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Evaluating Membrane Bioreactors for Removal of Contaminants of Emerging Concern using Analytical Chemistry and Fathead Minnow (*Pimephales promelas*) Exposure

by

Charles Christen

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Abstract

Wastewater originates from industrial, commercial, and residential sources. Each source has the potential to add contaminants of emerging concern (CECs) that may interact with an organism's cellular pathways and metabolic processes. Wastewater treatment plants (WWTP) are built to remove macro pollutants, nutrients, and microorganisms through three-stage processes but are not optimized for CEC removal. In the three-stage process primary treatment removes macro-pollutants, tertiary treatment disinfects, and the secondary treatment removes micro-pollutants such as CECs. Secondary treatment technologies range from well-established oxidation treatments to membrane bioreactors (MBR). Oxidation treatments use agitators to promote bacterial growth and nutrient removal, while MBRs uses a similar biological treatment but add membrane filtration. Studies have shown that the effluent released after oxidation treatments still contains CECs. While studies on MBR have shown better removal of CECs than oxidation treatments it is not known how the effluent affect exposed fish. The Hutchinson, MN WWTP splits its primary treatment effluent into both a MBR treatment and oxidation treatment allowing for direct comparison of the efficacy of CEC removal. The objective of the current study was to compare CEC removal efficacy between these wastewater treatment technologies through analytical chemistry and replicate exposure of fathead minnows (*Pimephales promelas*). Fathead minnows were exposed for 21-days via a flow through system to four treatments including a reference control, primary treatment effluent, oxidation treatment effluent, and MBR effluent. The results show that both secondary treatments reduce exposure activity ratios for all measured chemicals when compared to the primary treatment effluent. While fish did not survive in primary treated effluent the survival rate increased to >94% for fish exposed to MBR and oxidation treatment effluent. The reduction in exposure activity ratios for bisphenols and alkylphenols were similar between MBR and oxidation treatments, but there were notable differences in pesticide removal. MBR treatment resulted in significant decreases in gene expression for aerobic metabolism regulators in the liver when compared to control in the first exposure. Meanwhile results in the second exposure show significant differences in gene expression for reproductive pathways when the oxidation treatment was compared to control. The molecular endpoints are not reflected at the tissue or organ levels indicating that the impacts are subtle. Oxidation treatment, though it is not removing CECs as well as MBR, is providing better outcomes for fathead minnows. These results demonstrate that both treatments greatly improve the quality of effluent when compared to the influent. Meanwhile when comparing the secondary treatments CEC removal differs creating complex mixtures that are leading to better outcomes in fish treated with oxidation treatment effluent.

Acknowledgment

Funding for the present study was provided by the Minnesota Environment and Natural Resources Trust Fund as recommended by the Legislative-Citizen Commission on Minnesota Resources (grant M.L. 2021, First Special Session, Chp. 6, Art. 6, Sec. 2, Subd. 04f).

Table of Contents

Page

Chapter Page

List of Tables

List of Figures

Figure Page

Chapter 1: Introduction

Aquatic ecosystems, such as rivers, lakes, and oceans, are home to a diverse array of plants and animals, and play important roles in the global water cycle, climate regulation, and the overall health of the planet. However, aquatic ecosystems are vulnerable to environmental stressors that can have negative impacts on the plants, animals, and other organisms that call these ecosystems home. One such stressor is the addition of pollutants such as contaminants of emerging concern (CECs) into aquatic ecosystems.

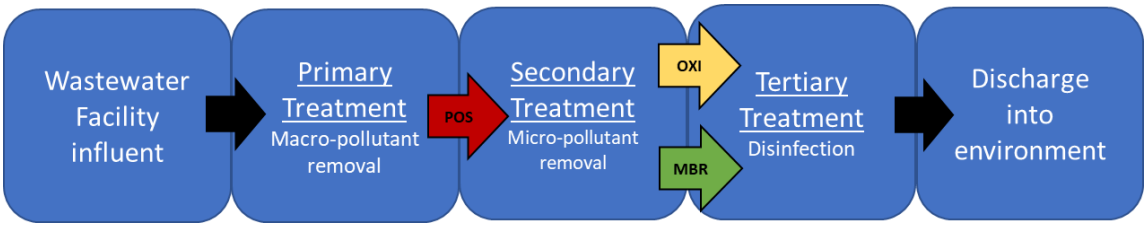
CECs are compounds that are associated with well-established positive and negative effects, can cause adverse effects at low concentrations, and are often not regulated (Noguera-Oviedo & Aga, 2016). Pharmaceuticals, personal care products (PCP), pesticides, and endocrine disrupting compounds (EDCs) can be classified as CECs (Sengupta et al., 2022). Many CECs are produced by households and industries, and after use they are discarded where they can enter the wastewater pathway.

Once in the wastewater pathway, CECs can be transported to wastewater treatment plants (WWTP), where their removal or degradation depends on the chemicals and effectiveness of the plants. However, numerous CECs have been detected in the complex mixtures of WWTP effluent, as shown by studies (Alan et al., 2008; Bolong et al., 2009; Gómez et al., 2012; Köck-Schulmeyer et al., 2013).

The primary function of WWTPs is to accelerate the natural process of water purification to remove nutrients and contaminants (United States Environmental Protection Agency [EPA], 1998). WWTP typically employ three-step process consisting of primary, secondary, and tertiary treatment. The primary treatment removes macro-pollutants, while the tertiary treatment focuses on disinfection. It is during the secondary treatment when contaminants are targeted for removal (Figure 1.1).

Figure 1.1

General wastewater treatment pathway



Note. Includes primary treatment that removes macro-pollutants, secondary treatment for nutrient and micro-pollutant removal, and tertiary treatment for disinfection. After treatment the water is discharged into the environment. Colored arrows represent treatments in this study primary effluent (POS) and two different secondary treatments oxidation ditch effluent (OXI) and membrane bioreactor (MBR).

In the United States, the most common secondary treatment method is the oxidation ditch (Noguera-Oviedo & Aga, 2016). The oxidation ditch treatment involves the use of activated sludge within circular channels that treat wastewater through aeration and biological degradation (Butler et al., 2017). Following treatment, the wastewater is sent to a clarifier allowing biomatter to settle before proceeding to tertiary treatment (United States Environmental Protection Agency (EPA), 1998). Studies have shown that the effluent produced by oxidation ditch treatment still contains CECs (Ahmed et al., 2017; Z. Li et al., 2019; Molé et al., 2019). Although the removal efficiency for surfactants can be over 95%, the treatment has low removal rates for pharmaceuticals, pesticides, and beta blockers (Ahmed et al., 2017). Given the limited efficacy of oxidation ditch treatment, alternative treatment options need to be explored.

Membrane bioreactor (MBR) treatment combines activated sludge with membrane filtration to achieve liquid-solid separation (O. Iorhemen et al., 2016; Mutamim et al., 2013; Tay et al., 2007). Advances in technology have reduced operational costs by creating fouling-resistant materials and adding chemicals to reduce fouling (Mutamim et al., 2013). Research has shown that MBR is effective in

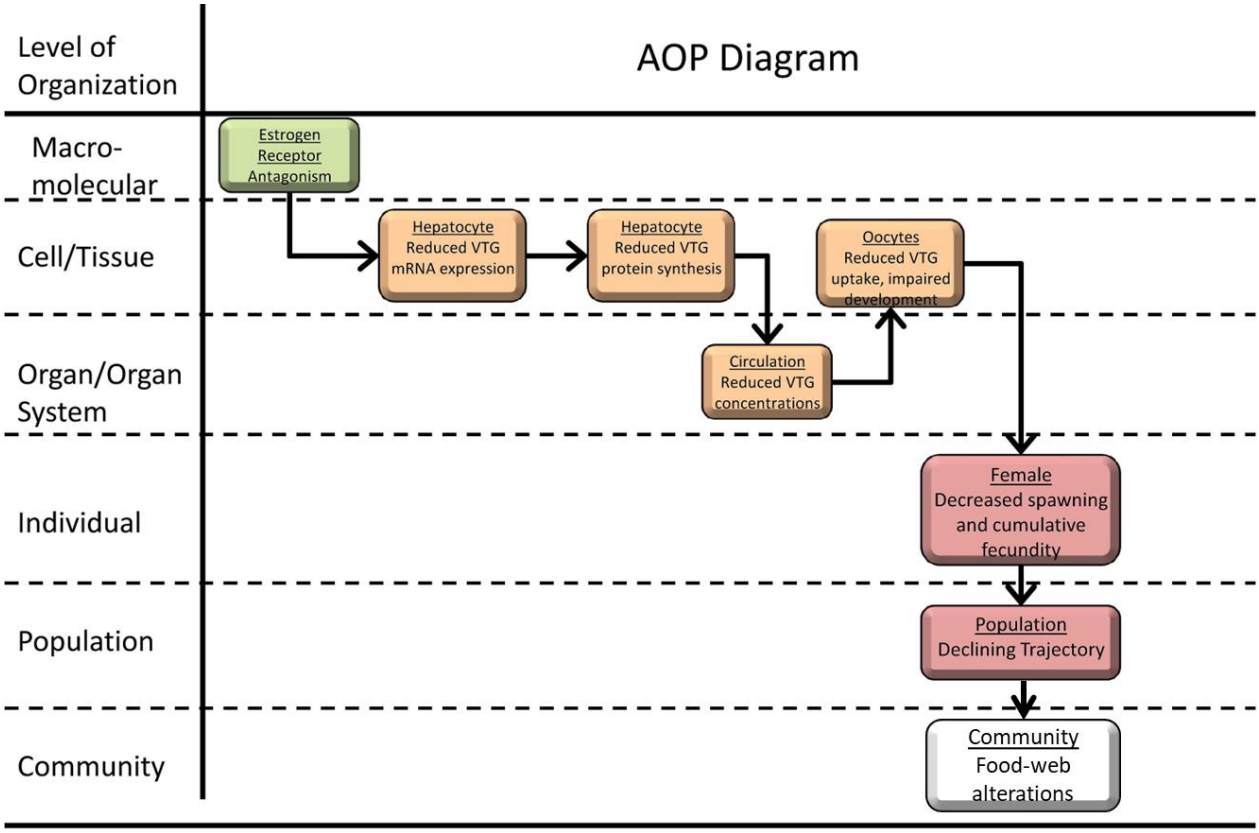
removing CECs such as EDCs, PCPs, and certain pharmaceuticals (Ahmed et al., 2017; Chen et al., 2008; Clara et al., 2005; Kim et al., 2014). For instance, Chen et al. (2008) reported that bisphenol A removal was better with MBR than conventional activated sludge treatment methods like oxidation ditch. While MBR has higher removal efficiency for EDCs and PCPs (90-100%) compared to conventional activated sludge (80-95%), its efficacy for removing pesticides (65%) and pharmaceuticals like anticonvulsants (14.9%) and lipid regulators (13.1%) is limited (Ahmed et al., 2017). Despite MBR's better performance in removing CECs, the impact of MBR on wildlife still requires further study.

Wastewater is a complex mixture of chemicals that poses a significant challenge in understanding its impact on aquatic life. CECs, have the potential to affect different levels of biological organization, from molecular to ecosystem wide. Adverse outcome pathways (AOPs) provide a useful framework for linking the initial exposure of chemicals to their eventual effects on populations (Ankley et al., 2010). AOPs rely on network analysis to predict the mechanisms underlying chemical toxicity by identifying a series of key events that link early stress responses to an adverse effect bridging the gap between chemistry and population effects (Zare et al., 2018). AOPs have the potential to detect early stress responses at subthreshold exposure levels, which may be insufficient to cause overt toxicity (Perkins et al., 2011). However, AOPs provide a framework to assess stressors or mixtures of stressors by bridging chemistry to population effects (Perkins et al., 2011).

AOPs are built on a foundation of well-documented molecular initiating events (MIE) caused by certain classes of chemicals and their corresponding adverse outcomes. For example, exposure to estrogenic antagonists like tamoxifen has been shown to decrease plasma vitellogenin (VTG) concentration in female fathead minnows, ultimately leading to decreased fecundity and population growth (D. Villeneuve, 2021)(Figure 1.2). By incorporating biomarkers such as growth, behavior, histopathology, hormone concentrations, and gene expression, AOPs offer powerful approach for assessing the cumulative effects of CECs on populations (Kramer et al., 2011).

Figure 1.2

Example AOP diagram for estrogen receptor antagonism



Note. AOP 30 estrogen receptor antagonism leading to reproductive dysfunction. Molecular initiating event by tamoxifen antagonizing estrogen receptor and ending with population declining (D. Villeneuve, 2021).

The aim of the current study was to evaluate the efficacy of different wastewater treatments, oxidative ditch vs. MBR, with respect to their ability to remove CECs. To achieve this, CEC concentrations were measured in the effluents of both treatments, and fathead minnows (*Pimephales promelas*) were exposed to each effluent. The current study assessed the long-term effects of CEC exposure on fathead minnows with AOP frameworks, focusing on endpoints related to contaminant exposure. It was hypothesized first that membrane bioreactor effluent will have lower concentrations of CECs than

oxidative ditch effluent and fathead minnows exposed to membrane bioreactor effluent will experience less stress than those exposed to oxidative effluent. Stress in this instance defined as a disturbance in homeostasis (Wendelaar Bonga, 1997).

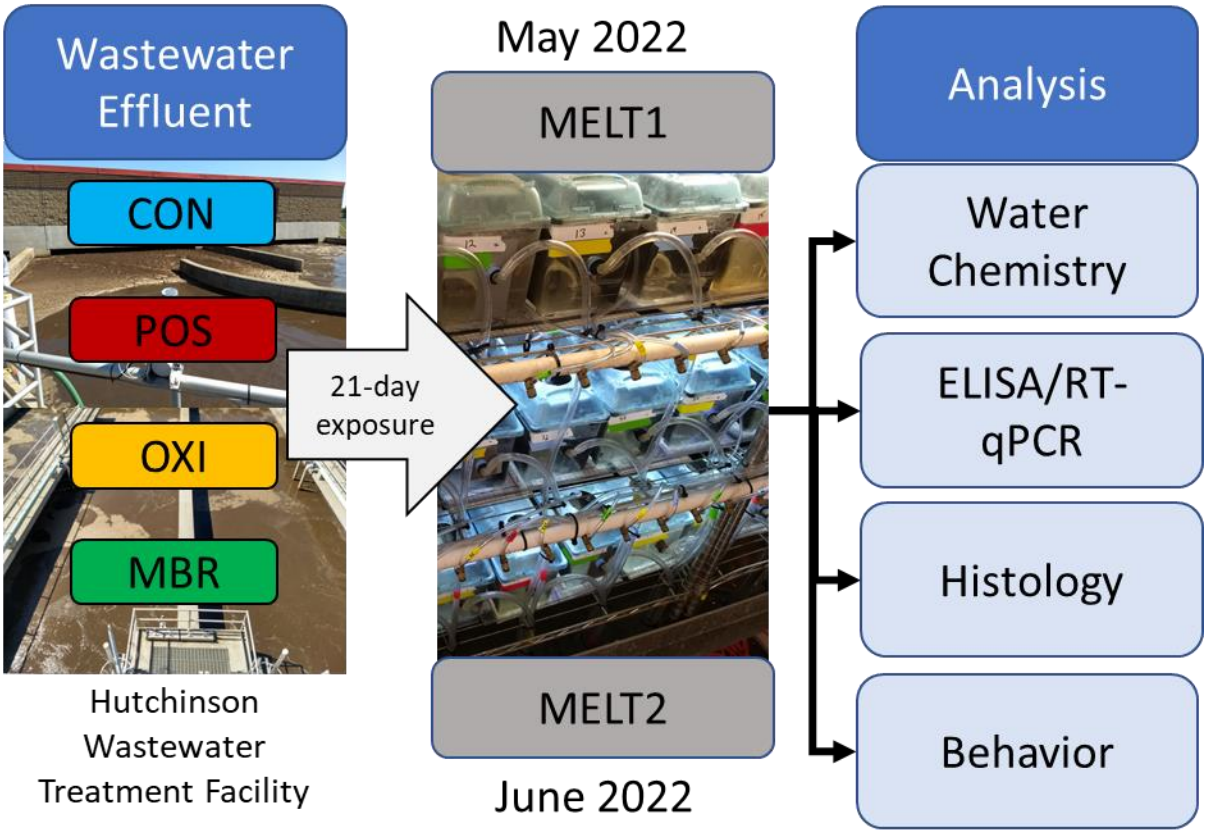
Chapter 2: Methods

2.1 Experiment Overview

The current study consisted of repeated 21-day exposures conducted at Hutchinson Wastewater Treatment Facility in Hutchinson, MN (44°52'28.7"N, 94°21'17.5"W) using the St. Cloud State University Mobile Exposure Laboratory Trailer (MELT) platform. Following modified OECD guidelines (OECD, 2012) and in accordance with St. Cloud State University's IACUC guidelines (approved Protocol # 8-133). Two separate MELT exposures (MELT1 and MELT2) were conducted during May and June 2022, with MELT1 including four treatments: carbon filtered tap water as reference control (CON), primary treatment effluent (POS), oxidative ditch effluent (OXI), and membrane bioreactor effluent (MBR). MELT2 included three treatments: CON, OXI, and MBR because of the low survival of the POS treatment it was removed. Water parameters (Table 2.1) were measured daily, and water was pumped to the trailer using Campbell Hausfeld 1/5 HP Utility Pump, maintaining a pressure of 600ccm.

