5-2019

"From Single Chemicals to Complex Mixtures": Effect of Contaminants of Emerging Concern on Three Life Stages of Pimephales Promelas

Utku Hasbay
haut1101@stcloudstate.edu

From Single Chemicals to Complex Mixtures: Effect of Contaminants of Emerging Concern on Three Life Stages of *Pimephales promelas*

by

Utku Hasbay

A Thesis

Submitted to the Graduate Faculty

of St. Cloud State University

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science in

Biology: Cellular and Molecular

May, 2019

Thesis Committee:
Heiko L. Schoenfuss, Chairperson
Satomi Kohno
Nathan Bruender
Edward J. Perkins

Abstract

Aquatic species are exposed to a diverse class of contaminants of emerging concerns (CECs) throughout different life stages. In this study, the effects of CECs in increasing complexity on three life stages of fathead minnows (*Pimephales promelas*) were assessed using existing Great Lakes tributaries' chemical occurrence and concentration data. Fathead minnows were exposed to either a water solvent control, or the following chemicals: 4-nonylphenol (surfactant), 5-methyl-1H-benzotriazole (corrosion inhibitor, anti-freezing agent), atrazine (herbicide), bisphenol-a (plasticizer), desvenlafaxine (antidepressant), fexofenadine (allergy medication), estrone (hormonal medication), metformin (antidiabetic medication), metolachlor (herbicide), *N,N*-diethyl-m-toluamide (insect repellent), sulfamethoxazole (antibiotic), tris(2-butoxyethyl) phosphate (flame retardant), fluoranthene (byproduct of organic raw material pyrolysis), imidacloprid (insecticide), triclosan (antibacterial), ibuprofen (anti-inflammatory medication), 17-beta estradiol (hormonal medication), 2,4,6-trinitrotoluene (energetic), 2,4-dinitroanisole (energetic), 2,6-diamino-3,5-dinitropyrazine-1-oxide (energetic), 2,4,6-trinitro-3-bromoanisole (energetic). All chemicals were used in exposures singly and in mixtures of different complexity. Concentration series for the exposures was centered on medium concentration which contained the highest environmentally measured concentrations while low exposure used $1/10^{th}$, ultra-low $1/100^{th}$ and the high exposure was set to 10x the medium exposure concentration. Adult and larval exposures were conducted simultaneously, while embryonic exposures were conducted at a later time using the same exposure waters. The apical endpoints for the study were survival, overall health, and several reproductive behaviors for adult fathead minnows. Survival, and feeding efficiency data were collected for larval fathead minnows. Lastly, time-to-hatch, and developmental abnormality for fathead minnow embryos were also recorded. Results suggest that the 96 hours CEC exposures affect different apical endpoints depending on the exposed life stage. As the complexity of the chemical exposure increased, alterations in endpoints such as courtship behavior in adult fathead minnows became more frequent. Medium and high concentrations elicited the greatest effects. In both single chemical and mixture exposures, concentration-dependent responses were not observed. This study highlights the need for complementary studies at different exposure time points as well as *in vivo* studies to identify potential "biological fingerprints" of single chemical effects in complex mixtures.

Table of Contents

Chapter Page

List of Tables

List of Figures

Chapter 1: Literature Review

Introduction

After decades of mostly unregulated disposal of contaminants of emerging concerns (CECs) such as pharmaceutical, hormonal, agricultural, and industrial compounds, many of these compounds have now reached detectable concentrations and, in some instances, have been linked to surface water toxicity (Lee et al., 2014). One substantial pathway for CECs to reach surface waters is wastewater treatment plant effluents (Heberer, Reddersen, & Mechlinski, 2002). CECs affect the health of aquatic organisms while there is a little information known about their inclusive harm to the ecosystem (Kolpin et al., 2002; Levine et al., 2017). To address this issue, a relatively new approach, adverse outcome pathway (AOP) analysis has been developed to understand the effects of recently detected or long-time prevalent CECs to aquatic organisms (Villeneuve et al., 2014). AOPs are beneficial to combine diverse biological findings in toxicology to interpret further potential responses of aquatic organisms that are under exposure risk of CECs to reduce organismal assessments (Ankley et al., 2010). To support these efforts, many studies have been conducted with single CECs to reveal their effect on aquatic organisms. However, there is a dearth of inclusive approaches to explain the effects of complex CEC mixtures, as they are common in the environment, across life stages (Oliveira, Domingues, Koppe-Grisolia, & Soares, 2009; Parrott, Alaee, Wang, & Sverko, 2013). To remedy this shortcoming, a collaborative effort involving federal agencies and universities was developed as part of the Great Lakes Restoration Initiative (GLRI) (Wattigney, Zheng, & Regin-Wilson, 2017). One outcome of the GLRI project was the collection of nearly 300 surface-water and 80 sediment samples from field sites around the Great Lakes (Elliot et al., 2017). A two-way cluster

analysis of the data matrix gained from these collections determined the presence and concentrations of mixtures of co-occurring CECs. Based on surrounding land-use characteristics, two principal mixtures, one representing urban CECs, and the other agricultural CECs, were identified (Elliott et al., 2017). Using these two mixtures of CECs as starting point, two long-term (over 300 days), multigenerational fathead minnow (*Pimephales promelas*) exposure experiments were conducted at the St. Cloud State University Aquatic Toxicology Laboratory to understand population level effects of CECs on fathead minnows (Cipoletti, 2018; Wang, 2017). The need for building a connection between these mixture exposure studies and the individual CECs represented in the mixtures, gave rise to the current study.

This study is aiming to link findings from the two multigenerational studies to short-term effects of CECs, singly and in mixture. To accomplish this objective, a series of short-term exposure experiments were conducted with the same CECs used in the long-term exposure studies. Fathead minnows were the organism of choice for these studies as they are a common native species of North America and widely used in toxicological research and testing (Nelson & Paetz, 1992). They can live in a variety of habitats including small ponds, streams, and lakes with high fish diversity (Becker, 1983; Pflieger, 1975; Smith, 1978). Fathead minnows are a critical member of their food web (Becker, 1983; Nelson & Paetz, 1992), as they can tolerate temperatures up to 33 Celsius (^{o}C) (Moyle, 1976), oxygen levels lower than 5.0 mg/L (Brungs, 1971) along with a wide range of pH levels (4-9.1) (McCarraher & Thomas, 1968; Mount, 1973). Assessing embryonic, larval, and adult life stages of fathead minnows, exposed to the same CECs, will aid in identifying the most sensitive stage at the molecular, organismal, and apical level of biological organization.

Fathead minnows were exposed to 20 chemicals singly, as well as in 20 mixtures of increasing complexity for 96 hours following established toxicity testing guidelines (Ankley & Villeneuve, 2006). Longer exposures can initiate many adverse pathways, but could also lead to compensation reactions within fish, while shorter exposures may not gather the full range effects (Villeneuve et al., 2009). Therefore, the goal of the current study was to assess the complexity of CEC exposure effects by adding different chemical classes together in a mixture. Each CEC and its mixtures were studied at five concentrations to assure that a range of measured environmental concentrations were covered within each exposure. CECs used in this study fell into one of several broad use categories.

Urban Contaminants and their Effects on Aquatic Organisms

Among CECs, pharmaceuticals stand out due to their ubiquitous presence in aquatic environments and their inherent biological activity. One of these ubiquitous CECs is the pharmaceutical ibuprofen. Previous studies researching toxicity of ibuprofen found read-across evidence for adverse effects in on fathead minnows. Prostaglandin E metabolite levels were significantly decreased in gill tissues of fathead minnows exposed to 370 and 470 μg/L of ibuprofen due to its non-selective inhibitory effects on cyclooxygenase enzymes (Patel et al., 2016). Evaluation of 25 mg/L ibuprofen exposure using zebrafish, *Danio reiro*, showed increasing activity of glutathione S-transferase and glutathione peroxidase enzymes in whole body homogenate (Bartoskova et al., 2013). In addition, Japanese medaka, *Oryzias latipes*, exposure to ibuprofen for six weeks increased their egg production while simultaneously reducing spawning events (Flippin, Huggett, & Foran, 2007). Another category of CECs used in the current study included steroidal hormones such as estrone (E_1), which preferentially binds to

estrogen receptor 1 (ESR1; former ERα) and activates it (Zhu, Han, Shim, Wen, & Jiang, 2006). E_1 was documented in a study in the Duluth-Superior Harbor at over 40 ng/L (Cavallin et al., 2016). A 21-day exposure of larval fathead minnows to 125 ng/L E_1 reduced predator avoidance and feeding performance (Bird, 2015). In addition to the feminizing effect of E_1 on male fathead minnows, E_1 can also be converted to 17β-estradiol, thus resulting in elevated measurements of that steroidal hormone in plasma of male fathead minnows (Ankley et al., 2017). *Rutilus rutilis* exposure to 17α-ethinylestradiol at concentrations as low as 50 ng/L showed reduced foraging success, which was calculated as the sum of D. magna captured within a given interval of time (Hallgren et al., 2014). Furthermore, a study on Bisphenol-A (BPA), reported reduced sperm abundance, and intersex in male white suckers (*Catostomus commersoni*) downstream of a wastewater treatment plant effluent that contained BPA along with other steroidal estrogens (Vajda, Barber, Gray Lopez, Woodling, & Norris, 2008).

Agricultural Contaminants and their Effects on Aquatic Organisms

Another CEC category included in the current study are agricultural sourced compounds such as the herbicide atrazine. Although fathead minnows exposed to atrazine at 20 or 250 µg/L did not exhibit any significant developmental alterations (Scahill, 2008), atrazine exposure affected testis maturity negatively, despite not showing a strong estrogenic effect in a 21-day exposure (Bringolf, Belden, & Summerfelt, 2004). Industrial chemicals such as fluoranthene were also a category of the CECs used in the current study. Previous studies have shown that fathead minnows exposed to fluoranthene below 8.6 µg/L could suffer lethal effects. In addition, fluoranthene exposure at environmentally relevant concentrations coupled with hypoxia causes lordosis in zebrafish larvae (Farr, Chabot, & Taylor, 1994; Matson, Timme-Laragy, & di Giulio,

2008). Among other CECs commonly measured in the environment and included in the current study was the insect repellent DEET (N,N-diethyl-m-toluamide). A 28-day exposure of common carp (*Cyprinus carpio L.*) to DEET caused changes in hematological parameters including increased red blood cells, decreased mean corpuscular volume, and mean corpuscular hemoglobin value in the concentration of 1 mg/L (Slaninova et al., 2014).

