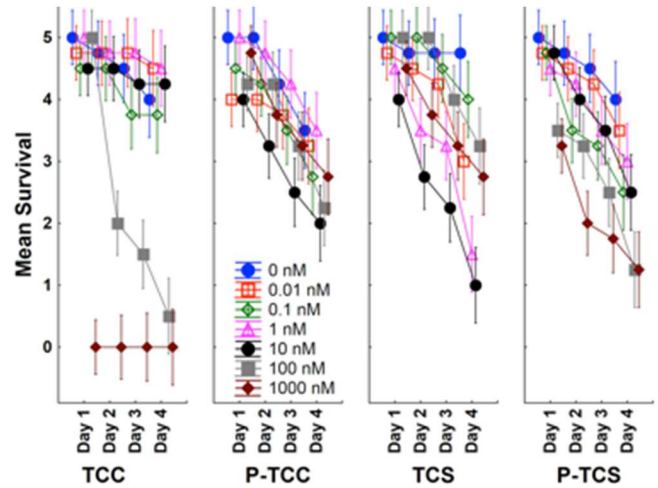
The effects of TCC and TCS and their photo-degradative products(P.TCC and P.TCS) on *Daphnid* survival in EPA water over a 5-day period are shown in Figure 20A. A significant concentration x chemical interaction ($F_{18, 84} = 3.87$, $P<0.001$) indicated that the toxic effects on survival were dependent on both the type of chemical exposure and the concentration. LSD tests indicated that the survival due to TCC exposure was significantly lower than the other three chemicals at the highest concentration, 1μM ($P<0.05$ in all cases). At the second highest concentration, 0.1μM, survival due to TCC exposure was significantly lower than TCS and P.TCC ($P<0.05$ in both cases), but not significantly different from P.TCS. The effects of chemical exposure were also dependent on duration of exposure and concentration (concentration x time interaction, $F_{18, 252}=3.17$, $P<0.001$). The time- and concentration-dependent effects can be seen in Figure 20B. There was a progressive time-dependent decrease in survival over the duration of exposure (time effect, $F_{3, 252}=141.43$, $P<0.001$) with significant differences among all 4 days (LSD test, $P<0.05$ in all cases). The overall effect of chemical exposure as seen in Figure 20C, was dependent on the type of chemical exposure, duration of exposure, and concentration (chemical x time x concentration interaction, $F_{54, 252}=2.11$, $P<0.001$). TCC caused the greatest reduction in survival among the four chemical exposures since the chemical x time x concentration effect after eliminating TCC from the analysis was no longer significant ($F_{36,189}=0.78$, $P>0.50$).



20A



20B



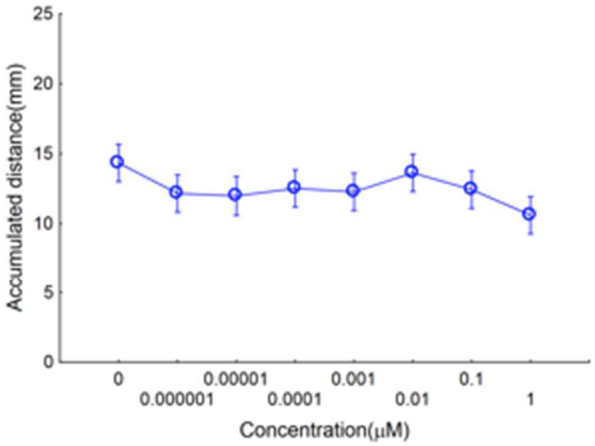
20C

Figure 20A: The concentration-dependent effects of four different chemical exposures on Daphnia pulex over a 5-day period **20B:** The time- and concentration-dependent effects of four different chemical exposures on Daphnia pulex over a 5-day period. **20C:** The overall effect of four different chemical exposures on Daphnia pulex over a 5-day period

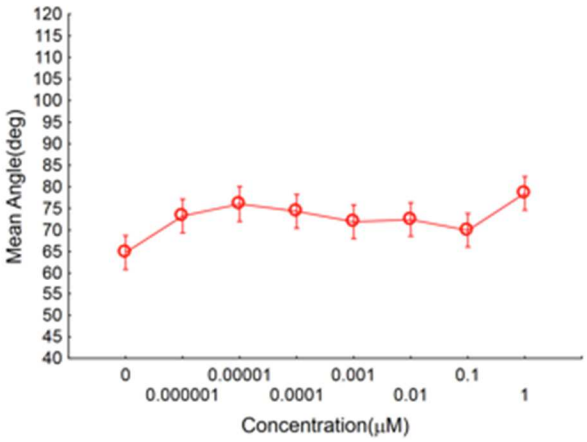
Behavioral Analysis of TCC and TCS – both parent and photolyzed

The effects of TCC, TCS, P.TCC and P.TCS (on swimming behavior were examined over a 24-hour period using the optical tracking method. Figure 21 through 26 illustrate the results from these experiments.

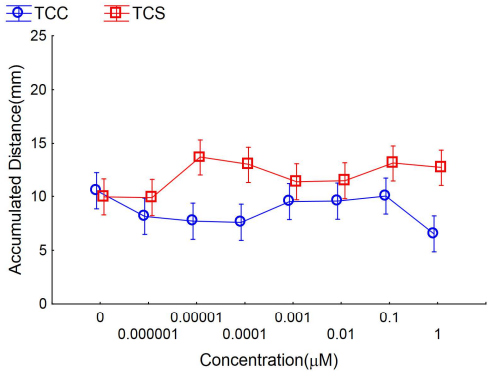
The concentration-dependent effects of TCC and TCS are shown in Figure 21. There was not a significant overall concentration effect of the parent compounds on accumulated distance (21A: $F_{7,80}$=0.40, P>0.50) or on mean angle (21B: $F_{7,80}$=0.54, P>0.50). Similarly, when comparing the chemicals, TCC and TCS, the concentration x chemical effect was not found to be significant for accumulated distance (21C: $F_{7,80}$=1.05, P>0.20) or for mean angle (21D: $F_{7,80}$=1.68, P>0.10). The results for the concentration-dependent effects of the photodegradates are shown in Figure 22, and the outcome was similar to that obtained for the parent compounds. Neither the concentration-effect of the photodegradates (accumulated distance-22A: $F_{7,79}$=0.30, P>0.50; mean angle-22B: $F_{7,79}$ =1.10, P>0.20) nor the concentration x chemical effect of the photodegredates (accumulated distance-22C: $F_{7,79}$=0.69, P>0.50; mean angle - 22D: $F_{7,79}$ =1.06, P>0.20) were found to be significant for either dependent variable, accumulated distance or mean angle.