Figure 2.1

Study overview



Note. Study overview with treatments reference control (CON), primary effluent (POS), effluent from oxidation ditch treatment (OXI), and membrane bioreactor treatment (MBR) used in repeated 21-day exposures to fathead minnows. After exposures analysis was conducted for water chemistry, reverse transcription quantitative polymerase chain reaction (RT-qPCR), enzyme-linked immunosorbent assay (ELISA), histology, and behavior.

2.2 Field Site

The Hutchinson Wastewater Treatment Facility, located in Hutchinson, MN, is designed to treat an average of 0.16m^3/s (3.67mgd) wastewater. The facility uses two different secondary treatments

after the primary treatment which is spilt in parallel: OXI and MBR. OXI is made up of two extended aeration units with an average flow of 0.107m^3/s (2.44mgd) and sludge age of 24 days. While MBR uses General Electric's (Boston, MA) ZeeWeed 500 ultrafiltration module with an average flow of 0.053m^3/s (1.22 mgd) and sludge age of 28 days. Both treatments undergo ultraviolet disinfection before discharging water into South Fork of the Crow River (*Hutchinson Public Works: Wastewater*, 2023).

2.3 Analytical Chemistry

Water samples were collected monthly from April to September 2022. Collection occurred weekly during the 21-day exposures of fathead minnows to CON, POS, OXI, and MBR treatments. Samples were filtered using a 0.7µm pore syringe filter and transferred into a 20mL glass amber vial for pesticide analysis, per USGS standard operating procedures (Wilde et al., 2014). The USGS National Water Quality Laboratory (NWQL, Denver, CO) analyzed the samples for 102 pesticides while SGS AXYS laboratories (Sidney, BC) analyzed for six bisphenols and four alkylphenols.

Exposure activity ratios (EAR) were calculated for each treatment using R studio package "toxeval" (DeCicco et al., 2022) with default benchmarks using R studio 2021.09.1 build 372.

$$EAR = \frac{Measured\ Concentration}{Benchmark\ (Toxcast)}$$

2.4 Experimental Design

The exposures were conducted in May 2022 (MELT1) and June 2022 (MELT2). Outdoor temperatures during MELT1 and MELT2 were recorded as mean (standard deviation) values of 15.8℃ (±7.9) and 22.5℃ (±6.1), respectively, while mean precipitation during MELT1 and MELT2 was recorded as 4.57mm (±0.42) and 0.76mm (±0.05), respectively, according to data reported by Brownton Wastewater Treatment Facility. Inside the trailer, mean temperatures were recorded as 22.5℃ (±4.0) during MELT1 and 23.8℃ (±2.6) during MELT2. The mean lighting intensity 12.3 lum/ft² (±9.2) during MELT1 and 7.4 lum/ft² (±5.3) during MELT2. For details refer to Table 2.1.

Table 2.1

Water quality parameters

Exposure		MELT1					MELT2		
Treatment	Facility Influent	CON	OXI	MBR	POS	Facility Influent	CON	OXI	MBR
Temp (°C)	15.4 (2.5)	20.6 (2.8)	19.8 (2.66)	19.0 (2.5)	21.1 (2.6)	21.4 (2.9)	22.0 (2.3)	21.7 (2.6)	19.8 (2.0)
Conductivity (SPC ms/cm)	-	1268.8 (216.7)	1300.7 (66.4)	1296.7 (79.7)	1347.9 (184.0)	-	1466.3 (248.2)	1482.3 (86.7)	1503.4 (90.1)
TDS	-	0.8 (0.1)	0.8 (0.04)	0.8 (0.1)	0.9 (0.1)	-	1.0 (0.1)	1.0 (0.1)	1.0 (0.1)
SAL	-	0.7 (0.04)	0.7 (0.04)	0.7 (0.04)	0.7 (0.1)	-	0.8 (0.05)	0.7 (0.05)	0.8 (0.05)
DO (mg/L)	9.4 (2.7)	2.1 (0.6)	2.2 (0.5)	2.4 (0.6)	1.9 (0.4)	7.47 (0.82)	2.2 (0.6)	2.1 (0.6)	2.5 (0.7)
pH	8.15 (0.28)	7.9 (1.3)	8.3 (0.3)	8.3 (0.2)	8.5 (0.2)	8.29 (0.16)	8.0 (0.2)	8.3 (0.3)	8.1 (0.2)
ORP	-	39.7 (11.6)	13.6 (10.1)	34.0 (15.7)	-19.4 (18.9)	-	36.5 (8.4)	17.3 (10.5)	38.0 (10.0)
Total Ammonia (ppm)	0.12 (0.04)	0.5	0.5	0.5	<6	0.13 (0.02)	0.25	0.25	0.25
Total Alkalinity (ppm)	-	240	240	240	240	-	240	240	240
Total Hardness (ppmCaCO3)	-	425	425	425	425	-	425	425	425
Total Chlorine (ppm)	-	0	0	0	0	-	0	0	0
Free Chlorine (ppm)	-	0	0	0	0	-	0	0	0
Intensity (lum/ft^2)	-	11.41 (10.37)	4.96 (4.31)	69.11 (51.61)	22.41 (19.51)	-	10.65 (15.21)	4.78 (3.92)	62.07 (45.68)

Note. Mean (±standard deviation) water quality parameters for each exposure, treatment, and facility influent. Facility measurements provided by Hutchinson Wastewater Treatment Facility. Total ammonia (0-6ppm) for treatments measured using API ammonia NH_3/NH_4 test strips (Chalfont, PA). Temperature,

Conductivity, Total Dissolved Solids (TDS), Salinity (SAL), Dissolved Oxygen (DO), pH, and Oxidation-Reduction Potential (ORP) measured using YSI meter. Total Alkalinity (0-240ppm), Total Hardness(0-1000ppm), Total Chlorine(0-10ppm), and Free Chlorine (0-10ppm) measure using HACH Aquacheck 7-way strips (Loveland, CO). Intensity measured using HOBO data logger.

Adult fathead minnows from Environmental Consulting & Testing (Superior, WI) were used for each 21-day exposure. Male and female pairs were randomly assigned into 2-liter aquaria, with 60 aquaria stacked in three rows of 20 and kept at a flow rate of 10mL/min for each treatment. Each aquarium treatment was assigned by randomly generating numbers to each treatment. Fish were acclimated for 24 hours before the experiment started and fed *ad libitum* with a combination of frozen blood worms and brine shrimp twice a day. The aquaria had constant aeration and a photoperiod of 16:8 hour light:dark.

On day 21, fish were not fed and were euthanized with neutral buffered 0.1% MS-222 (Argent Chemical Laboratories, Redmond, WA, Lot# TR0607N). Each fish was sexed based on secondary sex characteristics, with male fish receiving a score 0-3 for number of tubercles, dorsal pad, and coloration (Jensen et al., 2001). Total wet mass (Ohaus Scout Pro SP6001, 0.1g precision, Parsippany, NJ), standard length (SL, mm), and tail length (TL, mm) were measured before making a cut on the caudal peduncle vein. Blood glucose was measured using a TRUEbalance blood glucose meter (Moore Medical LLC, Farmington, CT) before collecting blood in a capillary tube. The capillary tube was then centrifuged using Hermle Z200A (Hermle Labortechnik, Wehingen, Germany) at 5,000 rpm for 5 min. After centrifugation, the percent hematocrit was determined with the Lancer CritoCap Micro-Hematocrit Capillary Tube reader (Sherwood Medical Industries, St. Louis, MO), and plasma was removed and stored in a 1.5mL tube at in -80℃ for later analysis.

Following blood collection, fish were submerged into 70% ethanol for disinfection, and the liver and gonad were excised. The liver and gonad were weighed (Ohaus Scout, 0.001g precision, Parsippany, NJ) and split in half. One half of each organ was placed in micromesh biopsy processing cassettes and then placed in 10% neutral buffered formalin, while the other half was placed into RNAlater® (Thermo Fisher Scientific, Waltham, MA).

Biological indices for the condition factor (CF), hepatosomatic (HSI), and gonadosomatic (GSI) were calculated using these equations:

$$CF = \frac{total\ mass}{standard\ length}$$

$$HSI = \left(\frac{liver\ mass}{fish\ mass}\right) * 100$$

$$GSI = \left(\frac{gonad\ mass}{fish\ mass}\right) * 100$$

Plasma, liver, and gonad samples were stored in -80℃ freezer for later analysis. The liver and gonad cassettes were kept in 10% neutral buffered formalin for later histological analysis.

2.5 Behavior

Behavioral responses link physiological function with ecological processes (Ford et al., 2021; Scott & Sloman, 2004). They are ideal for studying effects of environmental pollutant because behaviors relate to molecular initiation events that induce physiological changes (Steele et al., 2018). Changes in behavior are invaluable in predicting higher order outcomes in population and community levels (Bertram et al., 2022).

Tests were conducted in aquaria with PVC nest site placed near the back wall, a 1 cm grid covering the bottom of the aquaria, contained two concentric rings (13 cm diameter and 5.5 cm diameter). The tests were filmed from above the concentric rings using a GoPro Hero 8 (GoPro, San Mateo, CA)(Brodin et al., 2013).

On day 19, fish were not fed in the morning, and a boldness assay was conducted to test a fish's level of risk aversion. A foreign object (blue dice) was lowered into a central portion of rings as defined by the grid underneath the aquaria. Recording from above with a GoPro Hero 8 started before the dice was lowered and observation started when the dice was set. The observation period lasted for five minutes, and the following variables were measured: (1) time to first entry to ring, (2) total time in ring, (3) number of times in ring, (4) object bumps, and (5) charges on the object (Ward et al., 2017).

On day 20, fish were not fed in the morning, and nest defense assay was conducted in aquaria with surviving males. Nest defense was performed by placing another male or intruder within the ringed area, which was graded for secondary sex characteristics beforehand, with a score 0-3 on number of tubercles, dorsal pad, and coloration. Recording and observation from above with a GoPro Hero 8 started after the intruder was set in the ring. The observation period lasted for five minutes, and the following variables were measured: (1) time to first entry to ring, (2) approaches to intruder, (3) total time within first ring, (4) number of lateral displays, (5) time to first jar bump, and (6) total number of bumps (Ward et al., 2017).

2.6 Fecundity/Fertility

Reproductive impairment at the individual level was examined using fecundity and fertility. Throughout the experiment, PVC nests were examined daily for the presence of eggs by removing the PVC and checking the underside. When eggs were observed, eggs were counted and moved to aquaria without fish that contained treatment water. A new PVC nest was placed into the aquaria that had eggs. Three days after eggs were found, the PVC nests were examined for fertilization by looking for eye spots, and then eggs with eye spots were tallied (Ankley et al., 2001).

2.7 Histology

Toxicity can alter the structure and development of cells, which can be reflected in tissues. As toxins increase in concentration, vacuoles begin to form in the liver as it metabolizes toxins (Nayak et al.,

1996; Thomas et al., 2017). Meanwhile, the maturation of cells in the gonad is crucial for reproductive success (Pohl et al., 2018; Thomas et al., 2017; Zeilinger et al., 2009).

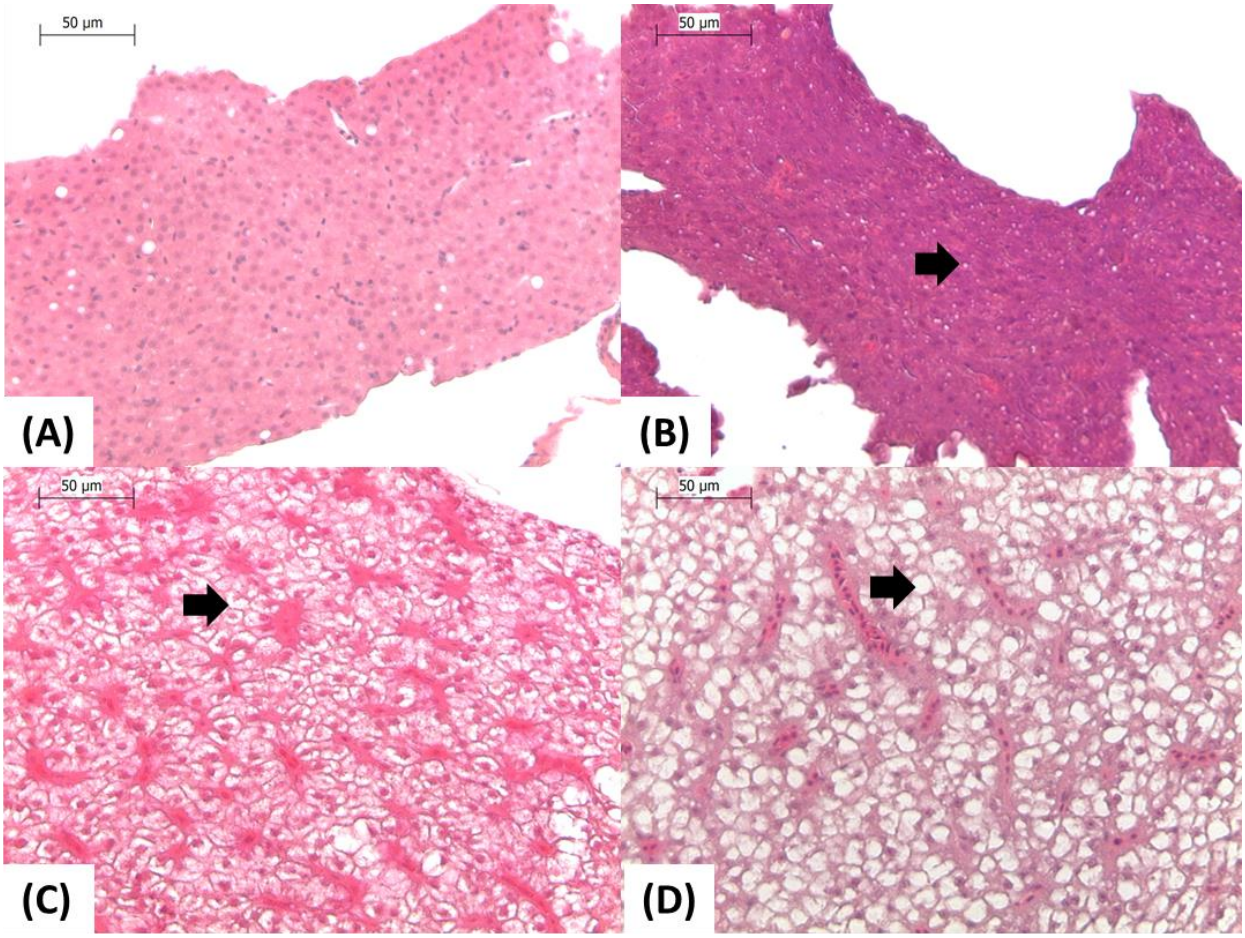
Histological analysis of liver and gonad tissues was performed to identify changes in liver vacuolation and to determine the distribution of cells in different stages of gametogenesis. Preparation of liver and gonad tissue samples followed published protocols (Nayak et al., 1996; Thomas et al., 2017).