Energetic Contaminants and their Effects on Aquatic Organisms

Lastly, a group of nitro toluene-based energetic or munition compounds were also included in the current study as their presence in some aquatic environments has raised concern. Munition components can find their way into wastewater effluent near munition production plants (Coiner, Pope, & Menl, 2010) and may cause adverse biological effects. For instance, 2,4,6-trinitrotoluene (TNT) has been documented to bioaccumulate in visceral tissues and spleen during a 12-hour exposure. Detrimental effects on exposed fathead minnows were recorded at concentrations as low as 2.58 mg/L (Smock, Stoneburner, & Clark, 1976; Yoo, Lotufo, Gibson, Steevens, & Sims, 2006).

Mixture Effects on Aquatic Organisms

Integrating these diverse categories of CECs will aid in enhancing our understanding of biological effects of complex mixtures and will guide the development of improved AOPs to examine whether single CEC effects are conserved in complex mixture exposures.

Although progress has been made in developing AOPs for single CEC exposures, many recent publications in ecotoxicology highlight the need for more data on mixture of CECs (Garcia-Reyero et al., 2009; Parrott & Bennie, 2009; Schultz et al., 2013). AOPs can confirm more realistic predictions for possible outcomes of complex mixtures from single CEC

exposures. The current study can bridge the knowledge gap between short term, single CEC and complex mixture exposures while revealing biological impacts on fathead minnows. Our results can potentially help determine the effects of CECs on fathead minnow population.

Chapter 2: Exposure to Increasingly Complex Mixtures of Cecs Does Not Alter Apical Effects in Exposed Fathead Minnows Proportional to the Number of Chemical Classes in the Mixtures

Introduction

Analysis of surface water and bottom sediment samples from the U.S. Great Lake area from 2010 to 2014 showed that urban and agricultural runoff are two key contributors to environmental contamination (Elliot et al., 2017). In the environment CECs are present as complex mixtures (United States Environmental Protection Agency [USEPA]. 1987). A complex mixture can be defined as a mixture of CECs from different classes which would be able to trigger different mechanisms of actions as individual constituents. To date it has been difficult to link mixture toxicity to each mixture components' toxicities and interactions (Groten, Feron, & Sühnel, 2001). Various studies tried to correlate single chemical exposure impacts using simple as well as complex mixture exposures, yet the data published provided contradictory conclusions (Brodin, Fick, Jonsson, & Klaminder, 2013; Jukosky, Watzin, & Leiter, 2007; Klaminder, Jonsson, Fick, Sundelin, & Brodin, 2014; Lin & Janz, 2006; Sun, Zha, & Wang, 2009). All these studies emphasize the extremely challenging nature of determining the biological effects of CECs in mixtures.

Hypothesis

Diverse classes of chemicals were used in this study to understand CEC effect on fish as we build the complexity of chemical exposures. It was hypothesized that exposure to increasingly complex mixtures of CECs will alter apical effects in exposed fathead minnows proportional to the number of chemical classes in the mixtures. Furthermore, this hypothesis was harmonized into three questions:

1. What life stage is most susceptible?
2. Are mixture effects greater than the effects of any single chemical?
3. Is there a biological “fingerprint” of a single chemical’s effect in complex mixtures?

Methods and Materials

Chemistry. All chemicals were prepared in concentrated stock solutions. Mixtures were aggregated using the single stock chemicals in concentrated stock solutions. Confirmatory chemistry through high-performance liquid chromatography was conducted by the United States Army Corps of Engineers laboratory in Vicksburg, Mississippi. Ultra-pure water was used to prepare both single and mixture stocks. Nominal exposure concentrations were separated by ten-fold increments. As only water was used to dissolve chemicals, a control group only receives filtered well water was included in all exposure experiments.

Table 2.1

List of Exposure Chemicals and Nominal Concentrations Showing all Chemicals Along with their Class Identification and Medium Nominal Concentration

Che. Class	Chemicals	Use Class	M. Con.(ng/L)
Urban	Desvenlafaxine	Antidepressant	583 ↓
	Fexofenadine	Antihistamine	1000
	Metformin	Antidiabetic	1210
	Sulfamethoxazole	Antibiotic	559
	Ibuprofen	Anti-inflammatory	440
	Triclosan	Antibacterial	0.5
	5-methyl-1H-benzotriazole	Industrial	6680
	Fluoranthene	Polycyclic aromatic hydrocarbon	0.1 ↓
Co-occuring	N,N-Diethyl-m-toluamide	Insect repellent	1600
	4-Nonylphenol	Surfactant	3710
	Bisphenol A	Plasticizer	600
	Estrone	Steroid/hormone	24
	Tris(2-butoxyethyl) phosphate	Flame retardant	21,000
Agriculture	Atrazine	Herbicide	400 ↓
	Metolachlor	Herbicide	170 ↓
	Imidacloprid	Insecticide	140
Energetic	2,4,6 trinitrotoluene	Munition	1500
	2,4-Dinitroanisole	Munition	1000
	2,6-diamino-3,5-dinitropyrazine-1-oxide	Munition	1000
	2,4,6-Trinitro-3-bromoanisole	Munition	1000

Table 2.2

Exposure Concentration Series Showing Concentration Series that was Established in Ten-Fold Increments. Medium Concentration was the Highest Detected Environmental Concentration (Elliott et al., 2017)

Ultra-low (1/100)
Low (1/10)
Medium (Environmental Concentration)
High (10x)

Table 2.3

Constituents of Exposure Mixtures Showing the Contributions of the Single Chemicals to Complex Mixtures

Che. Class	Chemicals	U-I	U-II	AgM	#01	#02	#03	#04	#05	#06	#07	#08	#09	#10	#11	#12	#13	#14	#15	#16	#17
Urban	Desvenlafaxine																				
	Fexofenadine																				
	Metformin																				
	Sulfamethoxazole																				
	Ibuprofen																				
	Tridosan																				
	5-met.-1H-benzotri.																				
	Fluoranthene																				
Co-occuring	N,N-Diet.-m-toluamide																				
	4-Nonylphenol																				
	Bisphenol A																				
	Estrone																				
	Tris(2-but.eth.) phos.																				
Agriculture	Atrazine																				
	Metolachlor																				
	Imidadoprid																				
Energetic	2,4,6 trinitrotoluene																				
	2,4-Dinitroanisole																				
	LLM-105																				
	2,4,6-Trinitro-3-bro.ani.																				

Each chemical listed in the second column was used in a single compound exposure experiment at four concentrations. Additional mixture composition was as follows: 1st mixture column: urban mixture (U-I); 2nd: urban mixture (U-II) which constitutes all the co-occurring chemicals in addition to the urban mixture-I. 3rd: agricultural mixture (AgM). The remaining columns highlight the additional seventeen mixtures prepared. Light green indicates absence of a

chemical class. Columns one through three, 17 and 19 were the exposures that were run in concentration series while rest of the mixtures were run at medium concentration. All exposures included a control group.

Water samples from each exposure was collected on day one and four for confirmatory analytical chemistry. Samples were collected in duplicate from the inflow of fish tanks in amber vials (20 mL Amber Borosilicate Vial, C&G Containers, Inc., Lafayette, LA). Twice a week (first and third day) exposure conditions were monitored for dissolved oxygen (mg/L, Pro 1020, YSI Incorporated, Yellow Springs, OH), temperature (°C), and pH along with other water quality indicators such as total chlorine, general hardness and alkalinity using test strips (5 in 1 Water Quality

Test Strips Cat. 27552-50, HACH, Loveland, CO).

Exposure design. Adult fathead minnow flow-through exposures were set up at St. Cloud State University in an IACUC approved facility (#8-82; 8-107). Adult fish were shipped from Environmental Consulting & Testing (Superior, WI) overnight to St. Cloud. The stock chemical solution was thawed on Monday morning and added into an opaque carboy (3 Gallon Carboy Glass, Northern Brewer, St. Paul, MN) which was filled with 25 L of heated (25-26 ºC) and filtered well water. This solution was then agitated to assure complete mixing. 10-fold dilutions were made for a concentration series exposure and the volume in each carboy were filled back up to 25 L to reach desired nominal concentration. This exposure solution was then directly pumped into the exposure aquaria via silicone tubing (Pentair Aquatic Ecosystems, Apopka, FL) for a daily exchange rate of 4 L/day. Each aquarium had six sections divided by an opaque glass piece and each section housed a male fathead minnow. Aquaria held 10 L of the

exposure solution n=6. For concentration series exposures one aquarium per concentration was used, while for mixed chemical exposures only medium concentration with two control aquaria per treatment were used (n=12 male per treatment). Each exposure replicates contained a nest tile made from a dense core PVC to allow the animal to establish a breeding territory. The aquaria were aerated (Sweetwater Air Diffusers AS1, Pentair Aquatic Ecosystems, Apopka, FL) to maintain dissolved oxygen levels greater than five parts per million. A grid outline was placed under the aquaria to allow for the quantification of behavioral responses. Each exposure was maintained for 96 hours, with being fed twice daily *ad libitum* with a 2:1 mixture of adult brine shrimp and blood worms (Brine Shrimp Direct, Ogden, UT). At the end of 96 hours, adult fathead minnows were euthanized, and their liver, gonad, muscle, gill and brain tissues were excised. Total length, standard length, weight (Ohaus Scout Pro 0.1g, Parsippany, NJ), secondary sex characteristics along with a blood sample per fish were also collected.

For larval fathead minnow exposures, fish (<24hrs post-hatch) were shipped overnight and upon arrival randomly distributed among exposure enclosures to provide 10 larvae per replicate (n=60 per treatment). Each section of the aquaria held 10 larvae (n=60 per treatment). Larval fish was fed twice daily *ad libitum* with hatched brine shrimp (Brine Shrimp Direct, Ogden, UT). Following the 96-hour, larval fish was tested for feeding or predator avoidance performance. After completion of the performance trials, fish were euthanized with 0.1% MS-222 (Argent Laboratories, Redmond, WA) in accordance to established IACUC protocols.