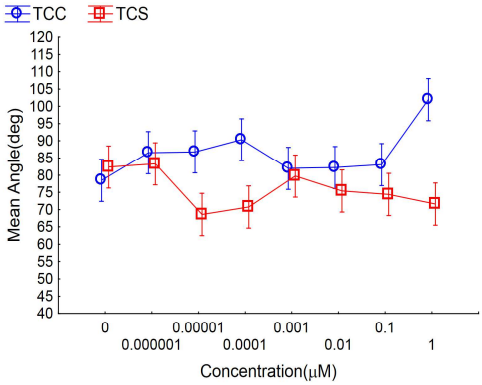
21A



21B

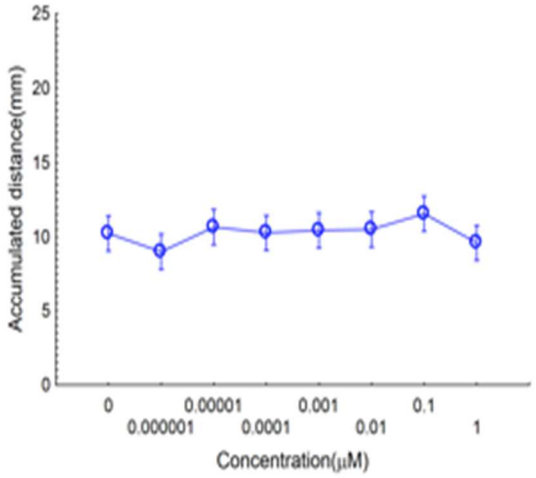


21C

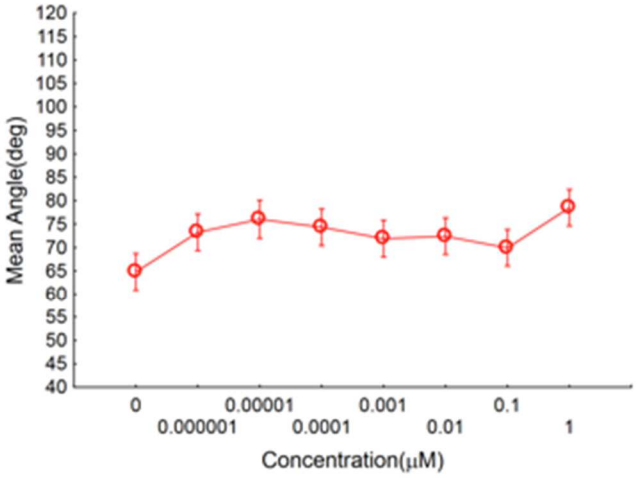


21D

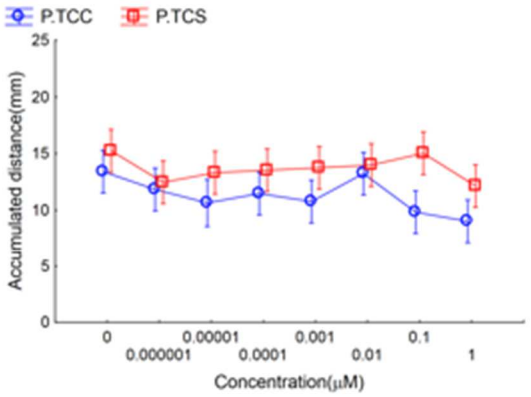
Figure 21: Concentration dependent effects of TCC and TCS. **21A**: Effects of TCC and TCS on accumulated distance. **21B**: Effects of TCC and TCS on mean angle. **21C**: Concentration x chemical effects on accumulated distance. **21D**: Concentration x chemical effect on mean angle.



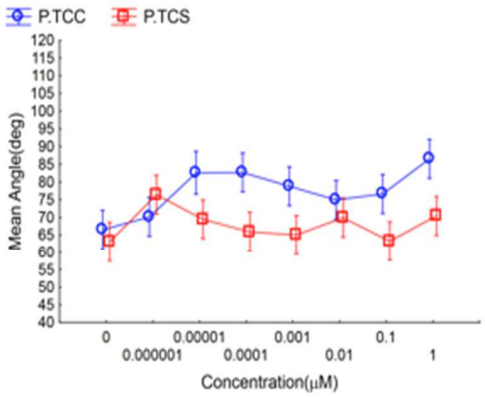
22A



22B



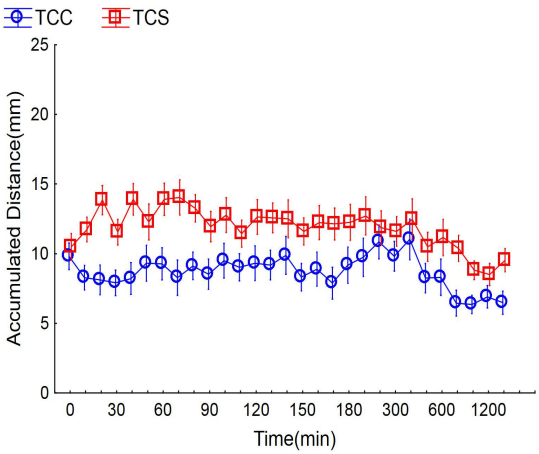
22C



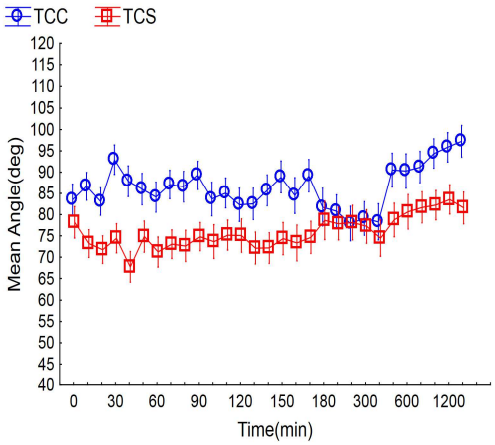
22D

Figure 22: Concentration dependent effects of photolyzed TCC and photolyzed TCS. **22A**: Effects of photolyzedTCC and photolyzed TCS on accumulated distance. **22B**: Effects of P.TCC and P.TCS on mean angle. **22C**: Concentration x chemical effects on accumulated distance. **22D**: Concentration x chemical effect on mean angle

