PATHWAY-BASED APPROACHES IN ECOTOXICOLOGICAL RESEARCH: EVALUATION OF COMPLEX MIXTURES ON FATHEAD MINNOW REPRODUCTION AND DEVELOPMENT

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Dedication

This thesis is dedicated to the late Dr. Gilman Veith,

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Abstract

Mixtures of natural and synthetic compounds are ubiquitously detected in wastewater treatment plant (WWTP) effluents and surrounding surface waters. Of particular concern are endocrine-disrupting compounds that can affect hypothalamic-pituitary-gonadal axis function in exposed organisms. Reproductive effects of exposure to a historically estrogenic WWTP effluent were examined in a 21-d real-time exposure using fathead minnows. Molecular and biochemical endpoints representing key events along adverse outcome pathways linking estrogen receptor activation and other molecular initiating events to reproductive impairment were examined. Analytical chemistry results were used to construct a chemical-gene interaction network to aid in targeted gene expression analyses. Estrone was consistently detected in the effluent and was subsequently used in an exposure aimed to implement whole-mount in situ hybridization with fathead minnow embryos to examine developmental effects at early-life stages. The results provide insights into the significance of pathway-based effects with regard to predicting adverse reproductive and developmental outcomes.

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List of Frequently Used Abbreviations

AOP	Adverse outcome pathway
AR	Androgen receptor
BLAST	Basic local alignment search tool
BPA	Bisphenol-A
CEC	Chemical of emerging concern
CTD	Comparative Toxicogenomics Database
Cyp	Cytochrome P450
DO	Dissolved oxygen
E1	Estrone
E2	17β-estradiol
EDC	Endocrine disrupting chemical
EDSP	Endocrine disruptor screening program
EE2	17α-ethinylestradiol
ELISA	Enzyme-linked immunosorbent assay
EPA	US Environmental Protection Agency
EQ	Equivalents
ER	Estrogen receptor
esr1	Estrogen receptor-a
GSI	Gonadosomatic index
HM	Hybridization mix
hpf	Hours post-fertilization
HPG	Hypothalamic-pituitary-gonadal
LC/MS	Liquid chromatography/mass spectrometry
LSW	Lake Superior water
MED	Mid-Continent Ecology Division
MIE	Molecular initiating event
NWQL	National Water Quality Laboratory
PBT	Phosphate buffered saline-Tween
QPCR	Quantitative polymerase chain reaction
SSC	Saline-sodium citrate
STITCH	Search tool for interactions of chemicals
T	Testosterone
TRB	17β-trenbolone
USGS	US Geological Survey
VTG	Vitellogenin
WET	Whole effluent toxicity
WISH	Whole mount in situ hybridization
WWTP	Wastewater treatment plant

Chapter 1: General Introduction

Unintended exposure of humans and wildlife to numerous chemicals associated with commerce occurs on a daily basis. Although the US Environmental Protection Agency (EPA) can impose regulations through the Toxic Substances Control Act on the release/use of more than 84,000 new or previously existing commercial chemicals in the US, in most instances insufficient data exist to make inferences about the possible human or ecological risks of most chemicals (US Environmental Protection Agency, 2012). For example, while the EPA has developed formal strategies for evaluating and regulating some chemicals (e.g., pesticides) (US Environmental Protection Agency, 2012), little is known concerning the potential effects associated with literally thousands of "chemicals of emerging concern" (CECs) that may enter the environment through commerce and other human activities. Chemicals of emerging concern theoretically could include a variety of chemicals, but often refer to pharmaceuticals/personal care products, some new-generation pesticides, and high-production volume chemicals used for purposes such as packaging, and flame retardants, that often enter the environment via point sources such as industrial and municipal effluents (Petrovic et al. 2003, Lee et al. 2004, Richardson et al. 2005, Schecter et al. 2010). In many cases, CECs accumulate in wastewater treatment plants (WWTPs) where they eventually enter the surrounding waterways, resulting in sometimes very significant exposures to aquatic species, including fish.