Embryonic exposures were conducted using an in-house breeding system. Eggs (<12hrs old) were collected and transferred to a 24 well plate (VWR International, Radnor, PA). Each well was filled two milliliters treatment solution. A total of 24 eggs were tested per treatment and

concentration exposure plates were maintained in a laminar flow hood to avoid contamination with airborne mold. Exposure solution in each well was exchanged by 50% by volume every day. After hatching, embryos were euthanized with MS-222 and 10% neutral buffered formalin was used for preservation.

Biological endpoints. Following exposure, adult fish were evaluated for their ability to defend a nest site (aggression assay) and their ability to court a female conspecific using established and published protocols (Cipoletti, 2018). The following formulas were derived to quantify fish behavior (Schultz, Bartell, & Schoenfuss, 2012).

Nest Defense Aggression = [(Sample size-mortality) / (time to onset attacks)] x number of attacks in the ensuing 300 seconds.

with: Number of attacks = Charges + butting

Courtship Performance Assay = [(Sample size-mortality) / (time to onset of courting+ (300 seconds - interest)] x number of courting behaviors in the ensuing 300 seconds.

with: Number of courting behaviors = broad side display + tubercle use

with: Interest = interaction time within 2 cm of female fish

In addition, a series of morphological parameters were calculated for each exposed male fish. These measures included the body condition factor (BCF), calculated as [body weight (g) / total length (mm)]; hepatosomatic index (HSI), calculated as [liver weight (g) / total weight (g)] * 100; and gonadosomatic index (GSI) calculated as [gonad weight (g) / total weight (g)] * 100. In addition, blind observations were made on secondary sex characteristics (Parrott, Wood, Boutot, & Dunn, 2003; Smith, 1978). A TRUEbalance blood glucose meter (Moore Medical LLC, Farmington, CT) was used to measure blood glucose (mg/dL). A blood sample was

collected in a micro-hematocrit capillary tube (Fisher Brand, Pittsburgh, PA) and percent hematocrit was obtained (Cox, 2016). Dissected gill, gonad and muscle tissues were flesh frozen in liquid nitrogen and stored at -80 ^{o}C, while liver, and brain tissues were stored in ribonucleic acid stabilization solution for further shipment to collaborating laboratories.

At the end of 96 hours of exposure larval fish were tested for foraging performance by using a feeding efficiency assay. For this assay, randomly paired larvae were placed in a well (Costar 3516, Corning Incorporated, Corning, NY) that contains 10 milliliters of exposure water. Pre-counted live hatched brine shrimp was administered to each of these wells and larvae were given one minute to capture prey items. Remaining brine shrimp were counted to calculate percentage of consumed prey.

For embryonic exposures developmental abnormalities were quantified in each exposure (Cipoletti, 2018). The analysis was based on the sum of the developmental ratings where 5 was minimum and 15 was the maximum possible abnormality. Lastly, time-to-hatch was recorded for each egg.

Statistical analysis. All biological endpoints were tested for the assumption of normality using the Shapiro-Wilk test as well as equal variances (Levene's test). All ratios (BCF, GSI, HSI) were arcsine transformed for analysis. Hematocrit was log-transformed prior to analysis. The variables BCF, GSI, HSI, hematocrit, glucose, and sum of sex characteristics were analyzed using a one-way analysis of variance (ANOVA) to test effects of treatment. Tukey's post-test was used for all pairwise comparisons. For all tests the statistical significance level of $p \leq 0.05$ was used.

Results

Exposure water chemistry.

Table 2.4

Confirmatory Chemistry Results for Single Stock Chemicals Showing

#	Chemical	Nominal (mg/mL)	Measured (mg/mL)	% of nominal
1	N,N-diethyl-m-toluamide	2.8	2.8	100.0
2	5-Methyl-1H-benzotriazole	5	4.96	99.2
3	DNAN	0.01	0.0101	101.0
4	2,4,6-trinitrotoluene	0.005	0.00472	94.4
5	Metformin	3.7	3.47	93.8
6	Sulfamethoxazole	0.6	0.56	93.3
7	Tris(2-butoxyethyl) phosphate	1.1	1.01	91.8
8	Bisphenol-A	0.11	0.0971	88.3
9	Estrone	0.1	0.115	115.0
10	Ibuprofen	0.02	0.0238	119.0
11	Triclosan	0.029	0.0234563	80.9
12	Imidacloprid	0.4	0.496	124.0
13	4-Nonylphenol	0.013	0.009	69.2
14	Metolachlor	0.53	0.28	52.8
15	TNBA	0.005	0.00121	24.2
17	Fluoranthene	0.0004	0.00003	7.5
18	Atrazine	0.34	0.0224	6.6
19	Desvenlafaxine	0.3	0.0000286	0.0
20	LLM-105	0.01	0.043	430.0
21	Fexofenadine	0.0007	0.00723507	1033.6

Confirmatory chemistry results for single stock chemicals showing the single chemical nominal exposure concentrations [Mg/Ml] for stock chemicals along with their confirmatory chemistry results. Green colored chemical stocks were within the 30% of the nominal concentration. 4-Nonylphenol was near this threshold. Eight chemicals had higher than 30% difference between their nominal and measured concentration (yellow).

Adult fathead minnow exposure effects. Body condition factor and hepatosomatic indices showed no significant effect between treatments across all exposure experiments. The gonadosomatic index differed significantly for medium concentration atrazine exposed male compared to control fish. Two mixtures also showed significant differences between treatments. The high concentration of the Urban Mix-II, and medium concentration of the Mix-9 both differed from their respective controls.

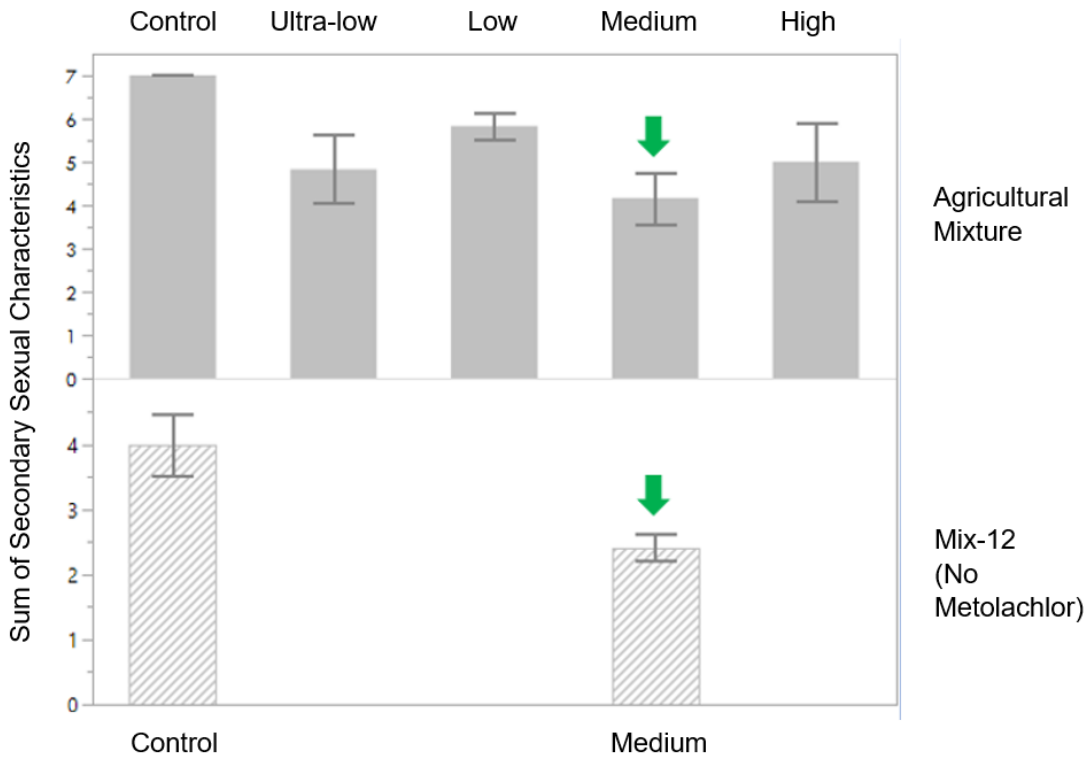

Figure 2.1. Adult fish secondary sexual characteristics

Adult Fish Secondary Sexual Characteristics showing the mean sum and standard error for secondary sexual characteristics in exposed fathead minnows. Green arrows indicate statistically significant difference. Agricultural mixture (n=6) and Mix-12 (n=12) showed significantly decreased secondary sex characteristic at medium concentration ($p \leq 0.05$).

Table 2.5

Adult Fish Survival Showing % Survival for each Treatment Chemical and Concentration

For instance, the 4-Nonylphenol exposure had one dead fish at high concentration.

Chemical Exposure	83%
4-Nonylphenol	High
5-methyl-1H-benzotriazole	Ultra-low, Medium
Bisphenol-A	Medium
Estrone	Low
Fluoranthene	High
N,N-Diethyl-m-toluamide	High
Tris(2-butoxyethyl) phosphate	Low, High
MIX-04 (no sulfamethoxazole)	Medium
MIX-16 (all chemicals)	Low

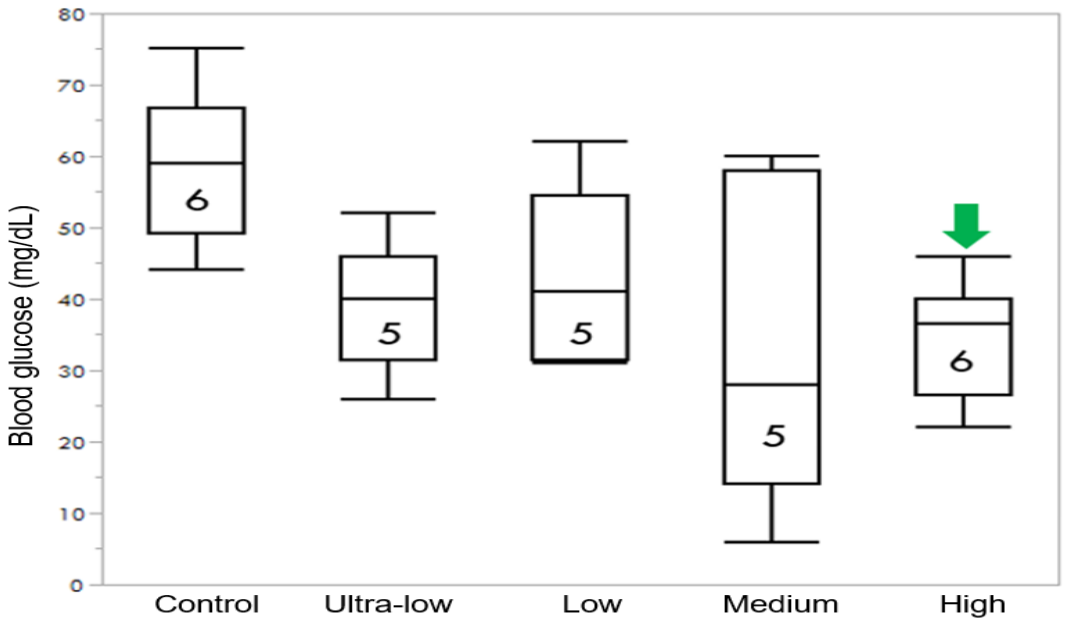

Figure 2.2. Bisphenol-A exposure glucose measurements

Bisphenol-A Exposure Glucose Measurements indicated by boxplot representations (25-75 percentile, mean, data range) of blood glucose concentrations (mg/dL) for male fish exposed

to Bisphenol-A at four concentrations. Green arrow indicates the statistically significant decrease at high concentration ($p \leq 0.05$). The sample size indicated in each box.