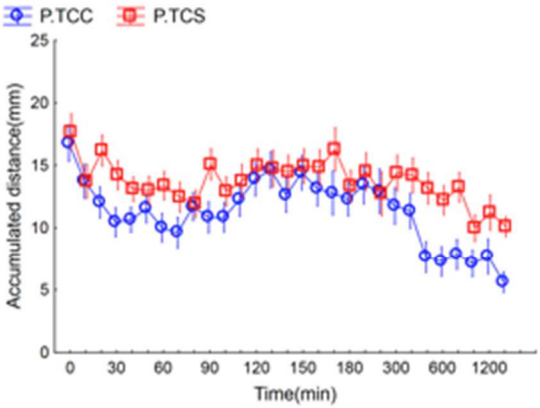
Figure 23 illustrates the time-course for the effects of the various chemical treatments on accumulated distance and mean angle when ignoring concentration. There were no significant differences between any chemical treatments at time 0 (LSD test, $P>0.50$ in all cases). A significant chemical-effect on both accumulated distance ($F_{1,80}=14.47$, $P<0.001$) and mean angle ($F_{1,80}=12.16$, $P<0.001$) was found when comparing TCC and TCS (see Figure 23A and 23B). A significant chemical-effect on both accumulated distance ($F_{1,79}=6.42$, $P<0.05$) and mean angle ($F_{1,79}=11.57$, $P<0.005$) was also found when comparing the photodegradates (see Figure 23C and 23D). As described above, the concentration x chemical effect was not significant for any of the chemicals. The interaction between chemical and time was significant for mean angle for the photodegradates ($F_{28,\ 2212}=4.14$, $P<0.001$, see Figure 23D), but was not significant for any of the other chemical x time interactions ($P>0.10$ in all cases). The interaction between chemical, concentration, and time was significant for mean angle in the parent compounds ($F_{196,2240}=1.19$, $P<0.05$), but was not significant for any of the other three-way interactions ($P>0.05$ in all other cases). Contrast analysis was used to examine the difference between the first four means and the difference between the last four means in each of the four plots (A-D) in Figure 23. Contrast analysis indicated significant differences ($P<0.001$) between parent compounds (A, B) and the photodegradates (C, D) in all cases except for the first four means of plots C and D ($P>0.05$ in both cases). Therefore, the chemical effect was the major significant effect observed, with more minor time- and concentration-dependent effects. As can be seen in time-course for the effects of parent compounds in 23A and 23B the mean values and standard errors for accumulated distance and angle over time are mostly separated. The major separation between the means and standard errors of the photodegradates occurs mostly after 300 minutes.



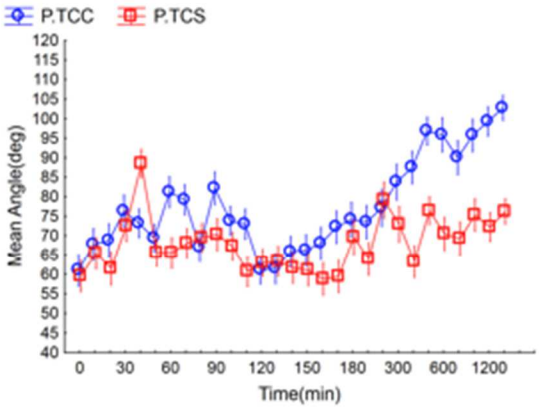
23A



23B



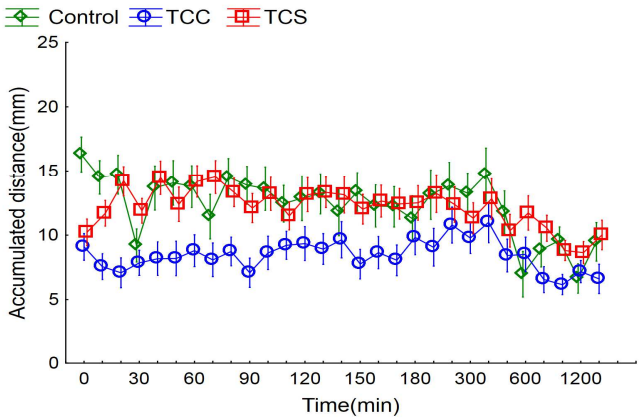
23C



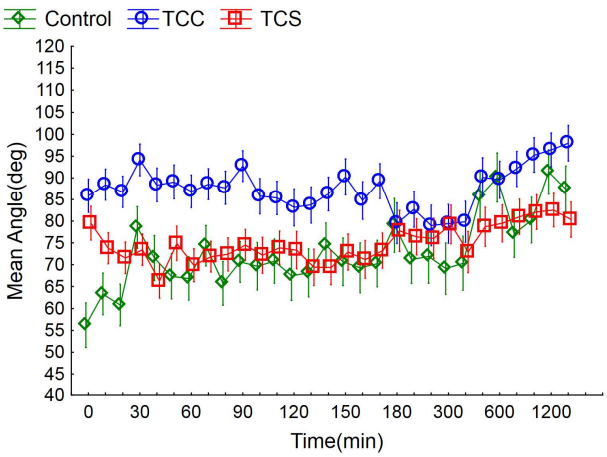
23D

Figure 23: time-course for the effects of the various chemical treatments on accumulated distance and mean angle when ignoring concentration. **23A**: time dependent effects of TCC and TCS on accumulated distance. **23B**: time dependent effects of TCC and TCS on mean angle. **23C**: time dependent effects of P.TCC and P.TCS on accumulated distance. **23D**: time dependent effects of P.TCC and P.TCS on mean angle

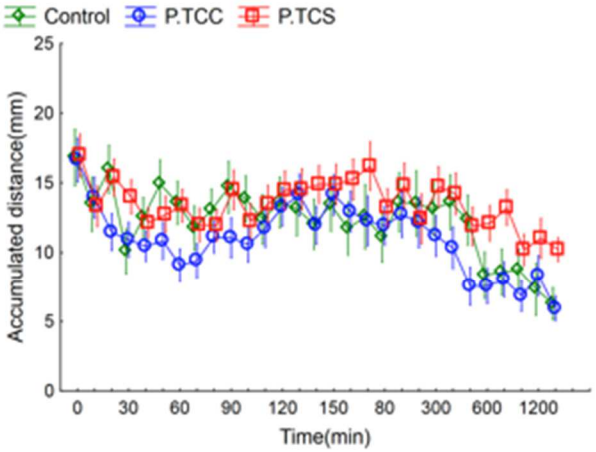
Since there were no significant concentration main-effects observed, and one of the concentrations was zero (controls with no chemical treatment), the no-treatment controls were pooled from the two experiments (parent and photodegredates) to create a separate pooled control group. This control data was incorporated into a new analysis to compare parent compounds and photodegradates. Figure 24 illustrates the chemical effects over time with the controls as a separate line. As can be seen in Figure 24A and 24B, the no-treatment controls closely follow the TCS parent compound, and the time course for these two groups is mostly separated from the TCC parent compound group. For the photodegradates in Figure 24C and 25D, the no-treatment group follows a more intermediate position between the TCC photodegredates and the TCS photodegredates over time. Figure 25A and 25B illustrates the effects of the highest concentration of TCC on accumulated distance and on mean angle respectively and the highest concentration of TCS on accumulated distance (25C) and mean angle (25D) relative to the pooled controls.