The tissues samples in histological cassettes containing 10% neutral buffered formalin were fixed using a Leica automated tissue processor ASP300 (Wetzlar, Germany). After fixation, the tissues were embedded in paraffin using Thermo Scientific Microm EC350-1 embedding station (Waltham, MA) and sectioned into 5µm sections using a Reichert-Jung cassette microtome (Reichert Ametek, Depew, NY). Finally, the tissue sections were stained using standard hematoxylin and eosin techniques in Leica Autostainer XL.

To assess the degree of vacuolation of hepatocytes in liver tissue, a semi-quantitative scale ranging from 1 to 4 was used. The scale was based on the percentage of vacuoles present in the total area. A score of 1 was assigned if vacuoles were visible in less than 5% of total area (Figure 2.2a), 2 if vacuoles were small but visible throughout in 25% of area (Figure 2.2b), 3 if there was broad presence of large vacuoles in 25–50% of the area (Figure 2.2c), and 4 if vacuoles were prominent and covered more than 50% of the field of view (Figure 2.2d). This method was adopted from Thomas et al. (2017) and illustrated in Figure 2.2.

Figure 2.2

Liver histology grading



Note. Liver histology micrographs represent four grades of liver vacuolation. (A) grade 1 few visible liver vacuoles; (B) grade 2 liver vacuoles visible but infrequent; (C) grade 3 liver vacuoles widespread; (D) grade 4 severe vacuolation. Liver vacuoles to the right of arrow heads. Image brightness and contrast was adjusted equally for all four micrographs using Microsoft PowerPoint.

The sex of fish was confirmed through gonad histology and then used as assigned sex for statistical analyses. The gametogenesis index of ovary or testes was calculated based on proportion of cell types visible in the field of view (female [Figure 2.3a]: perinuclear oocyte, cortical alveolar, early

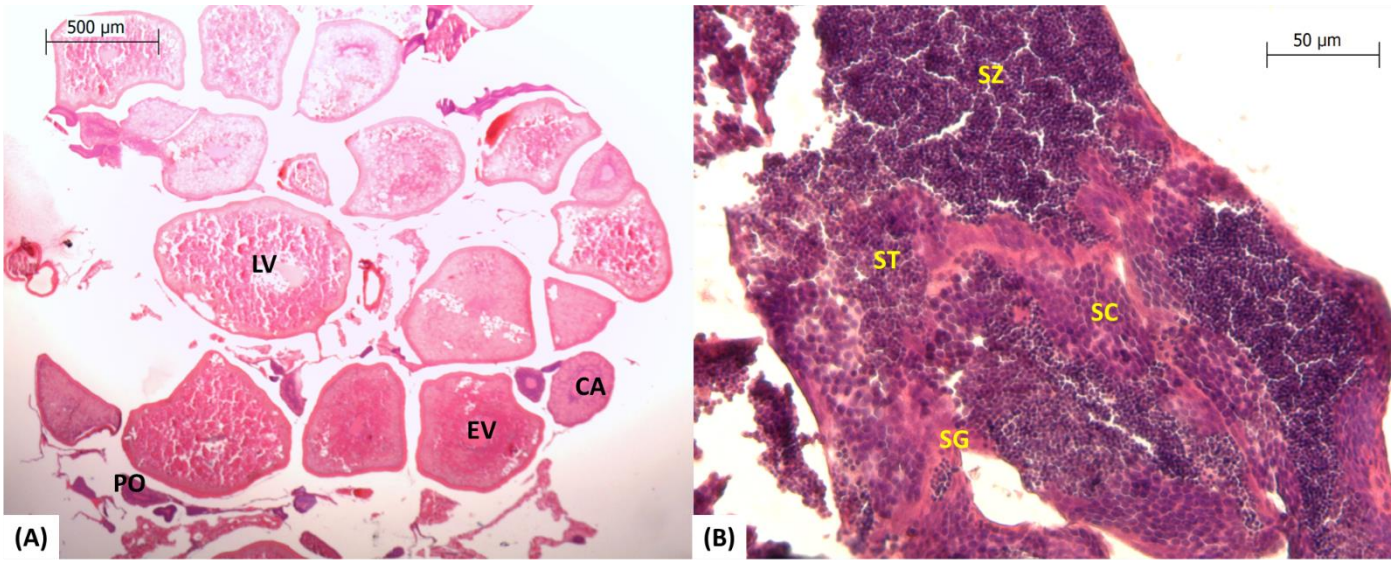
vitellogenic, late vitellogenic; male [Figure 2.3b]: spermatogonia, spermatocyte, spermatid, and spermatozoa)(Jensen et al., 2001; Thomas et al., 2017; Zeilinger et al., 2009)(Figure 2.3). Then rated on a scale of 1 = immature to 4 = only mature cells present with the overall maturity of sample calculated as follows:

$$Testis = \frac{\left((\%spermatogonia) + (\%spermatocytes * 2) + (\%spermatids * 3) + (\%spermatozoa * 4)\right)}{100}$$

$$Ovary = \frac{\left((\%primary\ oocyte) + (\%cortical\ alveolar * 2) + (\%early\ vitellogenic * 3) + (\%late\ vitellogenic * 4)\right)}{100}$$

Figure 2.3

Gonad histology grading



Note. Gonad histology representative for (A) female and (B) male gonad tissue. PO = perinuclear oocyte; CA = cortical alveolar oocyte; EV = early vitellogenic oocyte; LV = late vitellogenic oocyte; SG = spermatogonia; SC = spermatocyte; ST = spermatid; SZ spermatozoa. Images brightness and contrast

adjusted. Scale set at female = 500µm and male = 50µm. Image brightness and contrast was adjusted equally for both micrographs using Microsoft PowerPoint.

2.8 Biochemical Endpoints

2.8.1 *Enzyme-linked Immunosorbent Assay (ELISA)*

Hormones such as 11-ketotestosterone (11-KT) and estradiol (E2), as well as protein VTG, are commonly used biomarkers for assessing reproductive impairment in aquatic organisms (Kittelson et al., 2023; D. Villeneuve, 2021; D. L. Villeneuve, 2022). Meanwhile, cortisol in plasma is used as a biomarker of stress (Wendelaar Bonga, 1997).

Plasma samples were analyzed using competitive antibody-capture ELISA to quantify VTG (Korte et al., 2000; Parks et al., 1999). Standard preparation and sample analysis followed previously described methods (Minarik et al., 2014).

To measure the concentrations of plasma cortisol, 11-KT, and E2 instructions were followed using commercially available chemical kits (cortisol: Cayman Chemical #500370; 11-KT: Cayman Chemical 582751; E2: Cayman Chemical 501890).

2.8.2 *Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)*

Gene expression plays an important role in identification of AOP pathways and allows adverse outcomes to be monitored. At the molecular level expression changes can indicate early stress responses which may not be seen at higher levels of organization (Perkins et al., 2011). For this study genes in aerobic metabolism (PK, PFK1, LDH, PYGL, GSY1, CYCS, ACC, ACAD, and LPL), oxidative stress (SOD, NOX1, and MAPK13), and reproduction (ESR1, ESR2, AR1, CYP19a, and StAR) were measured. Aerobic metabolism was selected as if there are reductions in gene expression it can be attributed to stress and can be associated with survival. While oxidative stress is related to aerobic metabolism and

could be indicative of changes in other pathways. The reproductive genes are related to aerobic metabolism and can be associated with population growth.

To quantify mRNA abundances, RT-qPCR was performed following the MIQE guidelines (Bustin et al., 2009). Briefly, liver and gonad in RNAlater® were homogenized in TRIzol™ Reagent (Thermo Fisher Scientific, Cat#15596026) using Qiagen TissueLyser LT (Hilden, Germany) 50Hz for 5 minutes (Jiang et al., 2015). After homogenization, RNA was isolated using the modified Promega SV Total RNA Isolation System with DNase-I treatment (Madison, WI). RNA concentration and purity were determined using a NanoDrop-2000 spectrometer (NanoDrop, Thermo Fisher Scientific) with samples having a concentration of >10ng/µl and an absorbance 260/280>1.8. The RNA quality was further assessed by running samples on 1.5% agarose gel per standard protocol (Sambrook & Russel, 2001; D. L. Villeneuve et al., 2007).

The Applied Biosystems high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) was used to synthesize cDNA from 0.2µg of total RNA in 15µl reactions using Eppendorf Master cycler gradient (Hamburg, Germany). The resulting cDNA was stored at -20℃.

NCBI Primer-Blast (Ye et al., 2012)was utilized to design gene-specific primers for fathead minnow qPCR. Primers were selected to span exon-exon junctions with introns, and the annealing temperature was optimized using CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) with a temperature gradient (Table 2.2). Subsequently, primer pairs were then ordered from Eurofins Genomics (Louisville, KY).

Table 2.2

Gene primers with quality values

GENE	Accession No.		Primer	Annealing Temp (℃)	Effiency (%)	R^2
PFK-1	XM_039689986.1	F 5'-	CAACATCATTCAGCTTGGTGG	64	93.6	0.997
Phosphofructokinase-1		R 5'-	TTCAGATGAGTGTACTGCTGC			
PK	XM_039688700.1	F 5'-	GTTACGATGGGTGCTCGGAT	64	100.3	0.998
Pyruvate kinase		R 5'-	TTGAGTCTGGCGATGTTCATT			
LDH	XM_039663125.1	F 5'-	TGGCCTCCACAAAAGAGAAAC	64	101.6	0.997
Lactate dehydrogenase		R 5'-	GCCGTCACACTGTAGTCTTTAT			
SOD	XM_039667529.1	F 5'-	GTTTATTTCGATCAAGAGAGCGAC	64	76.1	0.998
Superoxide dismutase		R 5'-	TCCAAAAGCATGGACATGGAA			
ACAD Acyl-CoA dehydrogenases	XM_039683337.1	F 5'-	CGCTCCTTTTGTCTCCAAGAT	64	64.0	0.999
		R 5'-	ATCTCAATCCCCATCAGTCCC			
LPL	XM_039682610.1	F 5'-	CTTCTGGCTTAAGGAACATGGA	62	104.4	0.997
Lipoprotein lipase		R 5'-	CATGCCTTTGTTGAAGGTGTC			
GYS1	XM_039668436.1	F 5'-	TGCTGCTTCTGATTGGAGTC	62	95.1	0.996
Glycogen synthase		R 5'-	TTTTTATCCGTCAACTGGAGATGC			
PYGL Glycogen phosphorylase	XM_039656181.1	F 5'-	CAATAACGATCCTGTGATTGGC	64	99.5	0.998
		R 5'-	CTGTAGCTGGGATCACCTTC			

CYCS Cytochrome c	XM_039681838.1	F 5'- R 5'-	CGAACGGAAGCGCTAGAATG AGTATGCTGGGAGTGTGTAGT	64	109.1	0.996
MAPK13 mitogen-activated protein kinase 13	XM_039665341.1	F 5'- R 5'-	AACTAGAAAGCCCAGAGGCTA TACTGGCTCTGGGAAACAATG	64	94.0	0.999
ACC Acetyl-CoA carboxylase	XM_039658583.1	F 5'- R 5'-	CCCGAGAACCTCAAGAAACTC ACTGGGGAGAAAGGTTCTCTA	64	97.1	0.998
NOX1 NADPH oxidase	XM_039655173.1	F 5'- R 5'-	GGGAACTTTTAGGGTCTGCAT CAAATGAGCGACAGTGTGAAC	64	93.0	0.998
CYP19A Cytochrome P450 19 Subfamily A	AJ277866	F 5'- R 5'-	CCAGATACTCTCTCGATCAGT ATGTTTAACCTGGACAGATGC	64	95.4	0.999
ESR1 Estrogen receptor	AY775183	F 5'- R 5'-	GGTGTTGATGATCGGCCTCATA AGCCATCCCCTCGACACAT	64	92.2	0.999
AR1 Androgen receptor	XM_039650027.1	F 5'- R 5'-	TCCTCTTCAGCATCATTCCAG GCTCTTCCTGCCATAGTTGAT	64	96.4	0.999
StAR Steroidogenic acute regulatory protein	XM_039665495.1	F 5'- R 5'-	CATTTCCTACAGGCACGTGAG CTTCTGCAATCCGACTACTGAG	62	96.7	0.999
ESR2b estrogen receptor 2b	XM_039652091.1	F 5'- R 5'-	TTTAACCAGAGCAGTTCTGTCC AATGCCTGGAGAAGGATTCAA	64	102.0	0.999

RPL8 Ribosomal protein L8	AY919670	F 5'- R 5'-	CCCACAATCCTGAGACCAAG TTGTCAATACGACCACCACC	64	98.4	0.996
TBP TATA-Box Binding Protein	GCVQ01021764.1	F 5'- R 5'-	CATTCGATTAGAGGGCCTGG CCTGGGAAATAACTCTGGTTCA	64	96.0	0.999
G6PD glucose-6-phosphate-1-dehydrogenase	AF206637.2	F 5'- R 5'-	ATCTACGCCAAGATGATGAGC TGCGAACAAAATGCATCTGAC	64	97.6	0.998

Note. Gene targets for qPCR analysis. Gene name with accession number, forward and reverse primers. Annealing temperature. SYBR green efficiency percent and R^2 means for all plates and tissues.

The qPCR reactions were prepared using a SYBR Green reaction mix containing 20 mM Tris-HCl (pH 7.75), 50 mM KCl, 3 mM $MgCl_2$, 0.5% Glycerol, 0.5% Tween-20, 0.5x SYBR Green-I (Invitrogen, Thermo Fisher Scientific), 0.2 mM dNTP mix and 0.01 U/µl Ampli Taq Gold (Applied Biosystems, Thermo Fisher Scientific). Each 15 µl reaction contained 0.2 µM of each gene-specific primer and 1/50 volume of diluted cDNA. The reactions were performed with heat denaturing period at 95℃ for 5 min followed by 40 cycles of amplification with denaturing at 95℃ for 15 s and annealing/extension at the optimized temperature for 45 s (Table 2.2). The specificity of each PCR product was confirmed by analyzing the melting curve after each reaction. Data was analyzed using CFX manager with single threshold method.

To determine the absolute mRNA abundance, each signal was compared to a standard curve (10^2-10^8 copies/µl) within each PCR reaction plate. A standard curve for each gene was generated by creating known concentrations of plasmids that ligated each target gene (Kohno et al., 2003). PCR products were cloned for standard samples using the pGEM-T-Easy vector system (Promega) and Wizard Plus SV Minipreps DNA Purification System (Promega). The ligated plasmid PCR products were sequenced using Sanger sequencing (Eurofins Genomics) to confirm qPCR specificity. The plasmids were

diluted serially using TE buffer with 5 ng/µl tRNA (Sigma-Aldrich, Burlington, MA) to create the standard curve. Reactions for qPCR were run in triplicate, and the coefficient covariance (CV) was calculated. Samples with CVs greater than 1% in the Cycle-Quantification (CQ) value for standard samples and 20% in the Starting-Quantity (SQ) value of unknown samples were excluded from analysis.

To calculate the relative mRNA abundance, a normalization factor was used. The normalization factor was derived from the geometric mean of SQ of two internal control genes - ribosomal protein L8 (rpl8) and TATA-box binding protein (tbp) for liver samples (Table 2.3), and tbp and glucose-6phosphate-1-dehydrogenase (g6pd) for gonad samples (Table 2.4). The reference genes rpl8, tbp, and g6pd were analyzed using the Normfinder software (Andersen et al., 2004) to identify the most stable combination of genes.

Table 2.3

Results of normalization for liver samples

Liver Normalization Result

Gene name	**Stability value**	**Best gene**	TBP
G6PD	0.093	**Stability value**	0.030
TBP	0.030		
RPL8	0.062	**Best combination of two genes**	TBP and RPL8
		Stability value for best combination of two genes	0.035

Note. Normalization results for the liver samples using housekeeping genes G6PD, TBP, and RPL8.

Table 2.4

Results of normalization for gonad samples

Gonad Normalization Result

Gene name	**Stability value**	**Best gene**	G6PD
RPL8	0.108	**Stability value**	0.043
G6PD	0.043		
TBP	0.093	**Best combination of two genes**	G6PD and TBP
		Stability value for best combination of two genes	0.051

Note. Normalization results for the gonad samples using housekeeping genes G6PD, TBP, and RPL8.

2.9 Quality Assurance/Quality Control

To ensure accuracy and reproducibility of results, CV was calculated in all assays. In addition, for RT-qPCR R^2, SYBR efficiency, genomic DNA, and CQ of no template control were recorded. Blind data analysis was done to remove bias.