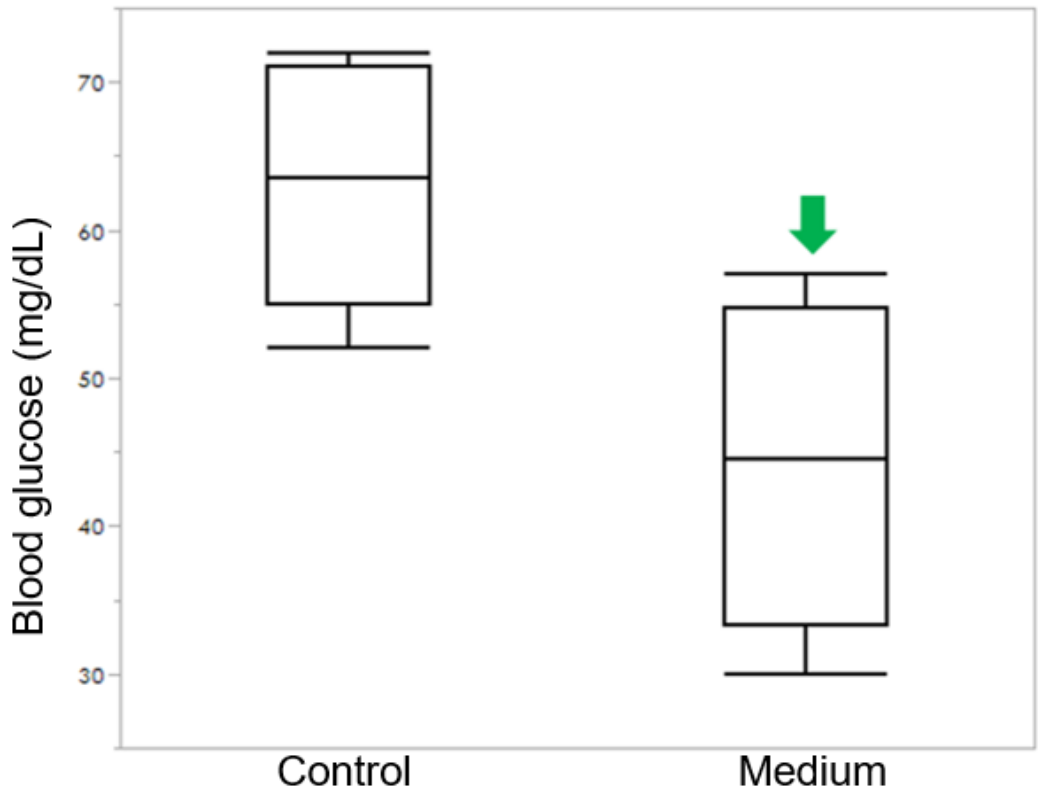

Figure 2.3. Mix-13 exposure glucose measurements

Mix-13 Exposure Glucose Measurements with boxplot (25-75 percentile, mean, data range) of blood glucose concentrations (mg/dL) for male fish exposed to Mix-13 at medium concentration (n=12). Green arrow indicates the statistically significant decrease ($p \leq 0.05$).

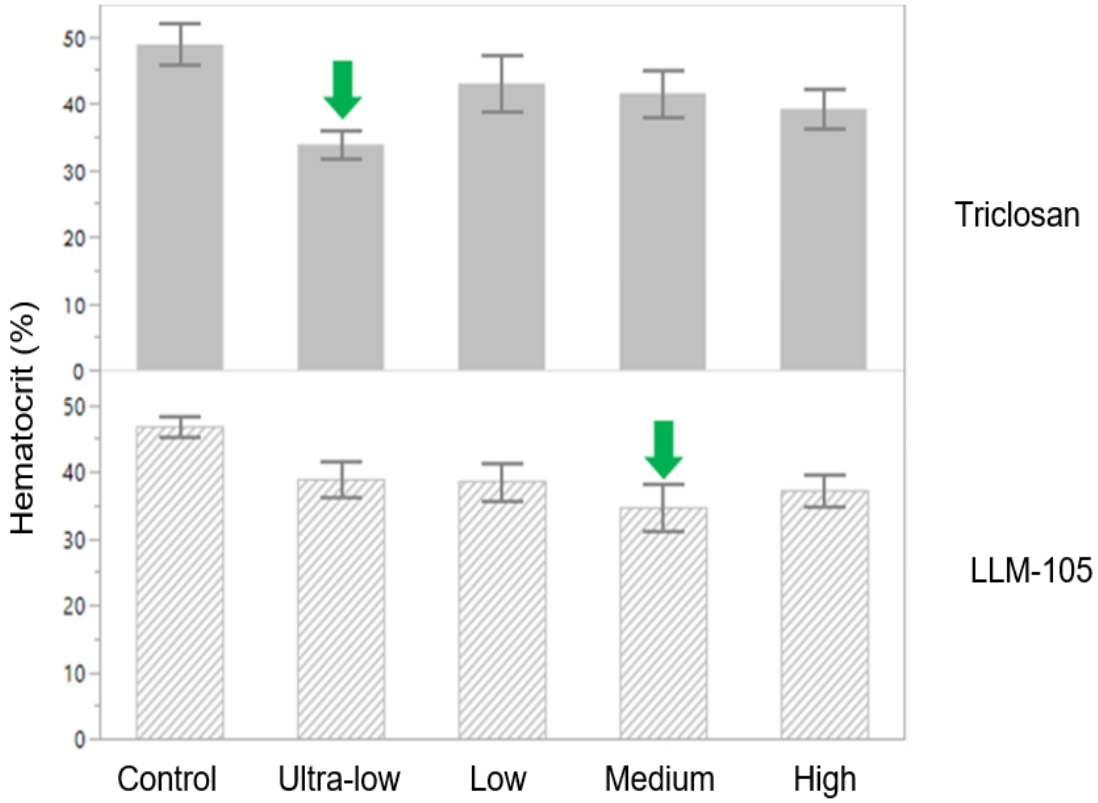

Figure 2.4. Hematocrit ratios of Triclosan and LLM-105 exposures

Hematocrit (% of plasma to red blood cells), of triclosan (n=6), and LLM-105 (n=6). Green arrows indicate that ultra-low of triclosan and medium of LLM-105 concentrations were significantly lower than control group ($p \leq 0.05$).

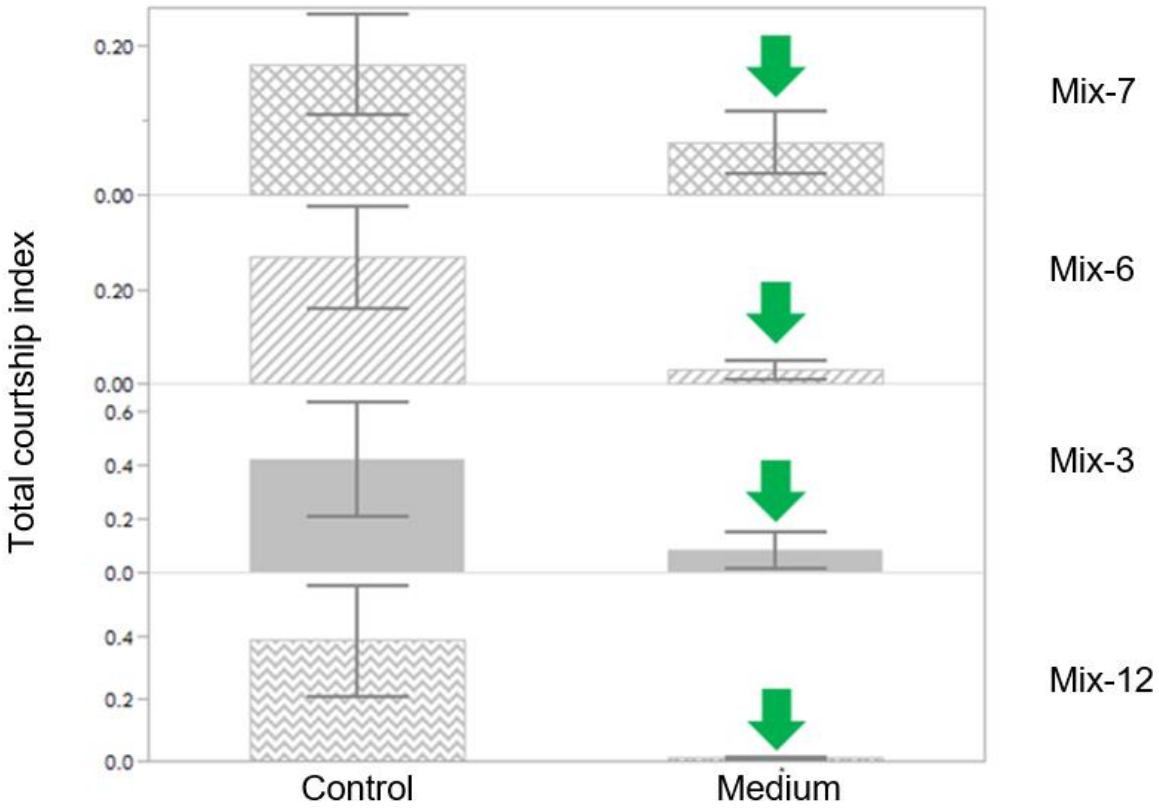

Figure 2.5. Courtship data for Mix-3, 6, 7 and 12

Courtship data with standard error bars showed significantly reduced overall response at medium concentration for Mix-7, Mix-6, Mix-3, and Mix-12 (n=12) ($p \leq 0.05$).