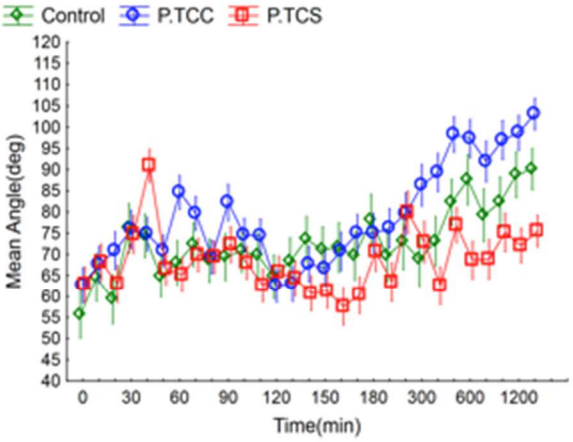
24A



24B

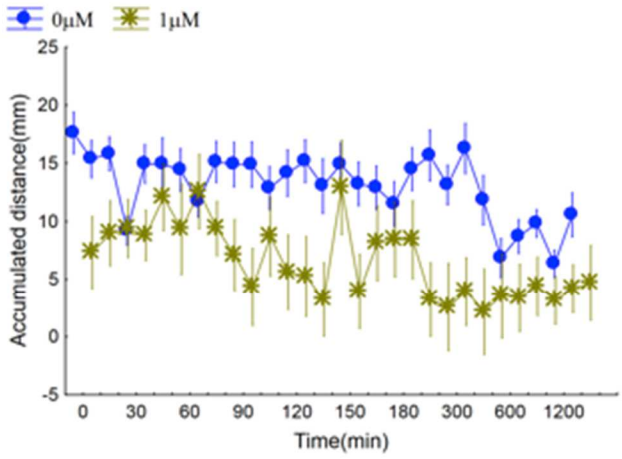


24C

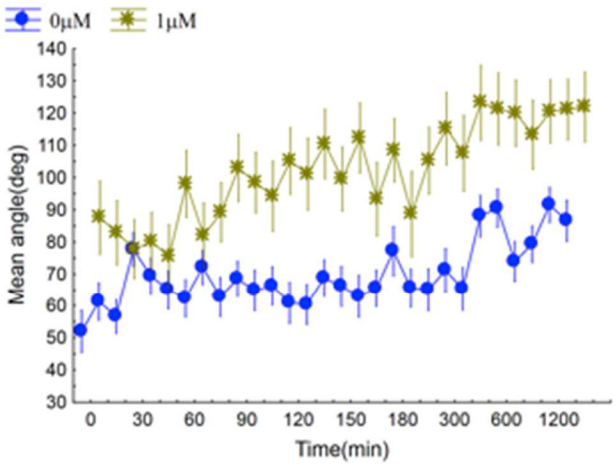


24D

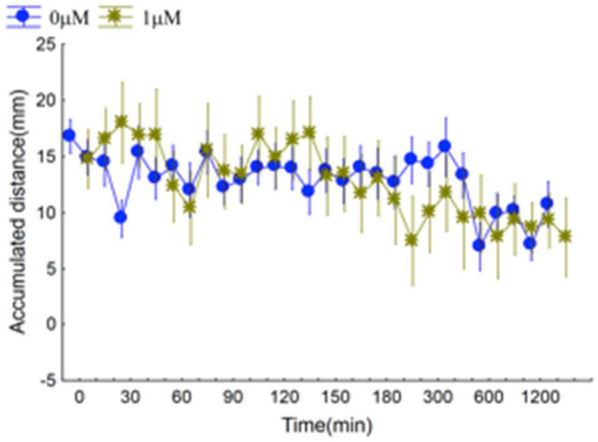
Figure 24: the chemical effects over time with the controls as a separate line. **24A**: effects of TCC and TCS on accumulated distance. **23B**: effects of TCC and TCS on mean angle. **24C**: effects of P.TCC and P.TCS on accumulated distance. **24D**: effects of P.TCC and P.TCS on mean angle