For each behavior assay (boldness and nest defense), 10% of assays were subjected to reanalysis. MELT1 boldness had a CV of 8.5%, while nest defense was 4.9%. MELT2 boldness was 2.8% and nest defense 5.1%.

Histological analysis was repeated for 10% of each tissue. The overall CV for liver of 9.4%, and gonad, it was 6.9%.

Repeated measures CV for each ELISA were calculated. The CV for VTG was 4.6%. Meanwhile, cortisol had CV of 4.5%, 11-KT had a CV of 3.9%, and E2 had CV of 3.2%. For data that fell below the limit of detection (LOD), it was changed to LOD/2 for each ELISA.

As stated earlier, RT-qPCR standards above 1% and samples above 20% CV were removed from analysis. R^2 and SYBR efficiency are recorded in Table 2.2. The gene SOD efficiency is lower than the 90%

acceptable level, but data was used due to the high R^2 and consistency of the efficiency. Genomic DNA was detected with mean CQ of 37.5 (±3.2) for liver and 38.6 (±2.5) for gonad. The CQ for all genes' no template controls when measurable was >38. Additionally, LOD and limit of quantification (LOQ) were calculated as 50 copies and 100 copies, respectively (Forootan et al., 2017). For data that fell below the LOQ, it was changed to LOQ/2.

2.10 Statistics

Power of analysis was conducted in JMP® Pro 16.0.0 using previous data on VTG, and the minimum sample size required for a significant result was determined to be 7 for females and 9 for males. Parametric methods, such as ANOVA, were used to analyze biological data if the assumptions of normality and variance were met. In cases where assumptions were not met, non-parametric methods such as Kruskal-Wallis rank test or Mann-Whitney U test were used, or data was transformed. Log transformation was applied to gonad mass, GSI, 11KT, ESR2, StAR, NOX1, MAPK13, LPL, LDH, GSY1, CYCS, AR1, and ACC. Square root transformation was applied to glucose, E2, and PYGL. Kruskal-Wallis rank test was used for VTG and CYP19a. Villeneuve et al. (2021a) statistical method for multiple endpoints in an AOP was followed. Tukey HSD was used for post-hoc analysis. Significance levels were set at $p \leq 0.05$. Statistical analysis was run in JMP® Pro 16.0.0 in Microsoft Windows 10 Home 64-bit 10.0.19044.0.

Chapter 3: Results

3.1 Analytical Chemistry

Out of 112 CECs from three chemical classes (See supplemental table S1 in Supplmental.pdf for full list of chemicals and groupings), 26 were detected in MELT1 and 28 were detected in MELT2 (Table 3.1). In MELT1, 19 of 102 pesticides, 4 of 6 bisphenols, and 3 of 4 alkylphenols were detected, while in MELT2, 21 of 102 pesticides, 4 of 6 bisphenols, and 3 of 4 alkylphenols were detected (Table 3.1).

Table 3.1

Chemicals detected in each treatment

Chemical Class	Chemical	MELT1				MELT2			
		CON	MBR	OXI	POS	CON	MBR	OXI	POS
Pesticide	2-4-D	X	X	X	X	X	X	X	X
	2-Hydroxyatrazine (OIET)	X	X	X	-	-	-	-	-
	Acetochlor	-	-	X	X	X	X	-	-
	Atrazine	X	X	X	-	X	X	X	X
	Azoxystrobin	X	X	X	X	X	-	-	X
	Carbaryl	-	-	X	X	-	-	-	-
	Deethylatrazine (CIAT)	-	-	-	-	X	X	X	-
	Dimethenamid	X	X	X	X	X	X	X	X
	Diuron	-	-	-	-	-	-	X	-
	Fipronil	X	X	X	X	X	X	X	X
	Fipronil amide	-	-	-	-	-	-	-	X
	Fipronil sulfide	X	X	X	-	X	-	X	X
	Fipronil sulfone	-	X	X	-	X	X	X	X
	Imidacloprid	X	-	X	-	X	X	X	-
	Metalaxyl	X	X	X	X	X	-	-	-
	Metolachlor	X	X	X	X	X	X	X	X
	Metolachlor SA	-	X	X	-	-	-	-	X
	Piperonyl butoxide	-	X	-	X	X	X	-	X
	Prometon	X	X	X	X	X	X	X	X
	Propiconazole	-	X	-	-	X	-	-	-
	Sulfentrazone	X	X	-	X	X	-	X	-
	Tebuconazole	-	-	X	X	-	-	-	X
	Triclopyr	-	-	-	-	X	-	X	-

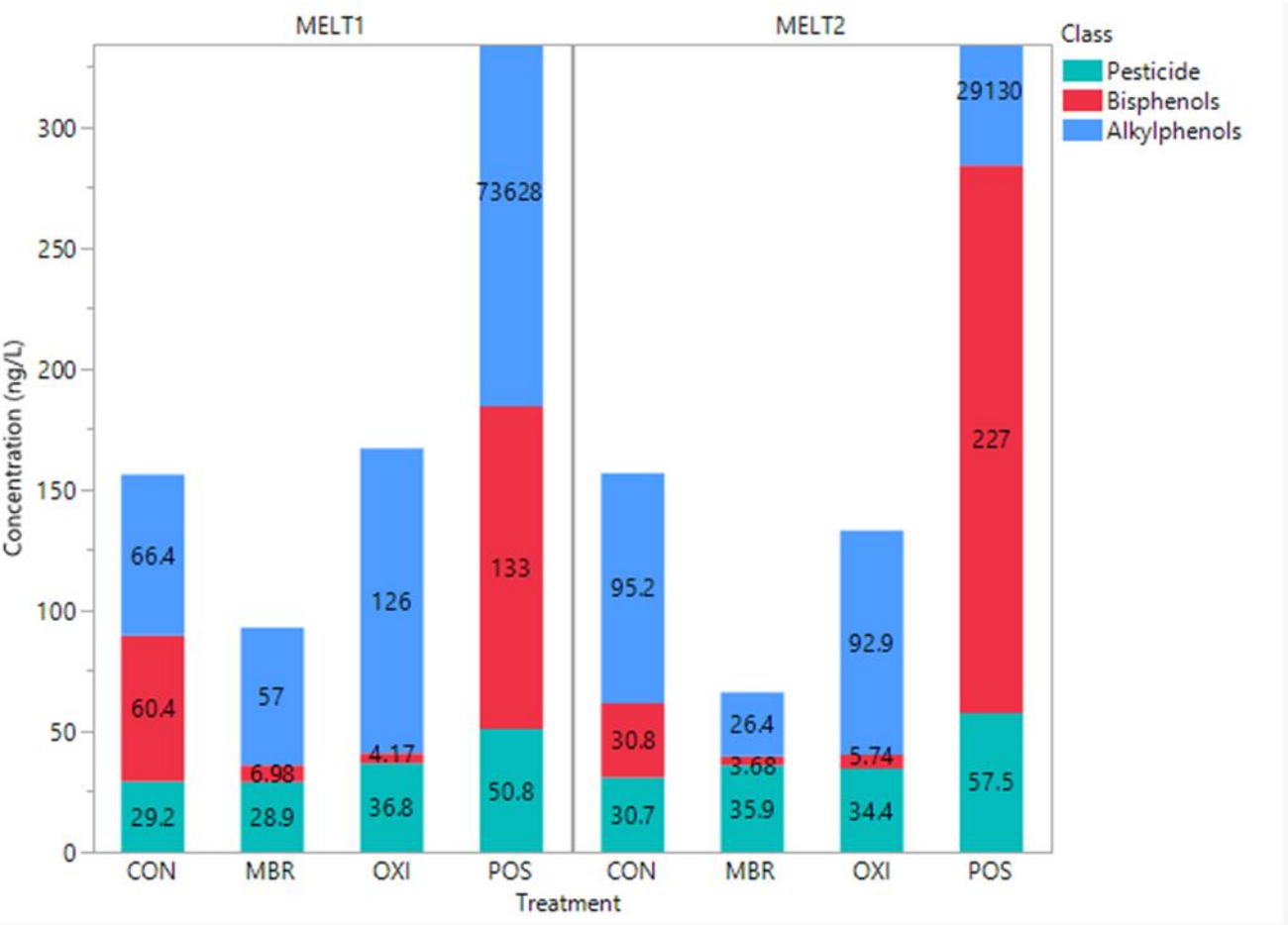
Bisphenol	Bisphenol A	X	X	X	X	X	X	X	X
	Bisphenol AF	-	X	-	X	X	X	-	X
	Bisphenol F	-	-	-	X	-	-	-	X
	Bisphenol S	-	-	-	X	X	-	-	X
Alkylphenol	4-Nonylphenol diethoxylates	X	X	X	X	X	X	-	X
	4-Nonylphenol monoethoxylates	X	X	X	X	X	X	X	X
	4-Nonylphenols	X	X	X	X	X	X	X	X

Note. CECs detected in each exposure and treatment. Separated into chemical class. Only chemicals that were detected are shown. X = Detected; - = Not detected.

The mean concentrations of each chemical class were calculated. In MELT1, the pesticide concentration (standard deviation) 29 (±43), 29 (±45), 37 (±67), and 51 (±72) ng/L for treatments CON, MBR, OXI, and POS, respectively. The concentrations of bisphenols 60 (±52), 7 (±6), 4 (±2), and 133 (±126) for treatments CON, MBR, OXI, and POS, respectively. The concentrations of alkylphenols 66 (±66), 57(±33), 126 (±72), and 73628 (±79173) for treatments CON, MBR, OXI, and POS, respectively. In MELT2, the pesticide concentrations 31 (±33), 36 (±40), 34 (±35), and 58 (±171) for treatments CON, MBR, OXI, and POS, respectively. The concentrations of bisphenols were 31 (±30), 4 (±1), 6(±3), and 227 (±172) for treatments CON, MBR, OXI, and POS, respectively. The concentrations of alkylphenols were 95 (±94), 26 (±17), 93 (±61), and 29130 (±31591) for treatments CON, MBR, OXI, and POS, respectively. (Figure 3.1)

Figure 3.1

Mean concentration (ng/L) vs. Treatment

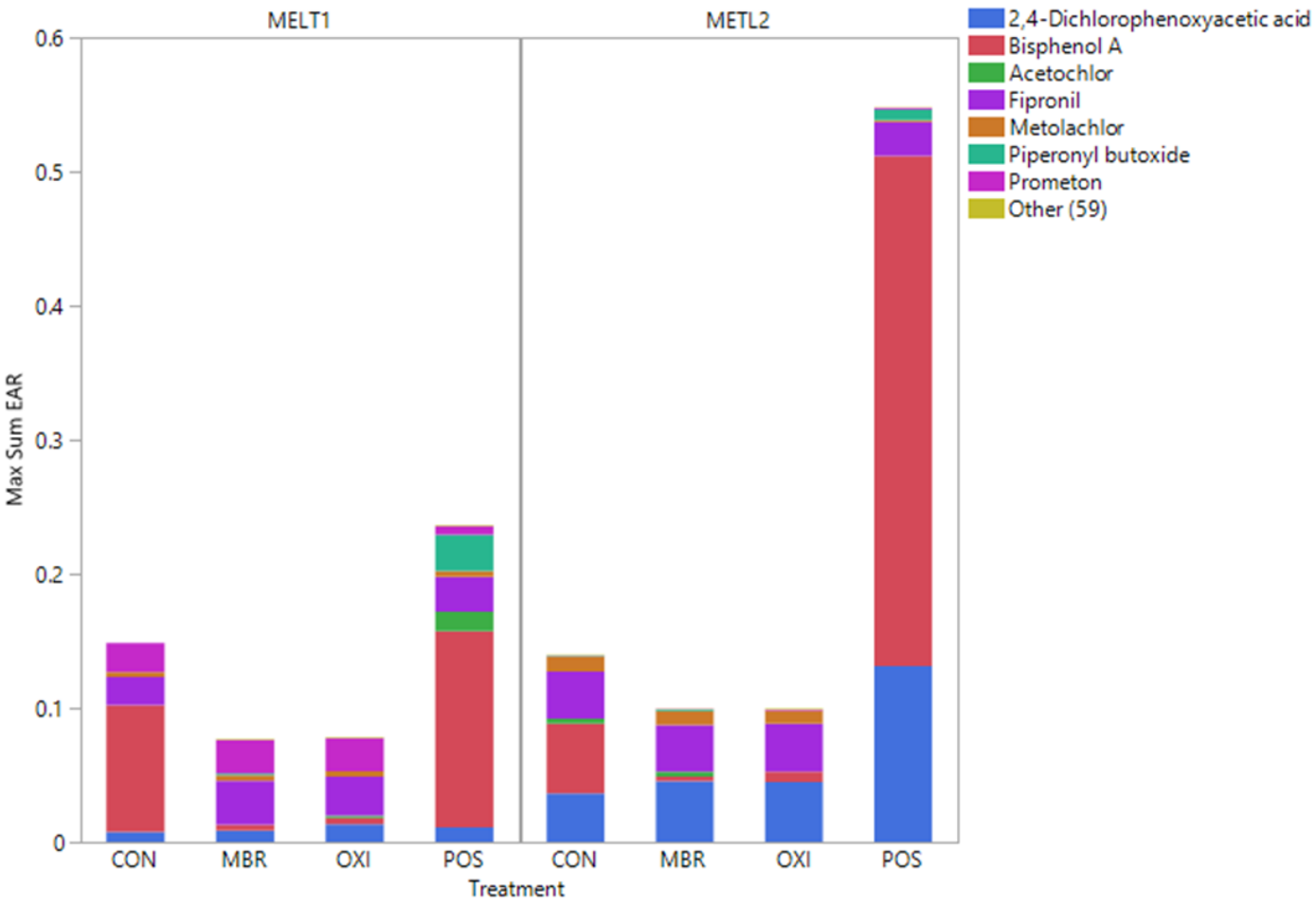


Note. Chemical class is stacked and labeled with the mean concentration. From bottom to top: Green = Pesticide; Red = Bisphenols; Blue = Alkylphenols.

To summarize the data and determine the possible biological effects, the maximum EAR was calculated for each detected chemical using R package “toxeval” (DeCicco et al., 2022). In MELT1, out of the 26 chemicals, 18 were suitable for maximum EAR calculation with a range of 0-0.15 (Figure 3.2). Similarly, in MELT2, 21 of 28 detected chemicals could be used for maximum EAR calculation with a range of 0-0.38 (Figure 3.2).

Figure 3.2

Max sum EAR vs. Treatment



Note. Max sum Exposure-Activity Ratio (EAR) vs. Treatment for MELT1 and MELT2. Shows sum of the max EAR for chemicals in each treatment. Sample size = 3.

3.2 Organism

The survival rates for MELT1 were 97% for CON, 97% for MBR, and 94% for OXI. The POS treatment had 0% survival after 24hrs and was, therefore, excluded, with aquaria being converted to other treatments. For MELT2, survival rates were 94% for CON, 94% for MBR, and 97% for OXI. Most endpoints differed significantly between male and female fish.