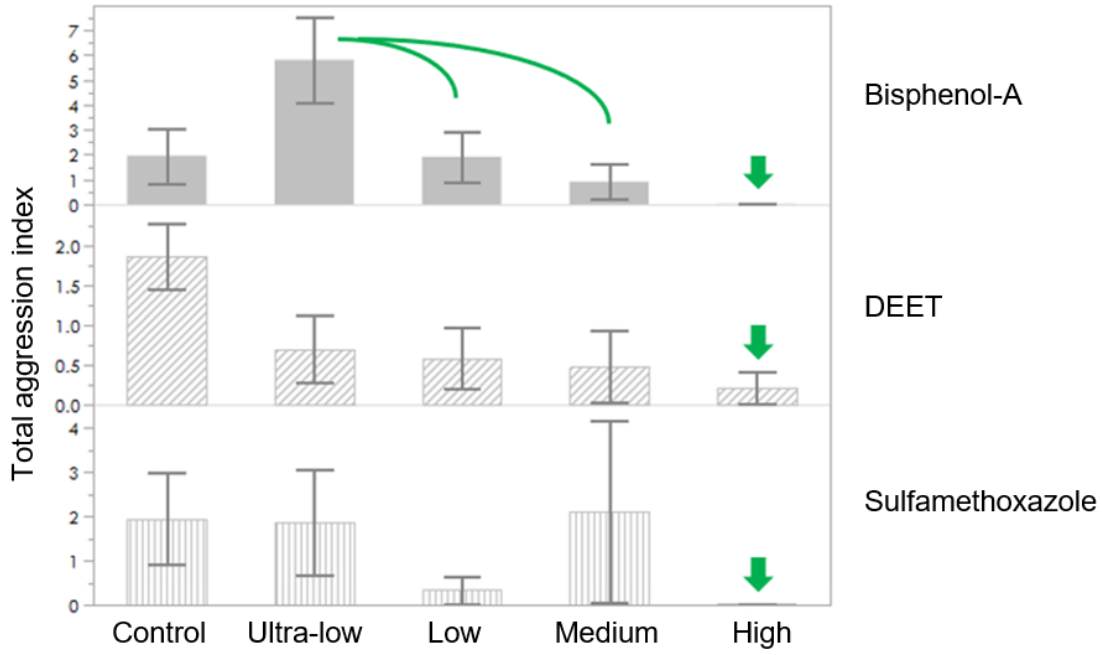

Figure 2.6. Aggression Index for Bisphenol-A, DEET, and Sulfamethoxazole

High Bisphenol A exposure (n_{medium}=5, n_{rest}=6), high *N*,*N*-Diet.-m-toluamide ($n_{ultra\text{-}low\ and\ high}$=5, n_{rest}=6), and high sulfamethoxazole (n=6) exposures showed significantly reduced nest defense aggression compared to the control ($p \leq 0.05$). Green arrows indicate significant changes. Ultra-low Bisphenol-A exposure significantly increased aggression in comparison to the low and medium concentrations.

Larval fathead minnow

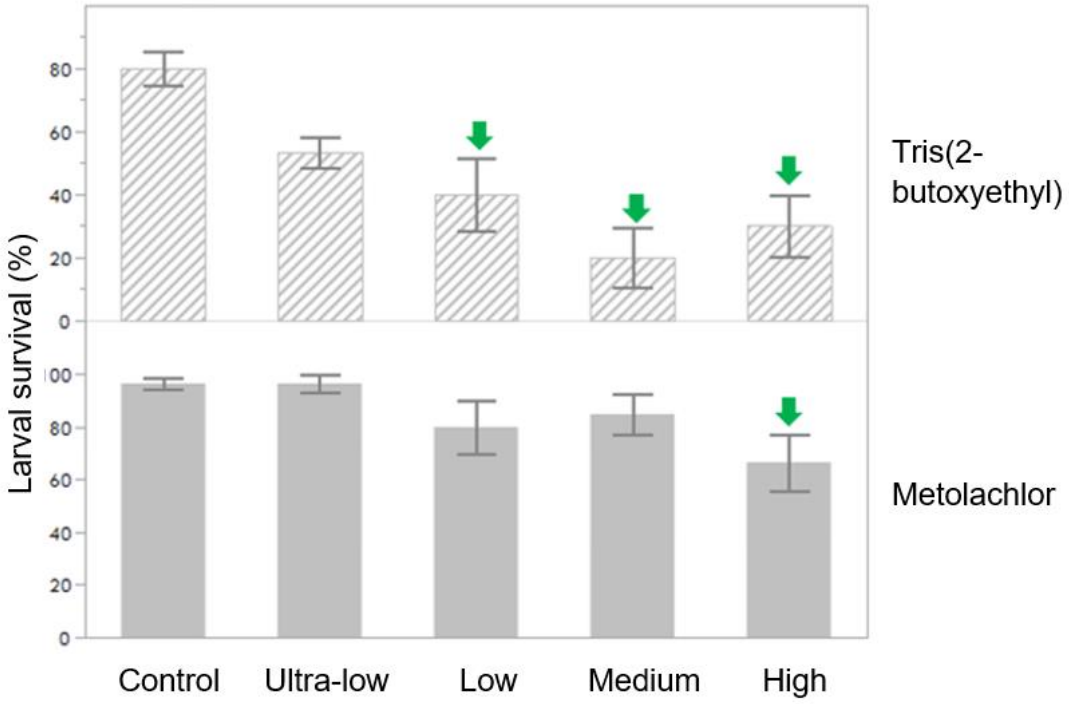

Figure 2.7. Larval Survival of Tris(2-butoxyethyl), and Metolachlor

Larval survival (%) was significantly impacted by tris(2-butoxyethyl) phosphate and metolachlor exposures with standard error. Green arrows indicate the significant decrease from control group ($p \leq 0.05$).

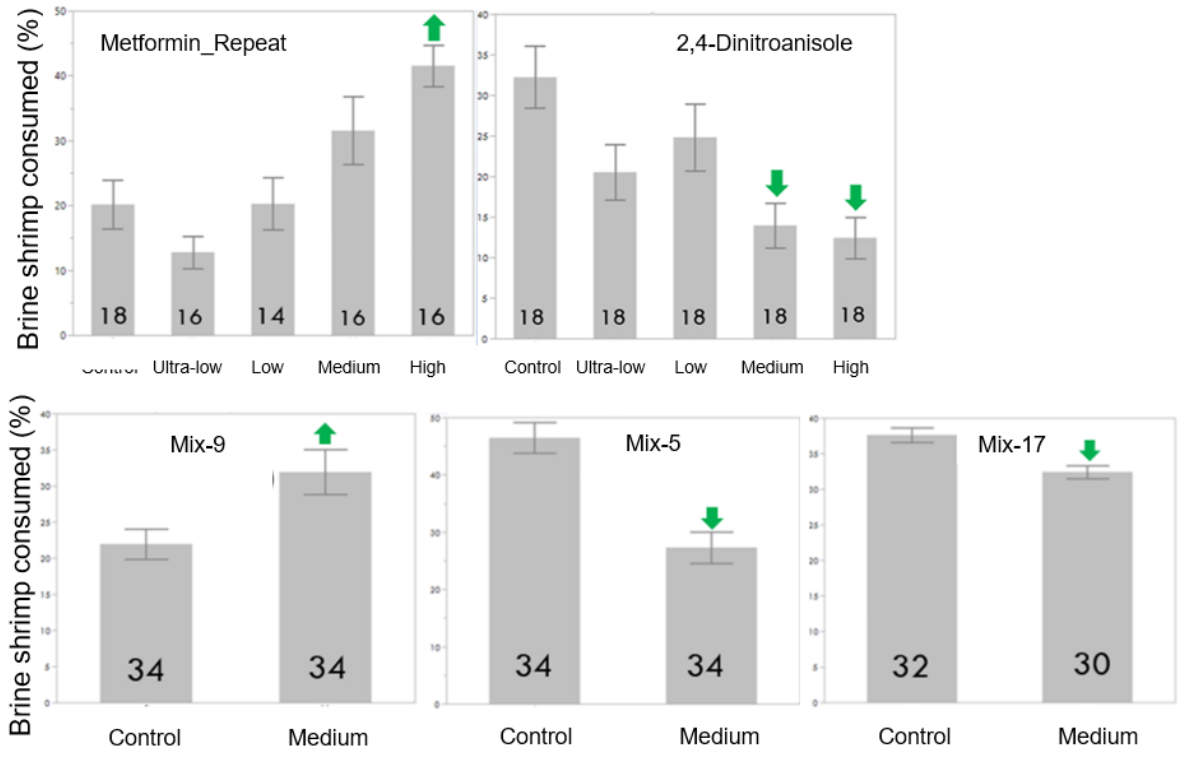

Figure 2.8. Larval feeding efficiency results

Percent of brine shrimp consumed during feeding performance assay with standard error bars. Numbers inside the bars indicate the sample size. Green arrows indicate the significant difference in comparison to the control group.