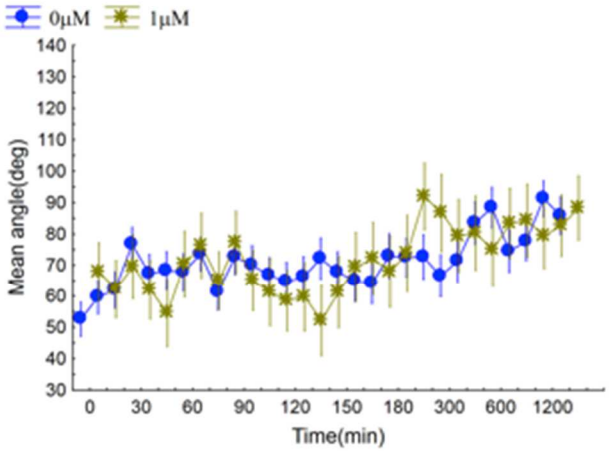
25A



25B



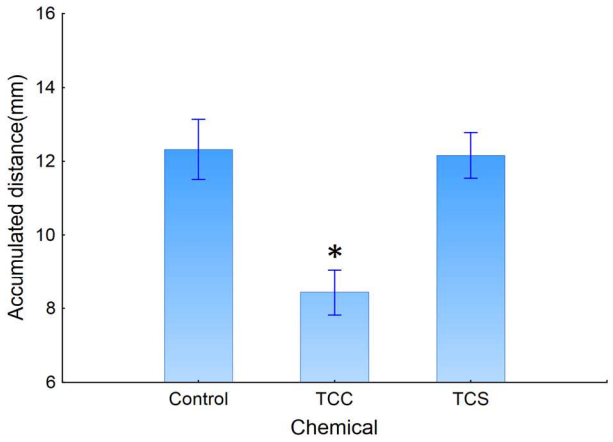
25C



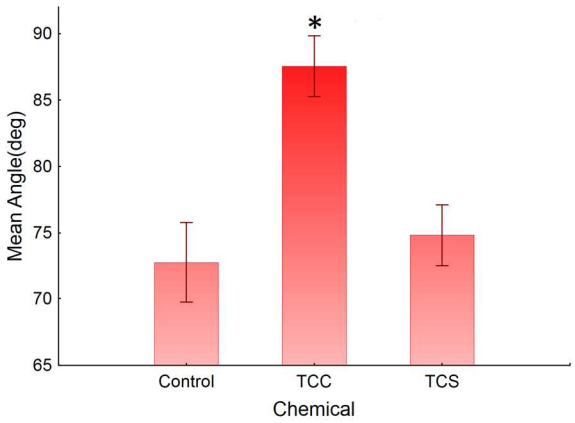
25D

Figure25A: effects of the 1µM TCC on accumulated distance. **25B**: effects of the 1µM TCC on mean angle**. 25C**: effects of the 1µM TCS on accumulated distance. **25D**: effects of the 1µM TCS on mean angle

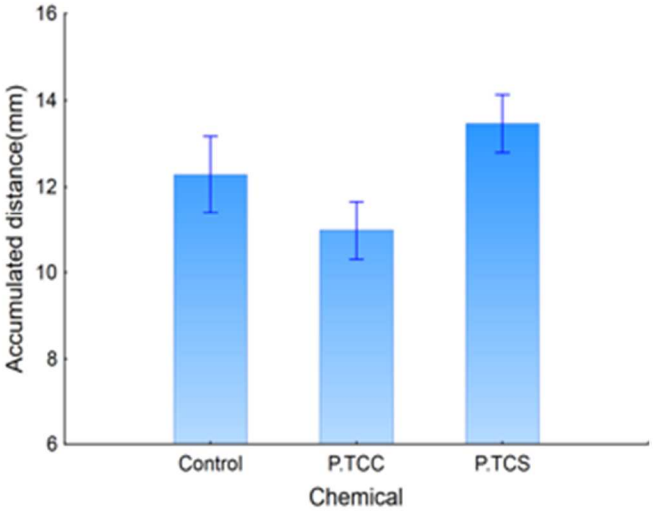
Figure 26 summarizes the effects of the various chemical treatments and the no-treatment control ignoring the concentration factor. There was a significant chemical effect for all chemical treatments (26A-D: $P<0.05$ in all cases). A post-hoc LSD test indicated that TCC was significantly different from control for both accumulated distance and mean angle ($P<0.05$ in both cases). This indicates that TCC significantly decreased accumulated distance and increased mean angle relative to control, inhibiting forward swimming behavior and increasing turning behavior (Figure 26A and 26B). TCS was not significantly different from control for either accumulated distance or mean angle (Figure 26C and 26D). For both accumulated distance and angle, the photodegradates were not significantly different from control (LSD, $P>0.05$). There was a non-significant trend towards an increase in mean angle of P.TCC relative to control ($P\sim 0.073$). The two photodegradates were significantly different from each other (LSD, $P<0.05$ in both cases).



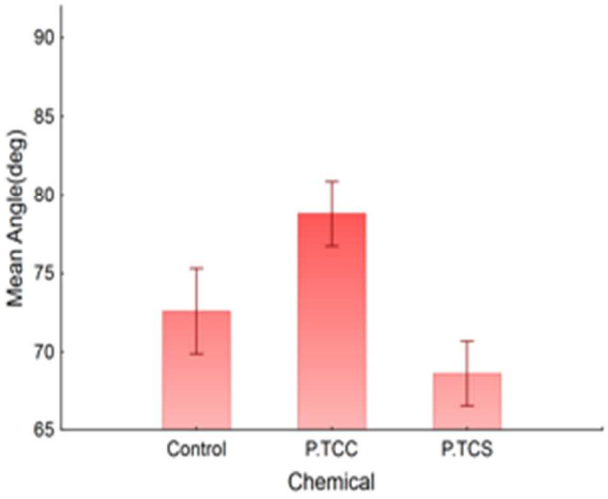
26A



26B



26C



26D

Figure26: the effects of the various chemical treatments and the no-treatment control ignoring the concentration factor. **26A**: effects of TCC and TCS on accumulated distance. **26B**: effects of TCC and TCS on mean angle. **26C**: effects of P-TCC and P-TCS on accumulated distance. **26D**: effects of P-TCC and P-TCS on mean angle

DISCUSSION

Triclocarban, Tricloan and photodegredates

Triclosan (TCS) and triclocarban (TCC) are halogenated aromatic hydrocarbons used in many antimicrobial products (e.g., soaps, detergents, toothpaste, plastics, etc.; Halden, 2014). These compounds have broad antimicrobial activity with multiple antimicrobial targets, and can be classified as bacteriostatic or as biocides depending on concentration (Russel, 2004; Escalada et al., 2005; Ahn et al., 2008; Dann and Hontela, 2011). As emerging contaminants, which are found extensively in surface waters and are known to bioaccumulate, these agents are receiving increasing scrutiny for their potential toxicity to wildlife and humans (Ahn et al, 2008; Dan and Hontela, 2011; Halden, 2014). Both TCS and TCC are also known to produce stable, but incompletely characterized photodegredates in the environment upon exposure to UV light (e.g., see Halden, 2014). Although toxicity to some aquatic life has been evaluated, to the best of our knowledge there have been no reports on behavioral effects of these agents in *Daphnia*.

Our preliminary behavioral study conducted in COMBO media compared the toxicity of TCC and TCS at two concentrations, 0.1µM and 10µM, over a 90-minute period of exposure. Evidence of acute toxicity was obtained in the swimming assay, indicating that both compounds caused a significant decrease in maximal accumulated distance and a significant increase in mean angle. These behavioral effects were not found to be concentration-dependent, suggesting that the two concentrations were equally toxic.