For the fathead minnows, there was no significant difference for treatments in wet mass (ANOVA p-value MELT1: Treatment = 0.94; MELT2: Treatment = 0.79), SL (ANOVA p-value MELT1: Treatment = 0.26; MELT2: Treatment = 0.45), and TL (ANOVA p-value MELT1: Treatment = 0.54; MELT2: Treatment = 0.81). Similarly, there was no significant difference in treatments in the TL/SL ratio (ANOVA p-value MELT1: Treatment = 0.11; MELT2: Treatment = 0.3075) or CF (ANOVA p-value MELT1: Treatment = 0.50; MELT2: Treatment = 0.97). The sum of secondary sex characteristics for male fathead minnows also did not significantly differ between treatments (ANOVA p-value MELT1: Treatment = 0.15; MELT2: Treatment = 0.20). (Table 3.2)

Table 3.2

Organism measurements

Organism			**MELT1**			**MELT2**		
Endpoint	**Sex**	**Statistics**	**CON**	**MBR**	**OXI**	**CON**	**MBR**	**OXI**
Wet Mass [g]	Female	Mean	1.4	1.4	1.4	1.3	1.2	1.3
		Std Dev	0.5	0.4	0.4	0.4	0.3	0.2
		N	14	17	20	20	12	15
	Male	Mean	3.1	3.1	3.0	2.7	2.6	2.4
		Std Dev	1.0	0.6	0.4	0.7	0.8	0.5
		N	17	17	13	28	20	20
TL [mm]	Female	Mean	49	49	49	47	47	46
		Std Dev	4	3	3	5	3	3
		N	14	17	20	20	12	15
	Male	Mean	60	62	60	58	58	58
		Std Dev	5	4	2	4	6	5
		N	17	17	13	28	20	20
SL [mm]	Female	Mean	45	45	44	39	38	38
		Std Dev	4	3	3	4	4	2
		N	14	17	20	20	12	15
	Male	Mean	50	51	49	48	48	48
		Std Dev	3	3	2	4	5	5
		N	17	17	13	28	20	20
TL/SL Ratio	Female	Mean	1.10	1.09	1.12	1.21	1.23	1.21
		Std Dev	0.04	0.04	0.07	0.07	0.05	0.06

		N	14	17	20	20	12	15
	Male	Mean	1.19	1.21	1.22	1.20	1.21	1.21
		Std Dev	0.05	0.05	0.02	0.05	0.03	0.03
		N	17	17	13	28	20	20
CF	Female	Mean	1.1	1.2	1.2	1.2	1.2	1.3
		Std Dev	0.2	0.2	0.2	0.1	0.1	0.2
		N	14	17	20	20	12	15
	Male	Mean	1.4	1.3	1.4	1.3	1.3	1.2
		Std Dev	0.2	0.2	0.1	0.2	0.2	0.2
		N	17	17	13	28	20	20
Sum SSC	Female	Mean	-	-	-	-	-	-
		Std Dev	-	-	-	-	-	-
		N	0	0	0	0	0	0
	Male	Mean	7	6	7	8	8	7
		Std Dev	2	2	2	2	1	2
		N	14	16	12	24	15	16

Note. Mean, standard deviation, and sample size for whole organism measurements for females and male fish. Split between MELT1 and MELT2 exposures as well as treatments. Tail Length (TL), Standard Length (SL), Condition Factor (CF), and secondary sex characteristics (SSC).

When comparing the results for the liver and gonad samples there was no significant difference in wet mass (Liver ANOVA p-value MELT1: Treatment = 0.57; MELT2: Treatment = 0.85; Gonad ANOVA p-value MELT1: Treatment = 0.79; MELT2: Treatment = 0.64) across treatments. While there were also no significant differences in treatment for HSI (ANOVA p-value MELT1: Treatment = 0.63; MELT2: Treatment = 0.99) and GSI (ANOVA p-value MELT1: Treatment = 0.70; MELT2: Treatment = 0.51). (Table 3.3)

Table 3.3

Organ measurements

Organ			MELT1			MELT2		
Endpoint	**Sex**	**Statistics**	**CON**	**MBR**	**OXI**	**CON**	**MBR**	**OXI**
Gonad Wet Mass(g)	Female	Mean	0.19	0.21	0.22	0.16	0.18	0.20
		Std Dev	0.12	0.11	0.14	0.11	0.08	0.10
		N	14	17	20	19	12	14
	Male	Mean	0.07	0.07	0.05	0.08	0.11	0.05
		Std Dev	0.05	0.04	0.03	0.18	0.19	0.03
		N	17	16	13	28	20	20
GSI (Gonad mass/Total mass)	Female	Mean	13.57	14.60	14.49	12.06	14.53	14.97
		Std Dev	5.95	6.24	7.79	7.19	5.68	7.10
		N	14	17	20	19	12	14
	Male	Mean	2.10	2.03	1.83	2.57	4.63	2.20
		Std Dev	1.11	0.96	0.93	4.56	8.37	1.64
		N	17	16	13	28	20	20
Liver Wet Mass(g)	Female	Mean	0.05	0.05	0.05	0.05	0.04	0.03
		Std Dev	0.02	0.02	0.02	0.05	0.03	0.02
		N	14	17	20	19	12	15
	Male	Mean	0.10	0.10	0.09	0.12	0.09	0.08
		Std Dev	0.03	0.04	0.03	0.16	0.04	0.04
		N	17	17	13	28	20	20
HSI (Liver mass/Total mass)	Female	Mean	3.43	3.67	3.46	3.57	3.35	2.63
		Std Dev	1.36	1.00	1.02	3.29	2.68	1.23
		N	14	17	20	19	12	15
	Male	Mean	3.38	3.31	2.99	4.09	3.39	3.13
		Std Dev	1.26	0.97	0.88	4.25	1.31	1.15
		N	17	17	13	28	20	20

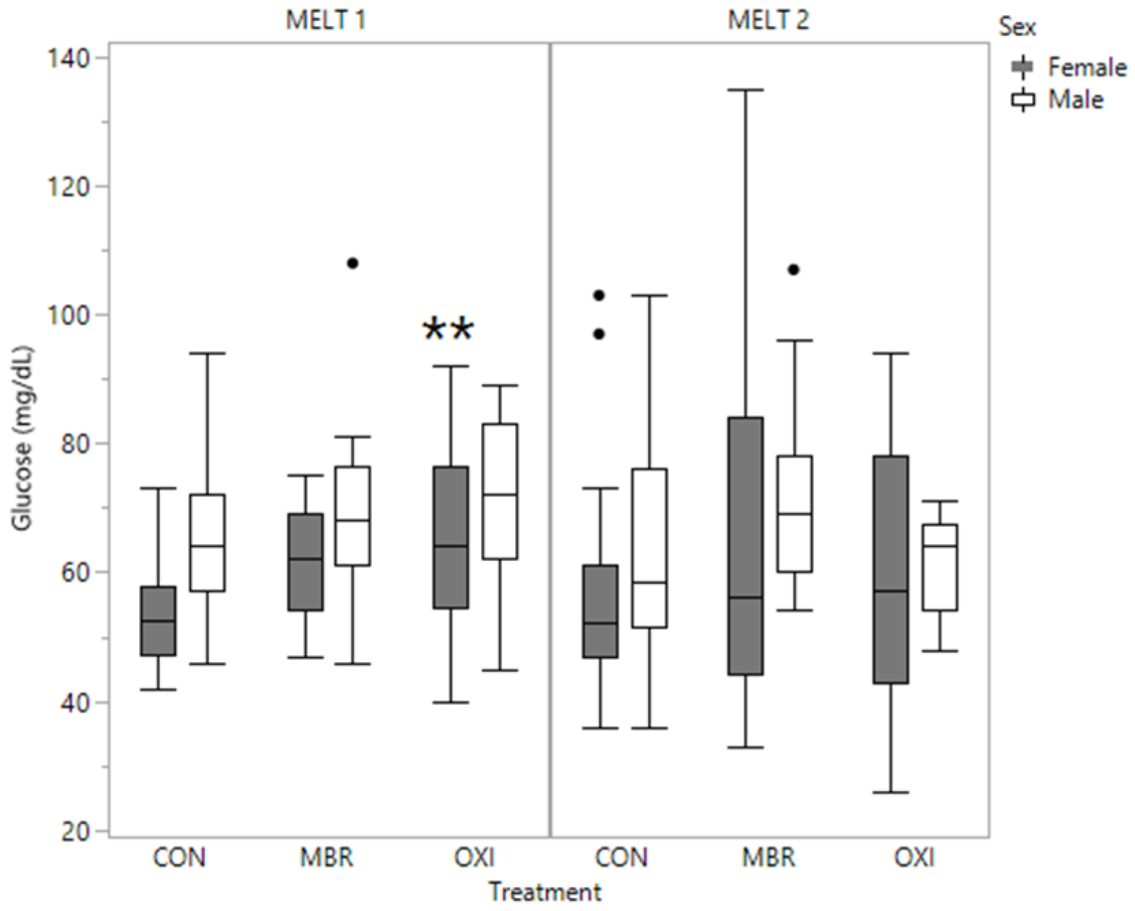
Note. Mean, standard deviation, and sample size for organ weights in females and male fish. Split between MELT1 and MELT2 exposures as well as treatments.

No significant difference was found between treatments in hematocrit levels (ANOVA p-value MELT1: Treatment = 0.86; MELT2: Treatment = 0.36). In MELT1, glucose concentration was significantly higher in OXI compared to CON treatment, while there was no significance between MBR and CON

treatments. In MELT2, there was no significant difference in glucose concentration between treatments (ANOVA p-value MELT1: Treatment = 0.01, vs. CON: MBR = 0.19, OXI = 0.008; MELT2: Treatment = 0.94). (Figure 3.3; Table 3.6)

Figure 3.3

Glucose (mg/dL) vs. Treatment



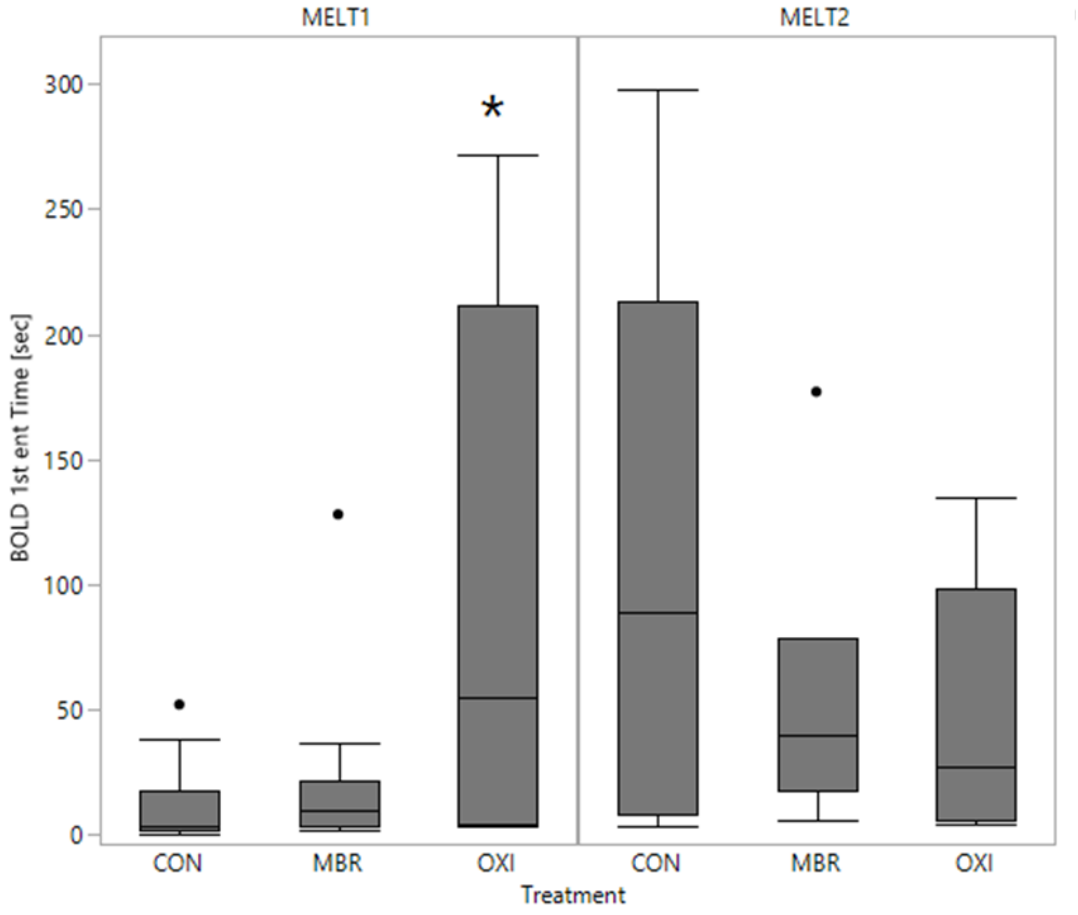
Note. Glucose (mg/dL) vs. Treatment split between exposures and sex. Box represents upper and lower quartile with median. Whisker indicates minimum and maximum observation. • outlier observations; ** denotes p <0.01 vs. CON.

3.3 Behavior

The analysis of boldness indicated no significant differences in treatments for total time in ring (ANOVA p-value MELT1: Treatment = 0.77; MELT2: Treatment = 0.46), number of times in ring (ANOVA p-value MELT1: Treatment = 0.69; MELT2: Treatment = 0.79), number of object bumps (ANOVA p-value MELT1: Treatment = 0.09; MELT2: Treatment = 0.43), and number of object charges (ANOVA p-value MELT1: Treatment = 0.84; MELT2: Treatment = 0.99). However, a significant increase for the time to first ring entrance in OXI vs. CON in MELT1 was observed (ANOVA p-value MELT1: Treatment = 0.006, vs. CON: MBR = 0.15, OXI = 0.01; MELT2: Treatment = 0.20). (Figure 3.4; Table 3.4)

Figure 3.4

Boldness time to first ring entry (sec) vs. Treatment

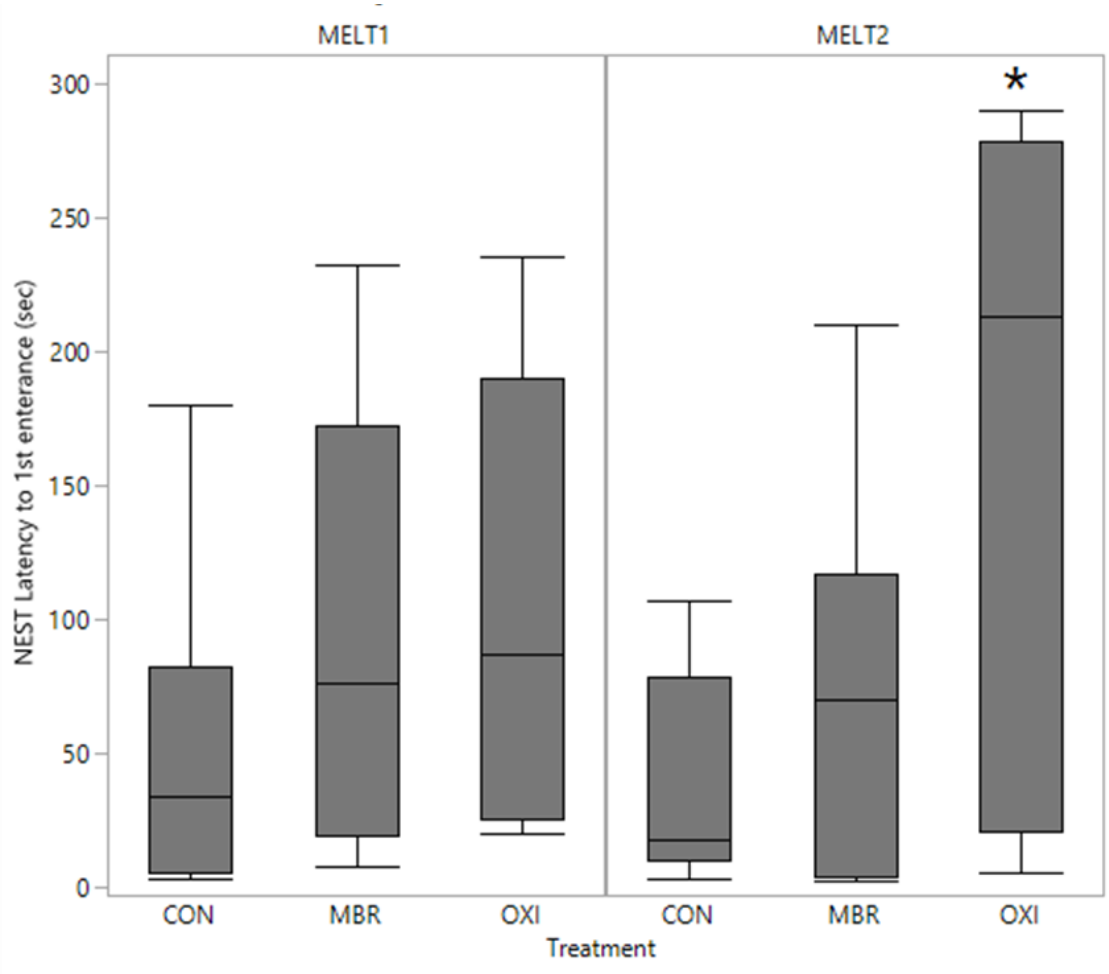


Note. Boldness time to first ring entry vs. Treatment split between exposures. Box represents upper and lower quartile with median. Whisker indicates minimum and maximum observation. • outlier observations; * denotes $p < 0.05$ vs. CON.