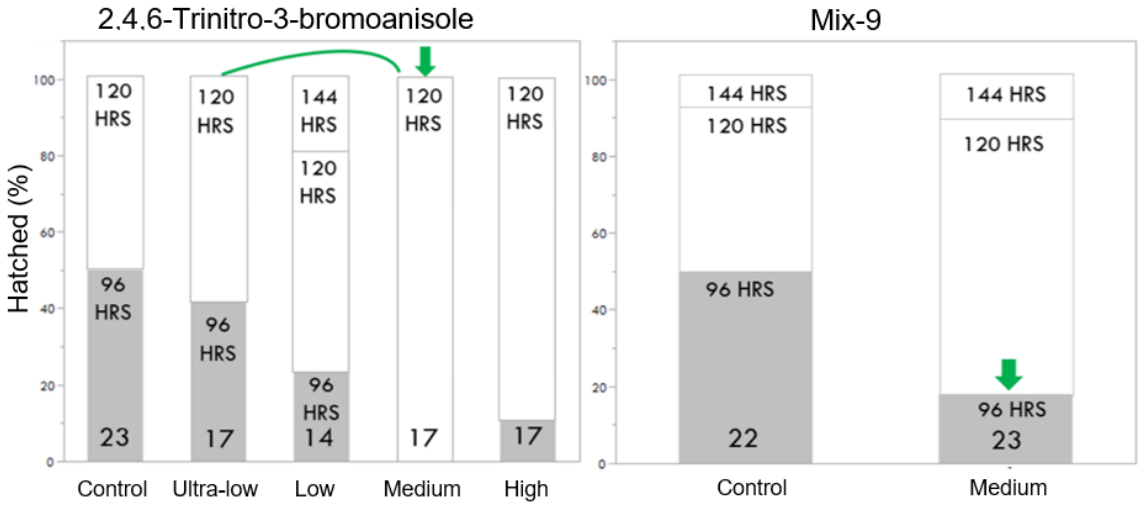

Figure 2.9. Embryonic development abnormality rates

At medium concentration for 2,4,6-Trinitro-3-bromoanisole percent hatched fathead minnow embryos were significantly reduced within 96 hours in comparison to the control and ultra-low groups. Similarly, medium treatment of Mix-9 had reduced percentage of hatched eggs within 96 hours in comparison to the control group. Grey area shows percent hatch embryo post 96 hours of fertilization. 120, and 140 hours percent hatching was also indicated on the bar graph. Numbers inside the bars indicate the sample size.

Figure 2.10. Hatching rate.

Embryonic abnormality quantification indicates that sulfamethoxazole medium and high concentrations had significantly higher abnormalities in comparison to control. The high concentration treatment was also significantly higher in comparison to the ultra-low and low concentrations. The triclosan exposure also significantly increased developmental deformities at low, medium and high concentrations. Low concentration treatment was also significantly higher in comparison to the ultra-low treatment. Mix-13 exposure medium concentration also had significantly high developmental abnormalities in comparison to the control group. Sample size indicated in each column.

Discussion

The current study provides foundational information to develop a comprehensive understanding of the combined effects of CECs on three life stages of the fathead minnow. Results are of particular relevance as the exposure concentrations were derived from an existing data set of environmentally measured concentrations in the Great Lakes of North America. CECs, both singly and in mixture altered apical endpoints. The goals of the study were to assess the effects of complex CEC mixture to (a) identify the most susceptible life stage, (b) determine

whether mixture effects are greater than the effects of any single chemical, and (c) a potential biological “fingerprint” of a single chemical effect can be recovered in a complex mixture.

Overall, the 96 hours long CEC exposure demonstrated that different apical endpoints are affected depending on the exposed life stage. As the complexity of the chemical exposure increased, alterations in endpoints such as courtship behavior in adult male fathead minnows became altered more frequently. Medium and high concentrations were also found to elicited greater adverse effects than low and ultra-low concentrations of the same compounds (Table 2.6).

Table 2.6

Summary of Apical Endpoints Showing the Significant Endpoints for all Three Life Stages Based on Exposure Concentrations

Blue colored boxes indicate the concentration of significant effect found. The vertical green lines separate the life stages, and the horizontal green line separates the single exposures from mixture exposures.

Chem. Class	Chemicals	Embryo		Larvae		Adult						Conc.
Urban	Desvenlafaxine											583 ng/L
	Fexofenadine											1000 ng/L
	Metformin											1210 ng/L
	Metformin_Repeat											1210 ng/L
	Sulfamethoxazole											559 ng/L
	Ibuprofen											440 ng/L
	Triclosan											0.5 ng/L
	5-met.-1H-benz.											6680 ng/L
	Fluoranthene											0.1 ng/L
Co-occuring	N,N-Diet.-m-tolua.											1600 ng/L
	4-Nonylphenol											3710 ng/L
	Bisphenol A											600 ng/L
	Estrone											24 ng/L
	Tris(2-but.eth.) phos.											21,000 ng/L
Agriculture	Atrazine											400 ng/L
	Metolachlor											170 ng/L
	Imidacloprid											140 ng/L
Energetic	2,4,6 trinitrotoluene											1500 ng/L
	2,4-Dinitroanisole											1000 ng/L
	LLM-105											1000 ng/L
	2,4,6-Trinitro-3-bro.ani.											1000 ng/L
	Ag Mix											
	Urb Mix I											
	Urb Mix II											
No energetics	MIX-14											
Med. con. no en.	MIX-15											
No AHR	MIX-7											
No ER	MIX-9											
No iboprofen	MIX-5											
No triclosan	MIX-6											
No sulfameth.	MIX-4											
No desvenlafax.	MIX-1											
No metformin	MIX-3											
No fexofena.	MIX-2											
No T.(2-b.e.) pho	MIX-10											
No metolachlor	MIX-12											
No atrazine	MIX-11											
No DEET	MIX-8											
No imidacloprid	MIX-13											
All at med. conc.	MIX-17											
All	MIX-16											
Ultra-low (1/ 100) Low (1/ 10) Medium (Enviornmental Concentration) High (10x)	Endpoints	Time-to-hatch	Dev. Ab.	Survival	Feed.	Survival	2nd Sex Charct.	Gluc.	Hemat.	Agres.	Court.	

The agricultural mixture in the study contained many herbicides, insecticides, and chemicals that have known for their estrogenic effects. The secondary sex characteristics resulted in significant decrease at medium agricultural mixture exposure. Another study did not report any significant change in secondary sex characteristics with 4-nonylphenol, and Nonylphenol Ethoxylate exposures on fathead minnows that was lasted 42 days (Miles-Richardson et al., 1999). Three weeks of an environmental estrogens exposure also did not report any significant change in secondary sex characteristics, while an effluent mixture reduced secondary sex characteristics (Martinovic, Hogarth, Jones, & Sorensen, 2007). The observed significant decrease at medium concentration in this study might be due to a combined effect of mixture constituents. Interestingly, life-long agricultural mixture exposure with similar chemicals also reported a significantly reduced expression of secondary sex characteristics (Cipoletti, 2018). It is important to reemphasize that high body condition factor of the control fish used in the agricultural mixture exposure might be resulted in suppression of significance expression in other treatments.

Adult fish exposed to Bisphenol-A responded with statistically significant decrease in blood glucose concentrations at high concentration. This could indicate altered blood glucose homeostasis through the disruption of the hypothalamus–pituitary–interrenal axis and complex metabolic dysfunctions and is consistent with previous studies (Aluru, Leatherland, & Vijayan, 2010). Furthermore, fathead minnows exposed to Bisphenol-A exposure also were less aggressive in their nest defense, a further indication that BPA exposure may initiate complex metabolic dysfunctions.

Adult fathead minnow exposure to LLM-105 and triclosan resulted in reduced hematocrit showing potential toxicological effect on the hematopoietic system by lowering the oxygen-carrying capacity. The results provide another evidence that why more detailed studies are needed using the neglected munition compounds to understand their toxicological effects. Triclosan exposure showed significantly reduced hematocrit at ultra-low treatment. This significance at ultra-low treatment might be due to increased need for an adaptive change at higher concentrations. The regenerative response within the biological mechanism might not be triggered at ultra-low concentration within 96-hrs (Canadian Environmental Protection Act, 2016; National Industrial Chemical Notification and Assessment Scheme, 2009).

The embryonic exposure results also support this argument by providing evidence for developmental abnormalities for other treatments in triclosan exposure. Additionally, previous studies recorded substantial variability based on severity of observed effects within triclosan exposure treatments, suggesting particularly sensitive fish might only suffer toxic effects (Schultz et al., 2012).

Sulfamethoxazole exposure resulted in significantly reduced aggression behavior. Interestingly, same treatment group also showed significantly increased neutrophil degranulation (Gordon, 2018), and an increase in the abundance of T cell-specific mRNA in the spleens (Johnson, 2018) suggesting overall impact in immune system of exposed fathead minnows.

Larval metolachlor exposure showed significantly reduced survival at high treatment. Significant change in larval survival was not observed in other agricultural CECs, more specifically herbicides. The low stock chemical confirmation could possibly explain why similar change was not observed in atrazine. Tris(2-butoxyethyl) phosphate also had significantly

reduced survival from low to high treatments. Another study on zebrafish embryos and larvae exposed to tris(2-butoxyethyl) phosphate resulted in decreased expression of proteins that plays role in cell proliferation and DNA repair, therefore increased number of apoptotic cells (Han et al., 2014). Effect on larval survival with the tris(2-butoxyethyl) phosphate exposure was also observed in adult fathead minnows.

Larval exposure to 2,4-dinitroanisole resulted in significantly decreased feeding performance at medium and high concentrations. The 2,4-dinitroanisole's biotransformation product 2,4-diaminoanisole also showed neurotoxicity as observed in the swimming behavioral tests (640 µM) of zebrafish (Olivares et al., 2016) and may suggest that larva fish were less able to control their swimming and/or approach to prey.

High treatment of metformin exposure effect on larval feeding performance was recorded which may be due to previously reported endocrine disrupting impact (Niemuth & Klaper, 2018), and estrogenic effects -only in juvenile- (Crago, Bui, Grewal, & Schlenk, 2016). Furthermore, a recent study that was presented at SETAC North America Annual Meeting pointed parallel effects on larval fathead minnow appetite (Pattulo, Nanna, Finegan, Barone, & Allen-Gil, 2018).

Even though Mix-16 and 17 were the same chemical mixtures, Mix-17 exposure resulted in a significant decline in feeding performance at medium concentration while Mix-16 did not alter feeding performance. This might be due to the initial stress introduced to the larval fish in addition to the chemical stress. Mix-17 was exposed to higher temperature difference during delivery period and therefore another challenge factor was set to pick more fit larval fish in the

sample size. It is possible that surviving larvae were more robust and inclined to eat more brine shrimp.