A more extensive concentration range that included UV-photodegradates of both TCC and TCS (provided by the laboratory of Yu-Ping Chin at Ohio State University), from 0.01nM to 1µM, was examined in the 5-day survival analysis study. This survival analysis used a standardized medium formulated by the EPA (http://water.epa.gov/scitech/methods/cwa/wet/upload/2007_07_10_methods_wet_disk2_atx7-

10.pdf). A significant time-and concentration-dependent decrease in survival was found that was also dependent on the specific type of chemical exposure. After 24-hours of exposure both TCC and P-TCS exhibited significant decreases in survival that progressively decreased over subsequent days of exposure. The highest 1µM concentration of TCC killed all animals within 24-hours of exposure. By the second day of exposure there was a significant decrease in survival of animals exposed to 0.1µM TCC. On the second day of exposure there was a significant decrease in the survival of animals exposed to TCS at the 10 nM concentration, and on the third and fourth day all of the concentrations from 1 nM to 1 µM showed a significant decrease in survival. On the second day there was a significant decrease in survival for the P-TCC exposed animals at 10 nM. On the first day of exposure there was a significant decrease in survivial for the P-TCS group at 0.1 and 1µM. By the fourth day survival was significantly decreased at 0.1 nM and 10 nM for P-TCS. These results indicate that TCC, TCS, P-TCC, and P-TCS exhibit significant toxicity at in the low nanomolar range.

Tamura et al., (2013) compared the toxicity of TCC, TCS, and other potential contaminants across three different aquatic organisms, green algae (*Pseudokirchneriella subcapitata*), *Daphnia magna*, and fish (*Oryzias latipes*). They calculated an EC_{50} for algae growth inhibition (72 hour exposure), an EC_{50} for Daphnia immobility (48 hour exposure), and an LC_{50} for fish (96 hour exposure). For TCC the rank order calculations for toxicity were: Daphnia (30nM) < green algae (90 nM) < fish (270 nM). For TCS the rank order calculations for toxicity were: green algae (17 nM) < Daphnia (620 nM) < fish (725 nM). There was a clear difference among species in terms of sensitivity to toxic effects and rank order was dependent on the type of chemical exposure, with fish generally being the least sensitive. In the first 24 hours of exposure for our survival experiment TCC also was more toxic than TCS. However, TCS

toxicity increased over time with a maximum effect on survival at 96 hours. In addition, the photolyzed TCC and photolyzed TCS exhibited significant toxicity at low nM concentrations.

A re-examination of the behavioral toxicity of TCC, TCS, P-TCC, and P-TCC in EPA medium covered the same concentration range as utilized in the survival analysis (0.01nM to 1μM). When the entire 24-hour time course was evaluated there were no significant concentration-dependent effects of any of the types of chemical treatments on swimming behavior. However, when the two parent compounds were compared there was a significant difference between TCC and TCS on both maximum accumulated distance and mean angle. When compared to pooled controls, it became apparent that TCC elicited the major behavioral effects relative to TCS, with a decrease in maximum accumulated distance and an increase in mean angle. These results would be consistent with that observed after the first day of exposure in the survival analysis. The outcome of the experiment comparing P-TCC and P-TCS is less clear since both treatments had mean values near the pooled controls for maximum accumulated distance, and some divergence from pooled controls at 24-hours with P-TCC higher and P-TCS lower than controls for mean angle. With the reduction in mean values for the P-TCS exposed animals after 24-hours of exposure in the survival experiment, an increase in mean angle might have been expected rather than a decrease in mean angle, but the effect on survival was not large at 24-hours and became larger with several more days of exposure.

The overall evaluation of TCC, TCS, and photolyzed compounds indicates that these are relatively toxic agents, which can affect survival in the nanomolar range. The 24-hour behavioral analysis was able to detect the effect of the highest 1 μM concentration of TCC that occurred within 24-hours, but the limitation in the evaluation of effects over time (24 hours), meant that later developing toxic effects on behavior could not be evaluated. The low concentrations

observed indicate that there should be concern about the environmental impact of TCC, TCS, P-TCC and P-TCS given there ubiquitous presence in water ways near urban areas with significant human populations.

CONCLUSION

In the study of contaminants with known biological targets, significant differences between behavioral and physiological response profiles for two different anticholinesterase agents were observed. One agent, physostigmine, that is rarely used therapeutically as a drug, and another agent, diazinon, which is an insecticide with a similar mode of action, exhibited a different time-course for their actions. The results with the anticholinesterase agents suggest that a more thorough analysis including other inhibitors commonly found as contaminants of emerging concern (e.g., chlorpyrifos) might provide new understanding of toxicokinetic factors that affect toxicity. A more complete understanding of toxicokinetic factors may enable better estimation of environmental risks. In addition the behavioral responses elicited by diazinon occurred at relatively low concentrations. In particular, the stimulatory effect of 0.125 nM diazinon on *Daphnid* swimming behavior may impact survival or fitness, and likely occurs at an environmentally relevant concentration.

The second phase of the study focused on contaminants of emerging concern with unknown biological targets. Triclosan and triclocarban are antibiotics commonly found in commercially available products, such as soap and toothpaste, and are now found in many waterways. These agents are known to be transformed into new and incompletely characterized chemicals. Experimental results obtained demonstrated that the parent compounds and the photolyzed compounds affected *Daphnid* survival at low environmentally relevant concentrations. The swimming assay was able to detect some early behavioral effects that may be useful indicators for the potential of some chemicals to affect fitness or survival. The mechanism for these toxic effects on *Daphnia* remains to be determined.

The advancement in technology and new analytical methods has enabled the detection of very low concentrations of chemical contaminants in the environment. More traditional toxicity

tests, such as LC_{50} test to estimate lethality have often focused on single contaminants and miss sub-lethal effects that may be expressed as changes in behavior. The potential risk from exposure to the contaminants of emerging concern must also address the issue of complex mixtures rather than being simply focused on single chemical agents. Since exposure may often involve complex mixtures rather than single chemical agents a more complete assessment of the impact of mixtures will be an ongoing challenge if we are to protect aquatic flora and fauna as well as human health.