The analysis of nest defense behavior found no significant differences in treatments for the total number of object approaches (MELT1 ANOVA p-value: Treatment = 0.20; MELT2 ANOVA p-value: Treatment = 0.45), number of lateral displays (MELT1 ANOVA p-value: Treatment = 0.44; MELT2 ANOVA p-value: Treatment = 0.15), time to first object bump (MELT1 ANOVA p-value: Treatment = 0.71; MELT2 ANOVA p-value: Treatment = 0.79), number of bumps (MELT1 ANOVA p-value: Treatment = 0.43; MELT2 ANOVA p-value: Treatment = 0.08), and total time of interactions (MELT1 ANOVA p-value: Treatment = 0.42; MELT2 ANOVA p-value: Treatment = 0.054). However, there was a significant difference in treatments for the total time within 3 cm object in MELT2 but not for treatments when compared to CON (MELT1 ANOVA p-value: Treatment = 0.68; MELT2 ANOVA p-value: Treatment = 0.04, vs. CON ANOVA p-value: MBR = 0.30, OXI = 0.25). Additionally, the results show a significant increase for the time to first approach in treatments when OXI is compared to CON for MELT2 (MELT1 ANOVA p-value: Treatment = 0.24; MELT2 ANOVA p-value: Treatment = 0.002, vs. CON ANOVA p-value: MBR = 0.24, OXI = 0.04). See Figure 3.5 and Table 3.4 for more details.

Figure 3.5

Nest defense time to first ring entry (sec) vs. Treatment



Note. Nest defense time to first ring entry vs. Treatment split between exposures. Box represents upper and lower quartile with median. Whisker indicates minimum and maximum observation. * denotes p <0.05 vs. CON.

Table 3.4

Behavior and reproduction measurements

Behavior/Reproduction			**MELT1**			**MELT2**	
Endpoint	**Statistics**	**CON**	**MBR**	**OXI**	**CON**	**MBR**	**OXI**
BOLD 1st ent Time [sec]	Mean	12	20	***97**	105	55	47
	Std Dev	18	33	107	104	62	54
	N	10	14	13	14	6	5
BOLD Total time [sec]	Mean	40	45	58	19	4	11
	Std Dev	73	68	87	50	13	29
	N	16	17	17	26	17	17
BOLD # times in ring	Mean	10	10	7	4	4	3
	Std Dev	12	14	6	9	6	5
	N	16	17	17	26	17	17
BOLD Bumps	Mean	0	0	0	1	0	0
	Std Dev	0	1	0	3	0	0
	N	16	17	17	26	17	17
BOLD Charge	Mean	0	0	0	0	0	0
	Std Dev	1	1	1	0	0	0
	N	16	17	17	26	17	17
NEST Latency to 1st entrance (sec)	Mean	54	101	102	36	74	***170**
	Std Dev	63	81	82	38	67	129
	N	12	14	7	15	11	6
NEST Total # of Approaches	Mean	2	6	2	3	2	1
	Std Dev	4	10	4	7	3	3
	N	15	17	13	24	16	15
NEST Total time within 3 cm of Jar (sec)	Mean	116	91	80	97	138	39
	Std Dev	134	95	103	117	119	78
	N	15	16	13	24	16	15
NEST # of lateral displays	Mean	1	0	1	1	1	0
	Std Dev	1	1	2	1	2	1
	N	15	17	13	22	16	15
NEST Latency to first jar bump with snout (sec)	Mean	81	104	127	147	113	108
	Std Dev	79	73	120	86	84	160
	N	6	10	4	6	7	3
NEST Total # of jar bumps	Mean	1	4	3	3	8	1
	Std Dev	4	6	8	8	15	2
	N	15	17	13	24	16	15
NEST Total duration of bout interactions (sec)	Mean	115	73	63	76	137	41
	Std Dev	133	101	96	110	119	80

Fecundity (# eggs)	N	15	16	13	24	16	14
	Mean	26	6	0	86	97	104
	Std Dev	68	23	0	134	120	152
Fertility (eggs with eyespots)	N	14	16	17	19	12	15
	Mean	17	3	0	53	57	50
	Std Dev	63	10	0	88	98	92
	N	14	15	13	17	12	14

Note. Mean, standard deviation, and sample size for behavior assays, fecundity, and fertility. Split between MELT1 and MELT2 exposures as well as treatments. Boldness (BOLD) and nest defense (NEST).

* denotes p <0.05 vs. CON

3.4 Fecundity/Fertility

Fecundity and fertility were not significantly affected by either exposure. There was no significant difference in fecundity or fertility for treatment in both exposures (ANOVA p-values: MELT1 - fecundity = 0.17, fertility = 0.44; MELT2 - fecundity = 0.93, fertility = 0.98). (Table 3.4)

3.5 Histology

No significant histological changes were observed in the liver or gonad across all exposures. Vacuole presence in livers was not significant in MELT1 treatments (ANOVA p-value MELT1: Treatment = 0.059; MELT2: Treatment = 0.99), and there were no significant differences in gonad maturity between treatments (ANOVA p-value MELT1: Treatment = 0.73; MELT2: Treatment = 0.84). (Table 3.5)

Table 3.5

Histology measurements

Histology			**MELT1**			**MELT2**		
Endpoint	**Sex**	**Statistics**	**CON**	**MBR**	**OXI**	**CON**	**MBR**	**OXI**
Gonad grade (1 immature-4 mature)	Female	Mean	3	3	3	3	3	3
		Std Dev	1	0	1	0	0	1
		N	14	17	18	17	11	13

	Male	Mean	3	2	2	2	2	2
		Std Dev	1	1	1	0	1	0
		N	15	16	12	25	20	20
	Female	Mean	2	2	2	2	2	2
		Std Dev	1	1	1	1	1	0
		N	14	17	19	18	12	14
Liver vaculole grade (1-4)	Male	Mean	3	3	3	3	3	3
		Std Dev	1	0	1	1	1	0
		N	15	16	12	28	18	19

Note. Mean, standard deviation, and sample size for histology gonad maturity and liver vacuolation in females and male fish. Split between MELT1 and MELT2 exposures as well as treatments.

3.6 Biochemical

3.6.1 *ELISA*

No significant difference in the induction of VTG and cortisol was found between treatments in either exposure (ANOVA p-value MELT1: Treatment = 0.09; MELT2: Treatment = 0.26 for VTG; ANOVA p-value MELT1: Treatment = 0.25; MELT2: Treatment = 0.82 for cortisol). (Table 3.6)

There were no significant differences between treatments for the sex hormones 11-KT (ANOVA p-value MELT1: Treatment = 0.09; MELT2: Treatment = 0.97) and E2 (ANOVA p-value MELT1: Treatment = 0.22; MELT2: Treatment = 0.95) in both exposures. The ratio of 11-KT/E2 also showed no significant differences in treatments for both exposures (ANOVA p-value MELT1: Treatment = 0.28; MELT2: Treatment = 0.86). (Table 3.6)

Table 3.6

Biochemical measurements

Biochemical			MELT1			MELT2		
Endpoint	**Sex**	**Statistics**	**CON**	**MBR**	**OXI**	**CON**	**MBR**	**OXI**
Hematocrit (%)	Female	Mean	31	29	31	34	28	25
		Std Dev	10	13	12	14	13	12
		N	11	12	19	16	8	10
	Male	Mean	46	46	43	47	45	46
		Std Dev	12	10	9	9	9	9
		N	16	15	13	28	18	19
Glucose (mg/dL)	Female	Mean	53	60	**65	57	64	58
		Std Dev	8	11	14	18	29	20
		N	12	15	20	19	12	15
	Male	Mean	65	70	72	64	71	61
		Std Dev	12	14	12	17	14	7
		N	17	17	13	28	20	20
Cortisol [ng/mL]	Female	Mean	57	75	98	30	22	48
		Std Dev	50	67	40	27	14	37
		N	7	8	13	11	5	7
	Male	Mean	43	69	47	22	24	24
		Std Dev	62	42	39	27	39	26
		N	15	15	13	26	17	19
VTG [ug/mL]	Female	Mean	615	424	506	500	630	427
		Std Dev	46	268	213	244	0	220
		N	10	10	16	14	4	8
	Male	Mean	557	571	563	545	396	564
		Std Dev	176	91	182	181	300	162
		N	14	15	13	27	17	19
E2 [pg/mL]	Female	Mean	1524	423	294	260	538	220
		Std Dev	1464	397	339	177	211	256
		N	5	4	7	5	2	3
	Male	Mean	104	180	170	130	83	124
		Std Dev	49	175	223	155	68	133
		N	14	15	13	25	15	17
11KT [pg/mL]	Female	Mean	371	414	618	607	30630	283
		Std Dev	176	172	635	824	-	120
		N	7	7	10	4	1	5
	Male	Mean	26593	11445	11717	17385	13909	16253
		Std Dev	20341	12161	13871	15821	16822	14870
		N	15	15	13	28	15	16

11-KT/E2 Ratio	Female	Mean	0	2	26	6	45	1
		Std Dev	0	1	62	11	-	0
		N	5	3	7	4	1	2
	Male	Mean	344	146	201	382	480	611
		Std Dev	338	247	459	750	857	954
		N	14	15	13	25	14	15

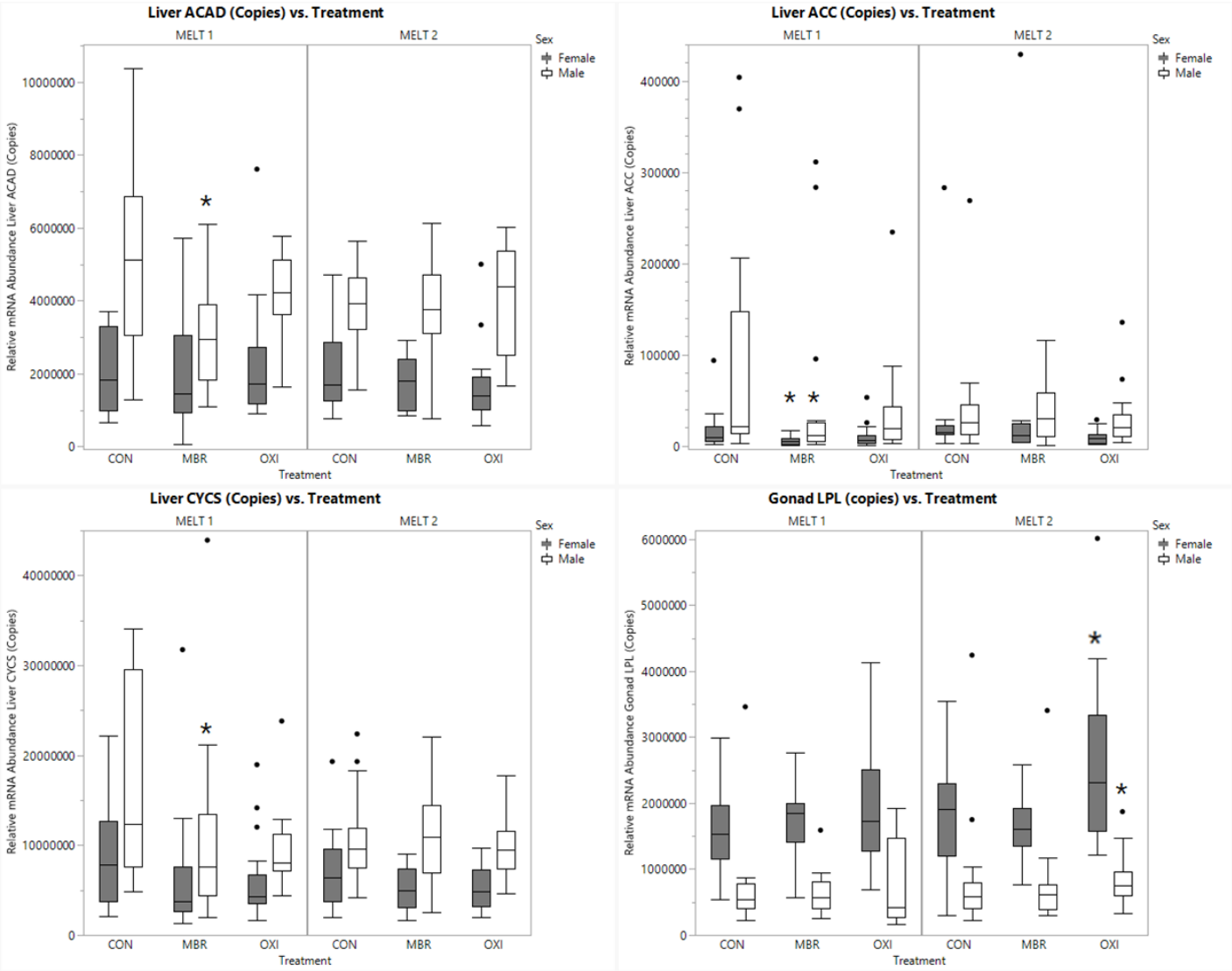
Notes. Mean, standard deviation, and sample size for biochemical measurements in females and male fish. Split between MELT1 and MELT2 exposures as well as treatments. Vitellogenin (VTG), estradiol (E2), and 11-ketotestosterone (11KT). **denotes p <0.01 vs. CON

3.6.2 *RT-qPCR*

When looking at the mRNA expression of aerobic metabolism regulators no significance was found in either exposure in liver tissue for genes PFK-1 (ANOVA p-value MELT1: Treatment = 0.14; MELT2: Treatment = 0.97), PK (ANOVA p-value MELT1: Treatment = 0.86; MELT2: Treatment = 0.23), GSY1 (ANOVA p-value MELT1: Treatment = 0.60; MELT2: Treatment = 0.85), PYGL (ANOVA p-value MELT1: Treatment = 0.19; MELT2: Treatment = 0.86), LDH (ANOVA p-value MELT1: Treatment = 0.93; MELT2: Treatment = 0.51), and LPL (ANOVA p-value MELT1: Treatment = 0.69; MELT2 Treatment = 0.80). In addition to that there was significant decreases for MELT1 when MBR was compared to CON for genes ACC (ANOVA p-value MELT1: Treatment = 0.02, vs. CON: MBR = 0.02, OXI = 0.10; MELT2 Treatment = 0.24), ACAD (ANOVA p-value MELT1: Treatment = 0.03, vs. CON: MBR = 0.02, OXI = 0.48; MELT2: Treatment = 0.84), and CYCS (ANOVA p-value MELT1: Treatment = 0.01, vs. CON: MBR = 0.02, OXI = 0.05; MELT2: Treatment = 0.51). (Figure 3.6; Table 3.7)

Figure 3.6

Graphs for liver ACAD, ACC, CYCS, and gonad LPL expression vs. Treatment



Note. Normalized relative mRNA abundance liver ACAD, ACC, CYCS, and gonad LPL vs. Treatment split between exposures and sex. Box represents upper and lower quartile with median. Whisker indicates minimum and maximum observation. • outlier observations; * denotes $p < 0.05$ vs. CON

Table 3.7

Gene measurements for aerobic metabolism in liver

Relative mRNA Abundance Liver Tissue			**MELT1**			**MELT2**		
Gene	**Sex**	**Statistics**	**CON**	**MBR**	**OXI**	**CON**	**MBR**	**OXI**
PK (Copies)	Female	Mean	1585236	1320096	1208641	1079109	804626	1447018
		Std Dev	1554361	1343318	821093	701630	427501	1379714
		N	14	17	19	19	11	14
	Male	Mean	4266407	4660845	4100492	6662356	6032891	8339738
		Std Dev	3733915	3033574	3850468	5597772	4533978	8273031
		N	17	17	13	28	19	19
PFK1 (Copies)	Female	Mean	769078	582475	761634	741712	576554	711011
		Std Dev	637413	470673	496393	455587	320919	584736
		N	14	17	19	18	11	14
	Male	Mean	789567	713480	1092335	671895	729829	671295
		Std Dev	524605	371044	726059	479045	599046	448654
		N	17	17	13	28	19	20
LDH (Copies)	Female	Mean	177242	174056	257119	339704	212439	156390
		Std Dev	250875	263238	545962	499280	380384	120943
		N	14	17	19	18	11	13
	Male	Mean	577795	321041	361252	308277	303672	187696
		Std Dev	707835	364175	440083	320895	584992	179492
		N	17	17	13	28	19	20
PYGL (Copies)	Female	Mean	644356	964134	398287	548011	338087	619549
		Std Dev	634533	1684002	445339	794251	319334	781711
		N	14	17	19	19	11	14
	Male	Mean	933043	1342740	943010	1231502	1260982	1095644
		Std Dev	901210	1776707	697231	1354485	1356102	1065250
		N	17	17	13	28	19	20
GSY1 (Copies)	Female	Mean	6954	1257	7120	31238	48345	8546
		Std Dev	13318	958	18235	69296	64819	7749
		N	5	5	9	14	5	6
	Male	Mean	2369	908	3560	2922	4656	2281
		Std Dev	1656	903	5967	3302	7535	1768
		N	5	5	7	17	10	8
CYCS (Copies)	Female	Mean	8716958	6724967	5996244	7124896	5460582	5165475
		Std Dev	5555517	7176040	4536402	4208443	2452858	2395788
		N	14	17	19	19	11	14
	Male	Mean	16510415	***10617682**	9754573	10558215	11336974	9845045
		Std Dev	10898380	10068163	4862300	4520096	5260540	3675188
		N	17	17	13	28	18	20