Time-to-hatch for fish embryos may differ due to extraembryonic membrane disruption, or overall impact on completeness of developmental stages (premature hatching). 2,4,6-Trinitro-3-bromtoanisole exposure induced a delay in the hatching process (Ninness, Stevens, & Wright, 2006). There was not any observed developmental deformity, and the hatching date was not extensively later than the control, therefore it would not be expected that 2,4,6-Trinitro-3-bromtoanisole exposure has an adverse impact during cleavage, gastrulation, or organogenesis periods, yet it might be affecting the extraembryonic membrane structure.

The observed embryonic abnormalities in sulfamethoxazole and triclosan may be due to dysfunction in folate metabolism (Lee et al., 2012) and immune dysregulation (Johnson, 2018) respectively. Although the mechanism of action for triclosan is still not clear, its toxic effects for fish embryo is well documented (Horie, Yamagishi, Takahashi, Iguchi, & Tatarazako, 2018; Macedo, Torres, & Santos, 2017; Oliveira et al., 2009; Wirth, Botka, Perez, & King-Heiden, 2018).

The results of this study showed the possible effects of complex CEC mixtures at environmentally relevant concentrations from Laurentian Great Lakes Watersheds can adversely affect fish health as short as 96 hours. In addition, it provides data to better understand how to build an adverse outcome pathway for the complex CEC mixtures using single CEC exposures.

Chapter 3: Conclusion

Analysis of short-term laboratory exposure studies of fathead minnows to increasingly complex mixtures confirmed that it is challenging to predict organismal endpoints of complex mixtures from the single chemicals. This study provides additional evidence that there is no linear relationship for organismal endpoints to extrapolate from single chemical effects to effects of complex mixtures (Zenobio, Sanchez, Archuleta, & Sepulveda, 2014). Results of the study form a baseline for short term complex exposures and can provide a bridge to interpret effects observed in long term multi-generational exposures. Furthermore, the results indicate the need for complementary *in vivo* experiments to establish a connection between adverse outcomes and key initiating events. Results from the current study indicate that environmentally relevant concentrations of CECs may have detrimental biological effects on fathead minnows and continued contamination could lead to significant impact on fish survival. Despite adding more chemical classes, the complex mixtures did not result in additional effect, even though prepared mixtures lead to greater total contaminant concentrations. Lastly, this study highlights the need for further investigations of complex mixtures of CECs found in water resources to review current regulatory requirements and to preserve the interlocked lifecycles of aquatic organisms within the ecosystem.

References

Aluru, N., Leatherland, J. F., &Vijayan, M. M. (2010). Bisphenol A in oocytes leads to growth suppression and altered stress performance in juvenile rainbow trout. *PLOS ONE, 5*(5), e10741. doi: 10.1371/journal.pone.0010741

Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., . . . Villeneuve, D. L. (2010). Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology and Chemistry*, *29*(3), 730-741.

Ankley, G. T., Feifarek, D., Blackwell B., Cavallin, J. E., Jensen, K. M., Kahl, M. D., . . . Villeneuve, D. L. (2017). Re-evaluating the significance of estrone as an Environmental estrogen. *Environmental Science and Technology*, *51*(8), 4705-4713. doi: 10.1021/acs.est.7b00606

Ankley, G. T., & Villeneuve, D. L. (2006). Review of the fathead minnow in aquatic toxicology: Past, present and future. *Aquatic Toxicolog*, *78*(1), 91-102.

Bartoskova, M., Dobsikova, R., Stancova, V., Zivna, D., Blahova, J., Marsalek, P., . . . Faggio, C. (2013). Evaluation of ibuprofen toxicity for zebrafish (Danio rerio) targeting on selected biomarkers of oxidative stress. *Neuroendocrinology Letters*, *34*(2), 102-8.

Becker, G. C. (1983). *The fishes of Wisconsin*. Madison, WI: University of Wisconsin Press.

Bird, K. (2015). The effects of pharmaceutical and estrone exposure on predator avoidance performance and feeding efficiency in larval fathead minnows. *Culminating Projects in Biology. 7*. https://repository.stcloudstate.edu/biol_etds/7

Bringolf, R. B., Belden, J. B., & Summerfelt, R. C. (2004). Effects of atrazine on fathead minnow in a short-term reproduction assay. *Environmental Toxicology and Chemistry*, *23*(4), 1019-1025.

Brodin, T., Fick, J., Jonsson, M., & Klaminder, J. (2013). Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. *Sci*, *339*, 814-815.

Brungs, W. A. (1971). Chronic effects of low dissolved oxygen concentrations on fathead minnows (Pimephalesprome- las). *Journal of the Fisheries Research Board of Canada*, *28*, 1119-1123.

Cavallin, J. E., Jensen, K. M., Kahl, M. D., Villeneuve, D. L., Lee, K., Schroeder, A., . . . Ankley, G. T. (2016). Pathway-based approaches for assessment of real-time exposure to an estrogenic wastewater treatment plant effluent on fathead minnow reproduction. Environmental Toxicology and Chemistry, *35*(3), 702-716.

Canadian Environmental Protection Act (CEPA). (2016). Assessment report-triclosan. *Environment and climate change Canada*. Retrieved from http://www.ec.gc.ca/ese-ees/

Cipoletti, N. (2018). Complex agricultural mixtures: Assessing effects on aquatic species (Pimephales promelas and Lepomis spp.) through short-term field and multi-generational laboratory exposures. *Culminating Projects in Biology. 33.* http://repository.stcloudstate.edu/biol_etds/33

Coiner, R. L., Pope, L. M., & Mehl, H. M. (2010). *Assessment of energetic compounds, semi-volatile organic compounds, and trace elements in streambed sediment and stream water from streams draining munitions firing points and impact areas, Fort Riley, Kansas, 2007-08*. Reston, Virginia: U.S. Geological Survey Scientific Investigations Report 2010–5021, p. 55.

Cox, M. K. (2016). Modulation of estrogenic effects via temperature on two life stages of fathead minnow. *Culminating Projects in Biology. 16*. http://repository.stcloudstate.edu/biol_etds/16

Crago J., Bui C., Grewal S., & Schlenk D. (2016) Age-dependent effects in fathead minnows from the anti-diabetic drug metformin. *General and Comparative Endocrinology, 232,* 185-190. doi: 10.1016/j.ygcen.2015.12.030.

Elliott, S. M., Brigham, M. E., Lee, K. E., Banda, J. A., Choy, S. J., Gefell, D. J., . . . Jorgenson, Z. G. (2017). Contaminants of emerging concern in tributaries to the laurentian great lakes: I. Patterns of occurrence. *PLOS ONE, 12*(9), 121. https://doi.org/10.1371/journal.pone.0182868

Farr, A. J., Chabot, C. C., & Taylor, D. H. (1994). Behavioral avoidance of fluoranthene by fathead minnows (Pimephales Promelas). *Neurotoxicology and Teratology, 17*(3), 265-271.

Flippin, J. L., Huggett, D., & Foran, C. M. (2007). Changes in the timing of reproduction following chronic exposure to ibuprofen in Japanese medaka, Oryzias latipes. *Aquatic Toxicology, 81*(1), 73-78.

Garcia-Reyero, N., Kroll, K. J., Liu, L., Orlando, E. F., Watanabe, K. H., Sepúlveda, M.S., . . . Denslow, N. D. (2009). Gene expression responses in male fathead minnows exposed to binary mixtures of an estrogen and antiestrogen. *BMC Genomics, 10,* 308. doi: 10.1186/1471-2164-10-308

Groten, J. P., Feron, V. J., & Sühnel, J. (2001). Toxicology of simple and complex mixtures. *Trends in Pharmacological Sciences, 22*(6), 316-322.

Gordon, J. (2018). The effects of urban contaminants on neutrophils of fathead minnows (Pimephales promelas). *Culminating Projects in Biology. 37.* https://repository.stcloudstate.edu/biol_etds/37

Hallgren, P., Nicolle, A., Hansson, L. A., Bronmark, C., Nikoleris, L., Hyder, . . . A. (2014). Synthetic estrogen directly affects fish biomass and may indirectly disrupt aquatic food webs *Environmental Toxicology and Chemistry, 33*(4), 930-936.

Han Z., Wang Q., Fu J., Chen H., Zhao Y., Zhou B., . . . Yu, H. (2014). Multiple bio-analytical methods to reveal possible molecular mechanisms of developmental toxicity in zebrafish embryos/larvae exposed to tris(2-butoxyethyl) phosphate. *Aquatic Toxicology, 150,* 175-181. doi: 10.1016/j.aquatox.2014.03.013

Heberer, T., Reddersen, K., & Mechlinski, A. (2002). From municipal sewage to drinking water: fate and removal of pharmaceutical residues in the aquatic environment in urban areas. *Water Science and Technology, 46*(3), 81-88. https://doi.org/10.1897/02-237

Horie Y., Yamagishi T., Takahashi H., Iguchi T., & Tatarazako N. (2018). Effects of triclosan on Japanese medaka (Oryzias latipes) during embryo development, early life stage and reproduction. Journal of Applied Toxicology, 38, 544-551. doi: 10.1002/jat.3561.

Johnson, C. (2018). The effects of acute urban mixture exposure on the T cells of fathead minnows (Pimephales promelas). *Culminating Projects in Biology. 36.* https://repository.stcloudstate.edu/biol_etds/36

Jukosky, J. A., Watzin, M. C., & Leiter, J. C. (2007). The effects of environmentally relevant mixtures of estrogens on Japanese medaka (Oryzias latipes) reproduction. *Aquatic Toxicology, 86,* 323-331.

Klaminder, J., Jonsson, M., Fick, J., Sundelin, A., & Brodin, T. (2014). The conceptual imperfection of aquatic risk assessment tests: highlighting the need for tests designed to detect therapeutic effects of pharmaceutical contaminants. *Environmental Research Letters, 9,* 1-7.

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

Lee, K. E., Langer, S. K., Menheer, M. A., Hansen, D. S., Foreman, W. T., Furlong, E. T., . . . Gefell, D. J. (2014). Chemicals of emerging concern in water and bottom sediment in the Great Lake's basin, 2012—Collection Methods, Analytical Methods, Quality Assurance, and Study Data. *U. S. Geological Survey*, 1-5.