APPENDIX A

Effect	Physostigmine: ANOVA with repeated measure(Time) Dependent variable = Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	955568.7	1	955568.7	1110.341	0.000000
Concentration	29951.0	5	5990.2	6.960	0.000011
Error	92945.7	108	860.6		

Effect	Physostigmine: ANOVA with repeated measure(Time) Dependent variable = Mean angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	11181249	1	11181249	2419.464	0.000000
Concentration	38391	5	7678	1.661	0.150114
Error	499109	108	4621		

Effect	Diazinon: ANOVA with repeated measure(Time) Dependent variable = Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	256516.3	1	256516.3	761.2355	0.000000
Concentration	6932.3	7	990.3	2.9389	0.009865
Error	21566.3	64	337.0		
TIME	15805.0	23	687.2	12.7666	0.000000
TIME*concentration	15465.7	161	96.1	1.7846	0.000000
Error	79231.7	1472	53.8		

Effect	Diazinon: ANOVA with repeated measure(Time) Dependent variable = Mean angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	9767199	1	9767199	1960.265	0.000000
Concentration	171040	7	24434	4.904	0.000178
Error	318886	64	4983		
TIME	164454	23	7150	14.559	0.000000
TIME*Concentration	194432	161	1208	2.459	0.000000
Error	722906	1472	491		

Effect	Diazinon: ANOVA with repeated measure(Time) Dependent variable = Accumulated distance_120 to 1440 minutes				
	SS	Degr. of Freedom	MS	F	p
Intercept	105160.4	1	105160.4	491.7294	0.000000
Concentration	11182.9	7	1597.6	7.4702	0.000001
Error	13686.9	64	213.9		
TIME	1224.7	11	111.3	2.2037	0.012799
TIME*concentration	5341.2	77	69.4	1.3730	0.023198
Error	35567.0	704	50.5		

Effect	Diazinon Physiology: ANOVA with repeated measure(Time) Parameters = HR, ABR				
	SS	Degr. of Freedom	MS	F	p
Intercept	*21326834*	*1*	*21326834*	*265.8946*	*0.000003*
Concentration	22698	2	11349	0.1415	0.870872
Error	481247	6	80208		
Parameter	*512568*	*1*	*512568*	*11.9403*	*0.013542*
Parameter*Concentration	57842	2	28921	0.6737	0.544564
Error	257567	6	42928		
TIME	*283037*	*21*	*13478*	*6.2048*	*0.000000*
TIME*Concentration	*241555*	*42*	*5751*	*2.6477*	*0.000016*
Error	273694	126	2172		
Parameter*TIME	*288466*	*21*	*13736*	*8.9704*	*0.000000*
Parameter*TIME*Concentration	*232927*	*42*	*5546*	*3.6216*	*0.000000*
Error	192947	126	1531		

Effect	Fluoxetine: ANOVA with repeated measure(Time) Dependent variable = Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	*207941.0*	*1*	*207941.0*	*383.5964*	*0.000000*
concentration	2492.9	7	356.1	0.6570	0.707251
Error	34693.3	64	542.1		
TIME	*2497.4*	*23*	*108.6*	*2.1975*	*0.000908*
TIME*concentration	*11319.5*	*161*	*70.3*	*1.4229*	*0.000738*
Error	72734.8	1472	49.4		

Effect	Fluoxetine: ANOVA with repeated measure(Time) Dependent variable = Mean angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	*10268045*	*1*	*10268045*	*1539.379*	*0.000000*
concentration	84384	7	12055	1.807	0.101111
Error	426896	64	6670		
TIME	*57357*	*23*	*2494*	*5.344*	*0.000000*
TIME*concentration	*139352*	*161*	*866*	*1.855*	*0.000000*
Error	686888	1472	467		

Effect	Parent Triclocarban and Triclosan in COMBO: ANOVA with re Dependent variable = Accumulated distance				
	SS	Degr. of Freedom		MS	F
Intercept	25542.40		1	25542.40	325.0469
Concentration	5.09		1	5.09	0.0648
Chemical	391.75		2	195.87	2.4926
Concentration*Chemical	20.09		2	10.04	0.1278
Error	1414.45		18	78.58	
TIME	1380.86		9	153.43	2.8030
TIME*Concentration	387.06		9	43.01	0.7857
TIME*Chemical	2305.10		18	128.06	2.3396
TIME*Concentration*Chemical	592.72		18	32.93	0.6016
Error	8867.37		162	54.74	

Effect	Parent Triclocarban and Triclosan in COMBO: ANOVA with re Dependent variable = Mean Angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	1540369	1	1540369	1414.026	0.000000
Concentration	0	1	0	0.000	0.995802
Chemical	10631	2	5315	4.880	0.020268
concentration*Chemical	2476	2	1238	1.136	0.342931
Error	19608	18	1089		
TIME	40351	9	4483	7.396	0.000000
TIME*Concentration	8742	9	971	1.602	0.118512
TIME*Chemical	33797	18	1878	3.097	0.000070
TIME*concentration*Chemical	12055	18	670	1.105	0.352036
Error	98210	162	606		

Effect	Parent & Photolyzed Triclocarban and Triclosan(survival data): ANOVA with repeated measure(Days) Dependent Variable = Survival(number of animnals				
	SS	Degr. of Freedom	MS	F	p
Intercept	6032.893	1	6032.893	1720.757	0.000000
Concentration	227.513	6	37.919	10.816	0.000000
Chemical	11.036	3	3.679	1.049	0.375210
Concentration*Chemical	244.058	18	13.559	3.867	0.000012
Error	294.500	84	3.506		
DAYS	148.161	3	49.387	141.426	0.000000
DAYS*Concentration	19.933	18	1.107	3.171	0.000026
DAYS*Chemical	7.982	9	0.887	2.540	0.008324
DAYS*Concentration*Chemical	39.924	54	0.739	2.117	0.000058
Error	88.000	252	0.349		

Effect	Parent Triclocarban and Triclosan: ANOVA with repeated meas Dependent variable = Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	*294660.5*	*1*	*294660.5*	*602.7290*	*0.000000*
Concentration	1362.3	7	194.6	0.3981	0.900815
Chemical	*7074.2*	*1*	*7074.2*	*14.4703*	*0.000277*
Concentration*Chemical	3586.1	7	512.3	1.0479	0.404923
Error	39110.2	80	488.9		
TIME	*3456.9*	*28*	*123.5*	*2.7927*	*0.000002*
TIME*Concentration	9952.2	196	50.8	1.1486	0.085416
TIME*Chemical	1093.6	28	39.1	0.8835	0.641706
TIME*Concentration*Chemical	9699.6	196	49.5	1.1194	0.132019
Error	99027.9	2240	44.2		

Effect	Parent Triclocarban and Triclosan: ANOVA with repeated meas Dependent variable = Mean Angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	*18308716*	*1*	*18308716*	*2821.808*	*0.000000*
Concentration	24490	7	3499	0.539	0.802282
Chemical	*78879*	*1*	*78879*	*12.157*	*0.000797*
Concentration*Chemical	76403	7	10915	1.682	0.125184
Error	519063	80	6488		
TIME	*36915*	*28*	*1318*	*2.879*	*0.000001*
TIME*Concentration	*108012*	*196*	*551*	*1.203*	*0.033533*
TIME*Drug chemical	16095	28	575	1.255	0.167804
TIME*Concentration*Chemical	*106957*	*196*	*546*	*1.191*	*0.041525*
Error	1025926	2240	458		