ACC (Copies)	Female	Mean	17719	***7945**	10211	29383	58675	10010
		Std Dev	23691	6497	12298	61810	139228	8358
		N	14	17	19	19	9	14
	Male	Mean	94721	***50247**	40400	36624	37299	28804
		Std Dev	125732	95611	62837	50063	33581	30051
		N	17	17	13	27	19	20
ACAD (Copies)	Female	Mean	2120612	2023579	2255505	2079933	1779544	1749891
		Std Dev	1163737	1479362	1608018	1103977	706267	1148314
		N	14	17	19	18	11	14
	Male	Mean	5236148	***3134777**	4181566	3842973	3868792	4019354
		Std Dev	2632194	1512287	1107111	1062725	1270726	1497432
		N	17	17	13	28	19	20
LPL (Copies)	Female	Mean	12854784	8101114	8880450	6138783	5854813	7588874
		Std Dev	21170376	8659307	7948010	4169457	5625166	7446556
		N	14	17	19	19	11	14
	Male	Mean	16230540	10181723	9901212	14825765	16825192	15464015
		Std Dev	16588425	8493835	6882628	7624040	10461538	15442946
		N	16	17	13	28	19	20

Note. Mean, standard deviation, and sample size for relative mRNA abundance in liver tissue for genes related to aerobic metabolism for females and male fish. Split between MELT1 and MELT2 exposures as well as treatments. * denotes $p < 0.05$ vs. CON

The mRNA expression of aerobic metabolism regulators in the gonad showed no significance for either exposure in genes PYGL (ANOVA p-value MELT1: Treatment = 0.88; MELT2: Treatment = 0.89), ACAD (ANOVA p-value MELT1: Treatment = 0.73; MELT2: Treatment = 0.60), and CYCS (ANOVA p-value MELT1: Treatment = 0.60; MELT2: Treatment = 0.54). However, a significant increase did occur in gonad expression of LPL in OXI when compared to CON (ANOVA p-value MELT1: Treatment = 0.53; MELT2: Treatment = 0.02; vs. CON: MBR = 0.99, OXI = 0.03). (Figure 3.6; Table 3.8)

Table 3.8

Gene measurements for aerobic metabolism in gonad

Relative mRNA Abundance Gonad Tissue			MELT1			MELT2		
Gene	**Sex**	**Statistics**	**CON**	**MBR**	**OXI**	**CON**	**MBR**	**OXI**
PYGL (copies)	Female	Mean	2411207	2570832	2406808	1767092	1593864	2017577
		Std Dev	1603467	1669043	2128037	1996457	1565502	2035847
		N	14	17	20	18	12	14
	Male	Mean	458640	398493	1444887	637313	888526	534341
		Std Dev	544373	311361	3069699	770014	1789226	721414
		N	17	15	13	26	19	20
ACAD (copies)	Female	Mean	3846821	3281576	4311049	2630878	2372633	2244928
		Std Dev	1838159	1416269	2434460	1092346	1222020	1190307
		N	14	17	20	18	12	14
	Male	Mean	4913661	5427686	4939745	4652041	4374042	4171032
		Std Dev	1657174	2043724	2764427	1947344	2253658	1840695
		N	17	15	13	26	19	20
CYCS (copies)	Female	Mean	23711389	21957589	23514379	19009813	15640767	17028983
		Std Dev	13024732	6858168	10299781	7385446	7190025	5990455
		N	14	17	20	18	12	14
	Male	Mean	20086945	19541668	14964634	22786252	20472287	23334603
		Std Dev	8265593	6653248	8282110	9534421	7883893	10239372
		N	17	15	13	26	19	20
MAPK13 (copies)	Female	Mean	599557	759178	633781	855506	687661	626648
		Std Dev	295643	567813	301588	523619	445448	416930
		N	14	17	20	18	12	13
	Male	Mean	1052293	973243	822149	1540568	1270772	1678221
		Std Dev	507920	637922	408817	792474	822672	859380
		N	17	15	13	26	19	19
LPL (copies)	Female	Mean	1583855	4916259	1879133	1854707	1632519	***2639687**
		Std Dev	651841	9515734	840134	790366	475427	1302066
		N	14	17	20	18	12	14
	Male	Mean	738174	635836	804938	770078	775322	***847686**
		Std Dev	729823	336756	655623	777517	678868	367589
		N	17	15	13	26	19	20

Note. Mean, standard deviation, and sample size for relative mRNA abundance in gonad tissue for genes related to aerobic metabolism and oxidative stress for females and male fish. Split between MELT1 and MELT2 exposures as well as treatments. * denotes $p < 0.05$ vs. CON

There was no significant difference in mRNA expression of enzymes related to oxidative stress in liver tissue in genes SOD (ANOVA p-value MELT1: Treatment = 0.63; MELT2: Treatment = 0.64), NOX1 (ANOVA p-value MELT1: Treatment = 0.16; MELT2: Treatment = 0.78), and MAPK13 (ANOVA p-value MELT1: Treatment = 0.37; MELT2: Treatment = 0.27). In gonad tissue, no significant difference for MAPK13 in both exposures (ANOVA p-value MELT1: Treatment = 0.28; MELT2: Treatment = 0.26). (Table 3.9)

Table 3.9

Gene measurements for oxidative stress in liver

Relative mRNA Abundance Liver Tissue			**MELT1**			**MELT2**		
Gene	**Sex**	**Statistics**	**CON**	**MBR**	**OXI**	**CON**	**MBR**	**OXI**
SOD (Copies)	Female	Mean	20945726	24476971	18453585	31324096	23789222	26116286
		Std Dev	26332600	21973927	22618530	29578461	22046925	15105079
		N	14	17	19	19	11	13
	Male	Mean	45820018	31968310	40156417	48893132	45916161	42843476
		Std Dev	28726060	25051397	25034779	29807245	28337140	31475972
		N	17	17	13	28	18	20
NOX1 (Copies)	Female	Mean	18678	15135	19726	23983	27265	17179
		Std Dev	15327	16161	21193	19448	37621	15398
		N	14	17	19	19	11	14
	Male	Mean	32141	23365	37210	36834	42981	30217
		Std Dev	49904	19689	35046	67778	86381	24787
		N	17	17	13	28	19	20
MAPK13 (Copies)	Female	Mean	152407	162605	142857	247362	198637	119179
		Std Dev	167456	177591	192554	244492	176300	97803
		N	14	17	19	19	11	14
	Male	Mean	666727	350404	630021	356562	515838	317264
		Std Dev	684781	420682	1070420	254827	550702	250663

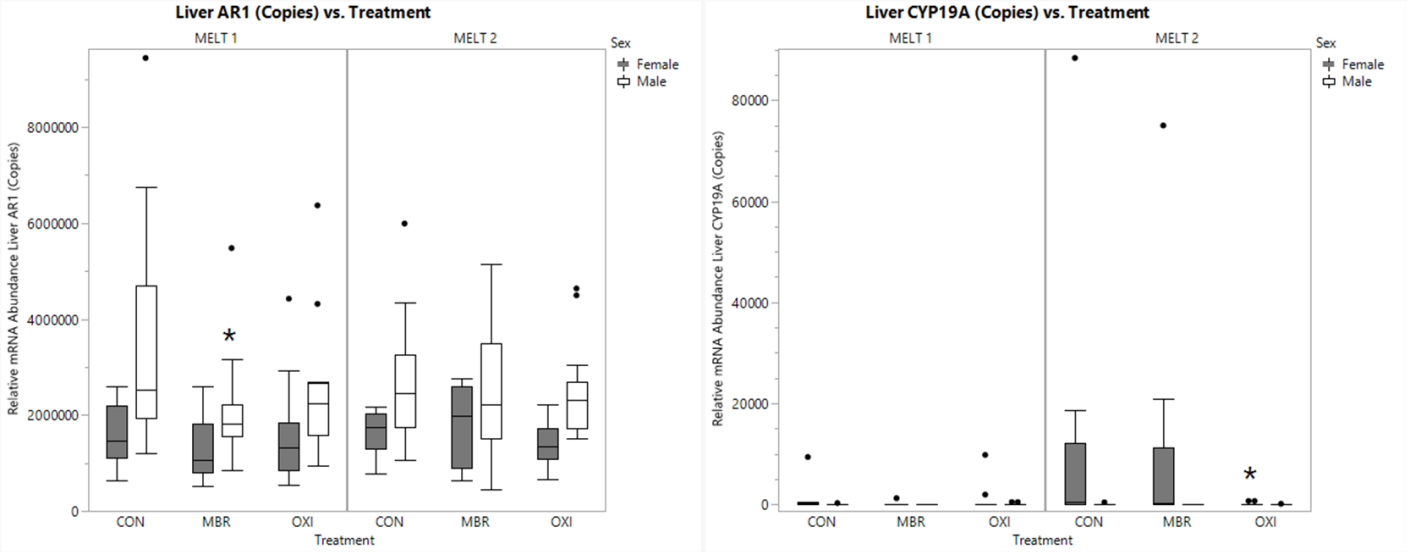
N	17	17	13	28	19	20

Note. Mean, standard deviation, and sample size for relative mRNA abundance in liver tissue for genes related to oxidative stress for females and male fish. Split between MELT1 and MELT2 exposures as well as treatments.

The mRNA expression of sex hormone associated proteins in the liver showed no significant difference in treatment in either exposure for genes ESR1 (ANOVA p-value MELT1: Treatment = 0.65; MELT2: Treatment = 0.50), ESR2 (ANOVA p-value MELT1: Treatment = 0.69; MELT2: Treatment = 0.06), and StAR (ANOVA p-value MELT1: Treatment = 0.19; MELT2: Treatment = 0.31). However, in MELT2 showed a decreased expression of CYP19a in the OXI when compared to CON (ANOVA p-value MELT1: Treatment = 0.47; MELT2: Treatment = 0.02, vs. CON: MBR = 0.93, OXI = 0.03). In addition, MELT1 showed a significant decrease in AR1 when MBR was compared to CON (ANOVA p-value MELT1: Treatment = 0.02, vs CON: MBR = 0.01, OXI = 0.29; MELT2: Treatment = 0.77). (Figure 3.7; Table 3.10)

Figure 3.7

Graphs for liver AR1 and CYP19A expression vs. Treatment



Note. Relative mRNA Abundance liver ACAD, ACC, CYCS, and gonad LPL vs. Treatment split between exposures and sex. Box represents upper and lower quartile with median. Whisker indicates minimum and maximum observation. • outlier observations; * denotes p <0.05 vs. CON.

Table 3.10

Gene measurements for reproduction in liver

Relative mRNA Abundance Liver Tissue			**MELT1**			**MELT2**		
Gene	**Sex**	**Statistics**	**CON**	**MBR**	**OXI**	**CON**	**MBR**	**OXI**
ESR1 (Copies)	Female	Mean	12344163	10856741	14652290	15935568	18868429	13637702
		Std Dev	12667331	13226585	11854293	13536317	11342693	10764531
		N	14	17	19	19	11	14
	Male	Mean	3992763	6372768	4725230	7538177	6213900	6177528
		Std Dev	3391360	6214280	4622428	4919141	4153150	3702902
		N	17	17	13	28	19	20

ESR2 (Copies)	Female	Mean	5228355	5661029	5423876	7327584	6760170	5357706
		Std Dev	3631897	4705185	3842707	4265419	6241361	3188499
		N	13	17	18	19	11	14
	Male	Mean	4855392	5384822	6254280	12008184	8437022	9027293
		Std Dev	2679626	1991753	3143986	7774581	4065657	3900181
		N	16	17	13	28	19	20
AR1 (Copies)	Female	Mean	1585782	1291417	1532386	1631120	1830698	1384606
		Std Dev	614914	669464	941855	408621	787040	465797
		N	14	17	19	19	11	14
	Male	Mean	3438551	***2033963**	2491716	2574809	2491471	2429483
		Std Dev	2185162	1050038	1427578	1138005	1222970	853741
		N	17	17	13	28	19	20
CYP19A (Copies)	Female	Mean	784	142	779	9916	10799	***174**
		Std Dev	2481	331	2455	23398	23505	308
		N	14	13	16	14	10	11
	Male	Mean	65	50	129	64	50	55
		Std Dev	59	0	240	73	0	20
		N	16	14	11	26	18	19
STAR (Copies)	Female	Mean	97	99	287	371	763	81
		Std Dev	163	112	449	475	1658	78
		N	12	14	16	17	7	13
	Male	Mean	10199	2219	3573	664	21600	258
		Std Dev	16701	5384	7177	1111	73205	386
		N	10	13	10	19	12	13

Note. Mean, standard deviation, and sample size for relative mRNA abundance in liver tissue for genes related to reproduction for females and male fish. Split between MELT1 and MELT2 exposures as well as treatments. * denotes $p < 0.05$ vs. CON

The mRNA expression of sex hormone associated proteins in gonad tissue did not show any significant differences in treatments across all exposures for the genes ESR1 (ANOVA p-value MELT1: Treatment = 0.79; MELT2: Treatment = 0.82), ESR2 (ANOVA p-value MELT1: Treatment = 0.39; MELT2: Treatment = 0.20), StAR (ANOVA p-value MELT1: Treatment = 0.32; MELT2: Treatment = 0.33), CYP19a (ANOVA p-value MELT1: Treatment = 0.38; MELT2: Treatment = 0.37), and AR1 (ANOVA p-value MELT1: Treatment = 0.86; MELT2: Treatment = 0.94). (Table 3.11)

Table 3.11

Gene measurements for reproduction in gonad

Relative mRNA Abundance Gonad Tissue			**MELT1**			**MELT2**		
Gene	**Sex**	**Statistics**	**CON**	**MBR**	**OXI**	**CON**	**MBR**	**OXI**
ESR1 (copies)	Female	Mean	514057	617516	650004	696300	872804	838462
		Std Dev	282947	567718	417032	461277	691519	508249
		N	14	17	20	18	12	14
	Male	Mean	526665	194905	103230	189720	209000	221854
		Std Dev	1578678	229571	77567	213486	453793	215532
		N	17	15	13	26	19	20
ESR2 (copies)	Female	Mean	439744	582572	807943	820148	912018	754570
		Std Dev	167177	259580	494362	348929	756555	287956
		N	14	17	20	18	12	14
	Male	Mean	1194629	982706	985100	1915325	1473627	2017909
		Std Dev	637328	209422	533734	737104	649000	962869
		N	17	14	11	26	19	20
AR1 (copies)	Female	Mean	2715946	3151767	3136384	3131875	2850816	2825721
		Std Dev	1431110	1427489	986745	1386740	797558	1222195

		N	14	17	20	18	12	14
	Male	Mean	1049628	852282	866636	1003028	959487	1065562
		Std Dev	410913	321005	437783	334650	417186	378833
		N	17	15	13	26	19	20
CYP19A (copies)	Female	Mean	932824	948346	831827	2001653	1946956	2380948
		Std Dev	1118056	996992	764526	2514948	2022088	2911969
		N	14	17	20	18	12	14
	Male	Mean	7802	15050	7519	12263	20258	12110
		Std Dev	10715	15253	7115	26215	52440	8109
		N	17	15	12	26	19	20
StAR (copies)	Female	Mean	48119	75140	44828	72241	46861	72704
		Std Dev	50783	149816	48124	78499	23724	73679
		N	14	16	20	18	12	14
	Male	Mean	1428987	533709	624048	2715715	1814631	3552235
		Std Dev	1547219	456352	783414	2623562	2205119	3957571
		N	15	15	12	26	19	20

Note. Mean, standard deviation, and sample size for relative mRNA abundance in gonad tissue for genes related to reproduction for females and male fish. Split between MELT1 and MELT2 exposures as well as treatments.