Lee, M. S., Bonner, J. R., Bernard, D. J., Sanchez, E. L., Sause, E. T., Prentice, R. R., . . . Brody, L. C. (2012). Disruption of the folate pathway in zebrafish causes developmental defects. *BMC Developmental Biology, 12,* 12. doi:10.1186/1471-213X-12-12

Levine H., Jørgensen N., Martino-Andrade A., Mendiola J., Weksler-Derri D., Mindlis I., . . . Swan S. H. (2017). *Human Reproduction Update, 23*(6), 646-659. doi: 10.1093/humupd/dmx022

Lin, L. L., & Janz, D. (2006). Effects of binary mixtures of xenoestrogens on gonadal development and reproduction in zebrafish. *Aquatic Toxicology, 80,* 382-395.

Macedo S., Torres T., & Santos, M. M. (2017). Methyl-triclosan and triclosan impact embryonic development of Danio rerio and Paracentrotus lividus. *Ecotoxicology*, *26*(4), 482-489. doi: 10.1007/s10646-017-1778-3

Martinovic, D., Hogarth, W., Jones, R., & Sorensen, P. (2007). Environmental estrogens suppress hormones, behavior, and reproductive fitness in male fathead minnows. *Environmental Toxicology and Chemistry*, *26*(2), 271-278. https://doi.org/10.1016/B978-0-7216-0693-4.50088-8

Matson, C. W., Timme-Laragy, A. R., & Di Giulio, R. T. (2008). Fluoranthene, but not benzo[a]pyrene, interacts with hypoxia resulting in pericardial effusion and lordosis in developing zebrafish. (2008). *Chemosphere, 74*(1), 149-54. doi: 10.1016/j.chemosphere.2008.08.016

McCarraher, D. B., & Thomas, R. (1968). Some ecological observations on the fathead minnow Pimephales promelas in the alkaline waters of Nebraska. *Transactions of the American Fisheries Society, 97*, 52-55.

Miles-Richardson, S. R., Pierens, S. L., Nichols, K. M., Kramer, V. J., Snyder, E. M., Snyder, S. A., . . . Giesy, J. P. (1999). Effects of waterborne exposure to 4 nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Environmental Research, 80*(2II), 129-145. https://doi.org/10.1006/enrs.1998.3945

Mount, D. I., (1973). Chronic effect of low pH on fathead minnow survival, growth and reproduction. *Water Research, 7*(7), 987-993.

Moyle, P. B. (1976). *Inland fishes of California*. Berkley, CA: University of California Press. Berkley, CA.

National Industrial Chemicals Notification and Assessment Scheme (NICNAS). (2009). *Triclosan (Priority existing chemical assessment report No. 30) [Internet]. Sydney (AU): Australian Government, Department of Health and Aging, National Industrial Chemicals Notification and Assessment Scheme*. Available from: www.nicnas.gov.au/publications/car/pec/pec30/pec_30_full_report_pdf.pdf

Nelson, J. S., & Paetz, M. J. (1992). *The fishes of Alberta*. Edmonton, Alberta: The University of Alberta Press, Edmonton.

Niemuth N. J., & Klaper R. D. (2018). Low-dose metformin exposure causes changes in expression of endocrine disruption-associated genes. *Aquatic Toxicology, 195,* 33–40. doi: 10.1016/j.aquatox.2017.12.003.

Ninness M. M., Stevens E. D., & Wright P. A. (2006). Removal of the chorion before hatching results in increased movement and accelerated growth in rainbow trout (Oncorhynchus mykiss) embryos. *Journal of Experimental Biology, 209*(Pt .10), 1874-1882.

Olivares C. I., Sierra-Alvarez R., Abrell L., Chorover J., Simonich M., Tanguay R. L., & Field J. A. (2016). Zebrafish embryo toxicity of anaerobic biotransformation products from the insensitive munitions compound 2,4-dinitroanisole. *Environmental Toxicology and Chemistry, 35*(11), 2774-2781. doi: 10.1002/etc.3446

Oliveira, R., Domingues, I., Koppe-Grisolia, C., & Soares, A. M. (2009). Effects of triclosan on zebrafish early-life stages and adults. *Environmental Science Pollution and Research, 16*(6), 679-688.

Parrott, J. L., Wood, C. S., Boutot, P., & Dunn, S. (2003). Changes in growth and secondary sex characteristics of fathead minnows exposed to bleached sulfite mill effluent. *Environmental Toxicology and Chemistry*, *22*(12), 2908-2915.

Parrott, J. L., Alaee, M., Wang, D., & Sverko, E. (2013). Fathead minnow (Pimephales promelas) embryo to adult exposure to decamethylcyclopentasiloxane (D5). *Chemosphere*, *93*(5), 813-818. doi: 10.1016/j.chemosphere.2012.10.053.

Parrott, J. L., & Bennie, D. T. (2009). Life-cycle exposure of fathead minnows to a mixture of six common pharmaceuticals and triclosan. *Journal of Toxicology and Environmental Health, 72*(10), 633-41. doi: 10.1080/15287390902769428

Patel, A., Panter, G. H., Trollope, H. T., Glennon, Y. C., Owen, S. F., Sumpter, J. P., . . . Rand-Weaver, M. (2016). Testing the “read-across hypothesis” by investigating the effects of ibuprofen on fish. *Chemosphere, 163,* 592-600. doi: 10.1016/j.chemosphere.2016.08.041

Pattulo, C., M. Nanna, M. P. Finegan, R. Barone, J. L., & Allen-Gil S. (2018). Effects of metformin on growth and appetite in juvenile fathead minnows (Pimephales promelas). In *SETAC NA Annual Meeting*, Sacramento, California, November 04 - 08, 2018.

Pflieger, W. L. (1975). *The fishes of Missouri*. Missouri: Missouri Department of Conservation.

Scahill, J. L. (2008). Effects of atrazine on embryonic development of fathead minnows (*Pimephales promelas*) and xenopus laevis. *Bios, 79*(4), 139-149.

Schultz, M. M., Minarik, T. A., Martinovic-Weigelt, D., Curran, E. M., Bartell, S. E., & Schoenfuss. H. L. (2013). Environmental estrogens in an urban aquatic ecosystem: II. Biological effects. *Environment International, 61,* 138-149. doi: 10.1016/j.envint.2013.08.006

Schultz, M., Bartell, S., & Schoenfuss, H. L. (2012). Effects of triclosan and triclocarban, two ubiquitous environmental contaminants, on anatomy, physiology, and behavior of the fathead minnow (*Pimephales promelas*). *Archives of Environmental Contamination and Toxicology, 63,* 114-1124. 10.1007/s00244-011-9748-x.

Slaninova, A., Modra, H., Hostovsky, M., Sisperova, E., Blahova, J., Matejova, I., . . Svobodova, Z. (2014). Effects of subchronic exposure to *N*,*N*-diethyl-m-toluamide on selected biomarkers in common carp (Cyprinus carpio L.). *BioMed Research International*, 828515. doi: 10.1155/2014/828515

Smith, R. J. F. (1978). Seasonal changes in the histology of the gonads and dorsal skin of the fathead minnow, *Pimephales promelas*. *Canadian Journal of Zoology, 56,* 2103-2109.

Smock, L. A., Stoneburner, D. L., & Clark, J. R. (1976). The toxic effects of trinitrotoluene (TNT) and its primary degradation products on two species of algae and the fathead minnow. *Water Research, 10*(6), 537-543. doi:10.1016/0043-1354(76)90191-3

Sun, L, Zha, J., & Wang, Z. (2009). Effects of binary mixtures of estrogen and antiestrogens on Japanese medaka (*Oryzias latipes*). *Aquatic Toxicology, 93,* 83-89.

United States Environmental Protection Agency [USEPA]. (1987). *Guidelines for the culture of fathead minnows Pimephales promelas for use in toxicity tests*. Duluth, MN: Author.

Vajda, A. M., Barber, L. B., Gray, J. L., Lopez, E. M., Woodling, J. D., & Norris, D. O. (2008). Reproductive disruption in fish downstream from an estrogenic wastewater effluent. *Environmental Science and Technology, 42,* 3407-3014.

Villeneuve, D. L., Grump, D., Garcia-Reyero, N., Hecker, M., Hutchinson, T. H., LaLone, C.A., . . . Whelan, M. (2014). Adverse outcome pathway (AOP) development I: Strategies and principles. *Toxicological Sciences, 142*(2), 312-320. doi: 10.1093/toxsci/kfu199

Villeneuve, D. L., Mueller, N. D., Martinović, D., Makynen, E. A., Kahl, M. D., Jensen, K. M., . . . Ankley G. T. (2009). Direct effects, compensation, and recovery in female fathead minnows exposed to a model aromatase inhibitor. *Environmental Health Perspectives, 117,* 624-631.

Wang, L. C. (2017). Three generational exposure of pimephales promelas to an urban contaminants of emerging concern mixture. *Culminating Projects in Biology. 20.* https://repository.stcloudstate.edu/biol_etds/20/

Wattigney, W. A., Zheng, L., & Ragin-Wilson, A. (2017). The Biomonitoring of Great Lakes Populations Program. *Environmental Health, 79*(8), 42-44.

Wirth H., Botka R., Perez K. E., &King-Heiden T. (2018). Embryonic exposure to environmentally relevant concentrations of triclosan impairs foraging efficiency in zebrafish larvae. *Environmental Toxicology and Chemistry, 37*(12), 3124-3133. doi: 10.1002/etc.4281

Yoo, L. J., Lotufo, G. R., Gibson, A. B., Steevens, J. A., & Sims, J. G. (2006). Toxicity and bioaccumulation of 2,4,6-trinitrotoluene in fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistr*y, *25*(12), 3253-3260.

Zenobio, J. E., Sanchez, B. C., Archuleta, L. C., & Sepulveda, M. S. (2014). Effects of triclocarban, *N,N*-diethyl-meta-toluamide, and a mixture of pharmaceuticals and personal care products on fathead minnows (*Pimephales promelas*). *Environmental Toxicology and Chemistry*, *33*(4), 910-919.

Zhu B. T., Han G. Z., Shim J. Y., Wen Y., & Jiang X. R. (2006). Quantitative structure-activity relationship of various endogenous estrogen metabolites for human estrogen receptor alpha and beta subtypes: Insights into the structural determinants favoring a differential subtype binding. *Endocrinology*, *14*(9), 4132-4150. doi:10.1210/en.2006-0113. PMID 16728493.