Effect	Photolyzed Triclocarban and Triclosan: ANOVA with repeated n Dependent variable = Accumulated distance				
	SS	Degr. of Freedom	MS	F	p
Intercept	*428414.2*	*1*	*428414.2*	*691.5678*	*0.000000*
concentration	3010.2	7	430.0	0.6942	0.676702
chemical	*3980.2*	*1*	*3980.2*	*6.4251*	*0.013227*
concentration*chemical	1299.3	7	185.6	0.2996	0.952006
Error	48939.1	79	619.5		
TIME	*11078.3*	*28*	*395.7*	*6.3309*	*0.000000*
TIME*concentration	12375.9	196	63.1	1.0103	0.449406
TIME*chemical	1854.2	28	66.2	1.0596	0.380371
TIME*concentration*chemical	12803.2	196	65.3	1.0452	0.325785
Error	138240.5	2212	62.5		

Effect	Photolyzed Triclocarban and Triclosan: ANOVA with repeated n Dependent variable = Mean Angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	*14509824*	*1*	*14509824*	*2748.306*	*0.000000*
concentration	40838	7	5834	1.105	0.368328
chemical	*61080*	*1*	*61080*	*11.569*	*0.001055*
concentration*chemical	38996	7	5571	1.055	0.400250
Error	417085	79	5280		
TIME	*188631*	*28*	*6737*	*11.306*	*0.000000*
TIME*concentration	135512	196	691	1.160	0.070840
TIME*chemical	*69098*	*28*	*2468*	*4.142*	*0.000000*
TIME*concentration*chemical	137807	196	703	1.180	0.050920
Error	1318010	2212	596		

Effect	Parent Triclocarban and Triclosan(pooled control): ANOVA with repeated meas Dependent variable = Accumulated distance				
	SS	Degr of Freedom	MS	F	p
Intercept	*351809.6*	*1*	*351809.6*	*755.1304*	*0.000000*
chemical	*10673.8*	*2*	*5336.9*	*11.4552*	*0.000032*
Error	48918.7	105	465.9		
TIME	*5259.6*	*28*	*187.8*	*3.9750*	*0.000000*
TIME*chemical	3390.4	56	60.5	1.2812	0.078932
Error	138931.1	2940	47.3		

Effect	Parent Triclocarban and Triclosan(pooled control): ANOVA with repeated meas Dependent variable = Mean Angle				
	SS	Degr. of Freedom	MS	F	p
Intercept	*17958837*	*1*	*17958837*	*2825.548*	*0.000000*
chemical	*137044*	*2*	*68522*	*10.781*	*0.000055*
Error	667367	105	6356		
TIME	*63250*	*28*	*2259*	*4.659*	*0.000000*
TIME*chemical	*40084*	*56*	*716*	*1.476*	*0.012653*
Error	1425331	2940	485		

Effect	Photolyzed Triclocarban and Triclosan(pooled control): ANOVA with repeated m Dependent variable = Accumulated distance					
	SS	Degr. of Freedom		MS	F	p
Intercept	*435325.9*		*1*	*435325.9*	*800.6472*	*0.000000*
chemical	*3697.8*		*2*	*1848.9*	*3.4005*	*0.037107*
Error	56546.6		104	543.7		
TIME	*11048.1*		*28*	*394.6*	*6.4072*	*0.000000*
TIME*chemical	2895.4		56	51.7	0.8396	0.796489
Error	179328.7		2912	61.6		

Effect	Photolyzed Triclocarban and Triclosan(pooled control): ANOVA with repeated m Dependent variable = Mean Angle					
	SS	Degr. of Freedom		MS	F	p
Intercept	*15624897*		*1*	*15624897*	*3033.883*	*0.000000*
chemical	*63026*		*2*	*31513*	*6.119*	*0.003074*
Error	535614		104	5150		
TIME	*166020*		*28*	*5929*	*9.888*	*0.000000*
TIME*chemical	*84950*		*56*	*1517*	*2.530*	*0.000000*
Error	1746200		2912	600		

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ABSTRACT

CONTAMINANTS OF EMERGING CONCERN: EFFECTS OF KNOWN NEUROACTIVE AGENTS, ANTIBIOTICS, AND CHEMICALLY UNCHARACTERIZED PHOTODEGRADATES ON BEHAVIOR AND PHYSIOLOGY ON *DAPHNIA PULEX*

by

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Degree: Master of Sciences

Emerging contaminants such as pharmaceuticals and personal care products (PPCPs), herbicides, pesticides, plasticizers, fire retardants, polycyclic aromatic hydrocarbons (PAHs), and other organic waste are increasingly being detected in surface water and ground water. These contaminants can enter into the environment through wastewater treatment plant effluent and agriculture runoff. Many of these emerging contaminants tend to be biologically active at very low concentrations, typically occur in water as part of complex mixtures, and may impact biota at concentrations not detected using traditional toxicity tests (e.g. LC 50 tests).

This study focuses on utilizing novel *Daphnid* optical bioassays to examine the toxicity of selected emerging contaminants. The chemicals selected for this study are found in surface water. Chemicals that are known neuroactive agents were selected. Anticholineesterase, physostigmine(drug) and diazinon(insecticides)[cholinergic system], and the antidepressant, fluoxetine[serotonergic system]. Triclosan and triclocarban are antibiotics that are used in antibacterial soaps and a number of other products, and these chemicals and their transformation products resulting form photodegredation. They do not have any known biological targets within

Daphni. Two different optical tracking assays were used to evaluate changes in *Daphnid* swimming behavior *Daphnid* cardio-respiratory function as a result of exposure to diazinon,fluoxetine, triclosan, triclocarban and photodegredates of the two antibiotics.

AUTOBIOGRAPHICAL STATEMENT

I was born and bought up in the city of Mumbai, India. I have loved and admired science since I was a child, especially the biology and chemistry part and therefore decided to take pharmacy as my major. I graduated from University of Mumbai with a Bachelor's in Pharmacy and decided to pursue my masters in United States of America. I researched many programs and university and found that Wayne State University met most of needs and the best was that it was an interdisciplinary program. After doing rotations I joined the laboratory of Dr. Pitts and I was very happy to do so. This lab taught me what an interdisciplinary study actually means since it also involved understanding from the environmental engineering and biological sciences perspective.

When I look back to my first day in Wayne State University and when I look at myself now I can see how much this journey has made me learn and grow as an individual. I am very excited to be graduated and climb the ladder further ahead in my career path. I thank everyone who has helped me through this journey