Chapter 4: Discussion

In the current study, replicate in-situ experiments exposing adult fathead minnows to split wastewater effluent were conducted to evaluate the effectiveness of secondary treatments, MBR and OXI. Treatment efficacy was evaluated using a combination of analytical chemistry and the AOP framework to assess the impacts of effluent exposure on fish. The AOP framework uses endpoints at different levels of biological organization, including macro-molecular, cell/tissue, organ/organ system, and organismal, while EARs were used to identify potential molecular initiating events. EARS can provide insights into molecular mechanisms of toxicity as they are based on molecular assays of single chemicals. While biological responses based on 21-day exposures of whole animals provide a more comprehensive understanding of the overall toxicity.

The results of the current study demonstrate the effectiveness of both secondary treatments, MBR and OXI, in removing CECs from primary treatment effluent, as evidenced by the decrease in contaminant concentrations and the increase in survival rate from 0% in POS to over 94% in both MBR and OXI. Moreover, the reduction of contaminant concentrations resulted in a decrease in EARs, supporting the putative biological effectiveness of both treatments. However, it is worth noting that the two treatments differ in their removal of biologically active chemicals for which analytical data were obtained.

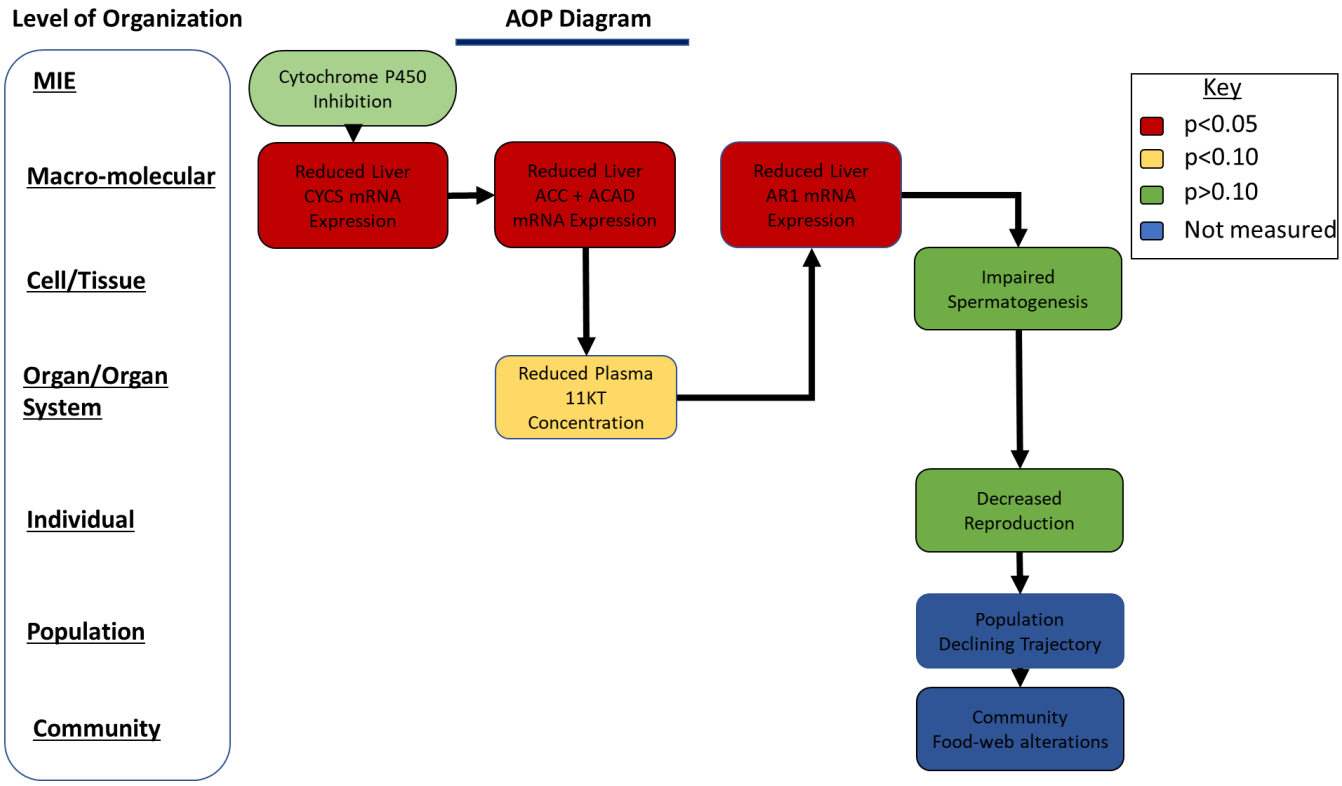
In MELT1, there were two chemicals with high enough concentrations to produce EARs that differed between MBR and OXI. Acetochlor was present in OXI (EAR=0.0019) and is known as a thyroid disruptor that effects development. A study of adult Chinese Rare minnows exposed to a maximum of 2000ng/L for 21-days documented no significant effects in liver tissue (W. Li et al., 2009) . Other studies have only observed effects on larval zebra fish acutely exposed as embryos with minimum concentrations of 1.2µg/mL (Jiang et al., 2015) and 50µg/L (Liu et al., 2017) well above the measured concentration in OXI of 0.0161µg/L in the current study. Although Jiang et al. (2015) reported that

acetochlor can upregulate expression of proteins related to oxidative stress and reproduction, the only significant result in the current study was an increase in glucose for OXI.

The MBR treatment in MELT1 shows that the presence of piperonyl butoxide is consistent with reports of this chemical inducing metabolic dysregulation. The piperonyl butoxide 4-week LOEC/NOEC based on fathead minnow growth is 27.4 and 7.8 µg a.i./L (Judkins & Shamblen, 2017) which is substantially higher than the 0.0048 µg/L found in MBR. The EAR calculated on the measured concentration in the current study (0.0010) is low suggesting that any biological response to exposure is unlikely. However, piperonyl butoxide is a pesticide used as a synergist that inhibits oxidases which can reduce metabolic activated toxicity and increase non-metabolic activated toxicity (Ankley et al., 1991). The inhibition of oxidases may be inducing metabolic dysregulation. As a molecular initiating event, piperonyl butoxide may decrease liver CYCS leading to the decreased expression of lipid metabolism regulators ACC and ACAD. This would mirror the AOP of peroxisome proliferator-activated receptor alpha agonism where reduction in cholesterol leads to decreases in plasma 11-KT and liver AR1 expression impairing spermatogenesis and decreasing offspring production (Kittelson et al., 2023) (Figure 4.1). Though 11-KT plasma concentration and histological changes to gonads in exposed male fathead minnows showed no significant differences, the gene expression that is known to alter circulating hormones and tissue do. The presence of piperonyl butoxide in the MBR treatment, but not in OXI or CON, highlights a difference in stressors contained in each treatment.

Figure 4.1

Proposed AOP diagram cytochrome P450 inhibition

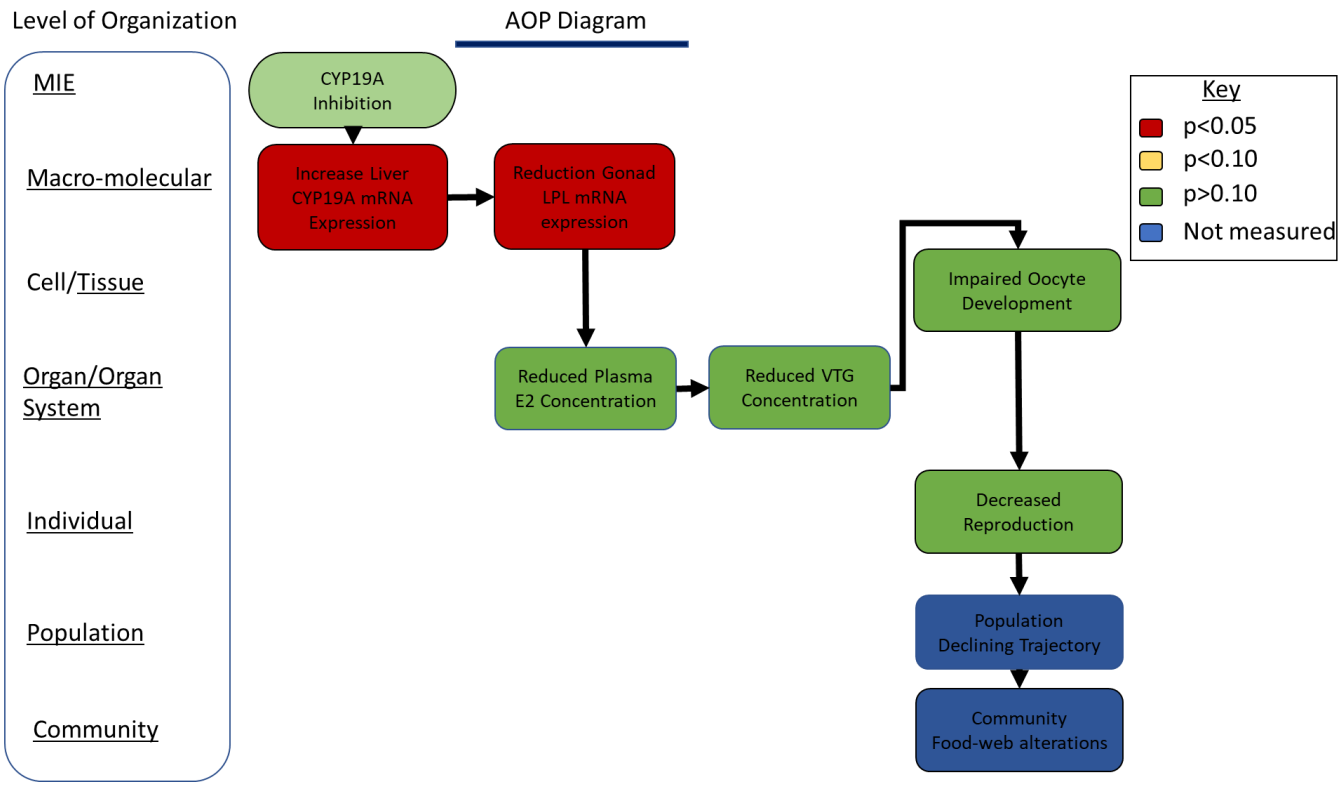


Note. Proposed adverse outcome pathway (AOP) for cytochrome P450 inhibition. The molecular initiating event (MIE) leads to reduction of liver cytochrome c (CYCS) mRNA expression. With a reduction of CYCS, lipid metabolism is impaired subsequently reducing acetyl-CoA carboxylase (ACC) and acyl-CoA dehydrogenase (ACAD) mRNA expression. As a result, 11-ketotestosterone (11KT) production is reduced, lowering plasma concentration of this hormone, and reducing androgen receptor 1 (AR1) mRNA expression. Spermatogenesis is then impaired leading to decreased fertility which may lead to a decline in population and potentially alterations of the food-web. Red = $p<0.05$; Yellow = $p<0.10$; Green = $p>0.10$; Blue = Not measured.

In MELT2, the chemical composition of treatments changed with the absence of acetochlor in OXI, though piperonyl butoxide was still present in MBR. Unlike MELT1, piperonyl butoxide also showed activity in CON, which could explain the different results between exposures. In OXI, significant decreases in liver aromatase (CYP19A) and increase in gonad LPL expression in females suggest the presence of aromatase inhibition in MBR and CON. Aromatase inhibition leads to CYP19A upregulation followed by increases in E2 production, plasma E2, and plasma VTG (D. L. Villeneuve, 2022; D. L. Villeneuve et al., 2021b) (Figure 4.2). Moreover, increased gonad LPL expression indicate that lipid reserves are being built up and affecting steroidogenesis, which is important for reproduction (José Ibáñez et al., 2008). Specifically, it was found that in the ovary of European sea bass LPL activity and expression were increased in fish with high GSI (José Ibáñez et al., 2008). Though the decrease of liver CYP19A and increase of gonad LPL in OXI indicate better reproductive outcomes, this finding is not fully supported as there were no effects observed for endpoints including plasma E2 concentrations, VTG biosynthesis, GSI, or fecundity. Analysis of pharmaceuticals (ongoing) in each treatment may also provide support for the decrease in CYP19A as studies have found that exposure to some pharmaceuticals may produce therapeutic effects with seemingly positive outcomes such as Eurasian perch exposed to oxazepam experiencing lower mortality (Klaminder et al., 2014).

Figure 4.2

Proposed AOP diagram for CYP19A inhibition



Note. Proposed adverse outcome pathway (AOP) for aromatase (CYP19A) inhibition. The molecular initiating event (MIE) leads to increase of liver CYP19A mRNA expression. With impaired steroidogenesis gonad lipoprotein lipase mRNA expression is reduced. Then estradiol (E2) production is reduced lowering concentration circulating in the plasma leading to a reduction of vitellogenin (VTG) concentration circulating in plasma. Reduction in VTG impairs the growth and development of oocytes leading to decreased fertility which would lead to a decline in population and alterations of the food-web. Red = $p<0.05$; Yellow = $p<0.10$; Green = $p>0.10$; Blue = Not measured.

Both exposures resulted in decreased exploratory behavior in OXI, indicating similar behavioral responses. Behaviors in fish can change to compensate for variations in metabolic requirements (Bertram et al., 2022). This could potentially link the increase in glucose in MELT1 to the reduction in exploratory behavior consequent to increased anxiety. Anxiety creates a tendency to avoid exploratory behaviors and may induce the fight or flight response (Heeren, 2020) initiated through the release of epinephrine in the short term and cortisol in the long term both leading to a rise in blood glucose (Wendelaar Bonga, 1997). When Simmons et al. (2017) exposed goldfish to WWTP discharge in the field for 21-days they documented in their activity assay that fish closer to WWTP discharge were observed to have a reduction in anxiety that was associated with serotonin reuptake inhibitors. However, to draw definite conclusion on how exposure to pharmaceuticals in the current study may have affected stress-related endpoints, more information on pharmaceutical concentration in the treatments will be needed (forthcoming). In another study (Mehdi et al., 2022), adult fathead minnows exposed to several dilutions of wastewater effluent coupled with changes in effluent temperature documented increased shelter-seeking behavior along with a metabolic cost to fish at 20℃. The authors hypothesized that this was a synergistic effect of effluent exposure and temperature (Mehdi et al., 2022), though in the present study temperature did not vary among treatments (Table 2.1).

It is important to note that the fish in the current study were exposed to 100% effluent for 21-days, while fish in the wild are typically exposed to a dilution of effluent. This could potentially affect the biological effects observed for the MBR and OXI treatments. Dilution of effluent in natural settings would result in lower concentrations of contaminants, which may temper the differences observed between treatments. Therefore, it is important to consider this factor when interpreting the results in relation to the environment.

Chapter 5: Conclusion

The current study demonstrates that MBR and OXI secondary treatments are effective in removing CECs from primary treatment effluent. While both methods show similar efficacy in reducing CEC concentrations and increasing survival rates, MBR was found to produce more stress on fish than OXI. However, this stress was limited to the macro-molecular level and did not affect other levels of biology. With these results my first hypothesis where membrane bioreactor effluent will have lower concentrations of CECs than oxidative ditch effluent is accepted. While my second hypothesis fathead minnows exposed to membrane bioreactor effluent experience less stress than those exposed to oxidative effluent is rejected and the alternative where fathead minnows exposed to oxidative effluent experience less stress than those exposed to membrane bioreactor effluent is accepted.

Therefore, wastewater treatment plants may still consider investing in MBR, but hybrid systems that utilize multiple treatments like the one at the Hutchinson Wastewater Treatment Facility may offer the best outcomes for removing pharmaceuticals and pesticides while producing less stressful environments for fish as the mixed final effluent will have a reduced chemical load and advantageous environmental conditions. Further research should also consider downstream effects on organisms in the environment who are exposed to diluted effluent. These studies should also expand on analytical chemistry and the range of organisms and endpoints studies.

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