We know that some CECs in effluents can negatively affect fish, but little is known about the potential impacts of the many untested and/or unregulated chemicals that enter the environment. Some of these chemicals could impair development,

reproduction, and overall organism health through their impacts on endocrine systems (e.g., Guillette et al. 1995, Guillette and Gunderson 2001, Hotchkiss et al. 2008). Chemicals that fall within this category have been broadly labeled as "endocrine disrupting chemicals" (EDCs). EDCs can include both synthetic and natural compounds that have the ability to alter the natural homeostatic state of endocrine systems by mimicking, stimulating, or inhibiting other naturally occurring endogenous hormones (Ankley and Johnson 2004, US Environmental Protection Agency, 2011b). The EPA Endocrine Disruptor Screening Program (EDSP) was established to address concerns regarding the possible adverse effects that EDCs may have on humans and wildlife. The EDSP provides a set of guidelines which are used to identify and test endocrine-active chemicals before they are released to the environment (US Environmental Protection Agency, 2011b). While the EDSP testing guidelines focus on investigating chemicals individually, in reality, many unidentified EDCs already exist in the environment, typically as components of complex mixtures.

While WWTP effluents are a common source of potentially toxic CECs (including EDCs) to the environment, (Martinovic et al. 2007, Vajda et al. 2008), often little is known as to the mechanisms/pathways through which these mixtures might cause adverse effects in fish. Traditional whole effluent toxicity tests with organisms can be informative with respect to impacts on standard apical endpoints (e.g., survival, growth, and reproduction) (US Environmental Protection Agency, 2002b), but these tests seldom assess the effects complex mixtures may elicit at the molecular level. The lack of molecular, mechanistic data limits the ability to extrapolate potential adverse effects of complex mixtures across species, time, and/or spatial gradients. Molecular endpoints

have been examined in many single-chemical and small-scale chemical mixture studies in the laboratory, but a streamlined approach to assessing impacts on these types of endpoints by complex mixtures, such as WWTP effluent, has yet to be established. By examining sub-lethal molecular changes, we can better evaluate relationships between biochemical responses and organismal-level adverse effects (e.g., impaired reproduction) of chemical mixtures, which can serve as a basis for prediction of potential impacts in untested species and/or under different exposure scenarios.

Adverse outcome pathways

The adverse outcome pathway (AOP) concept is an integrating principle increasingly used in the field of toxicology to link effects across levels of biological organization (Garcia-Reyero 2015). An AOP is a conceptual framework that represents a sequence of events that occurs after an external perturbation, such as chemical exposure, on a biological system (Figure 1) (Ankley et al. 2010). Adverse outcome pathways describe causal connections between a "molecular initiating event (MIE)" (e.g., the interaction between a chemical and a specific biomolecule) and observed adverse outcomes at the organism and/or population level (Ankley et al. 2010). Specifically, an AOP describes a relationship between an initial and/or persistent perturbation at the level of a MIE through a cascade of effects across levels of biological organization that lead to adverse apical effects in individuals and, ultimately, populations (Miller et al. 2007, Ankley et al. 2009, Ankley et al. 2010, Kramer et al. 2011, Villeneuve and Garcia-Reyero 2011). The insights gained from use of the AOP concept to assemble and depict biological data are important both experimentally and conceptually, in that they aid in the

application of predictive approaches to chemical risk assessments (Villeneuve et al. 2014a, Villeneuve et al. 2014b).

Understanding how chemicals act at the molecular-level allows for the utilization of pathway perturbation, as defined by AOPs, as a basis for making predictions regarding the potential impacts at the organismal and population levels. While AOPs often are derived from studies with single chemicals, in reality, most environmental settings involve multiple chemicals, making AOP application and interpretation much more complicated/challenging. For example, if a fish is exposed to a chemical mixture such as WWTP effluent, multiple endocrine-oriented MIEs may be affected depending on which chemicals are present, thereby initiating responses through multiple AOPs.

In order to understand the interactions among AOPs in the context of complex mixtures (such as WWTP effluent), it is useful to have some level of analytical definition of the chemical composition of the sample. Analytical chemistry data can be used to make predictions of biological effects, at the level of the MIE, that may be associated with exposure to these chemicals. A recently-described approach (Schroeder et al. 2015) showed how these types of predictions could be made based on chemical-gene/protein interaction data generated from sources such as the Comparative Toxicogenomics database (CTD) (Davis et al. 2013) and STITCH ('search tool for interactions of chemicals') (Kuhn et al. 2008), both of which compile chemical-gene/protein interaction data from published literature and may be valuable in determining potential MIEs (and, consequently, relevant AOPs). Specifically, by understanding chemical-gene interactions that may occur at the molecular level during exposure to a complex WWTP effluent, AOP-based influences can be made as to possible effects at the cellular, organ, and

organismal levels of biological organization. In this research project, I utilized the AOP concept to establish a systematic approach to assess the potential effects of a chemical mixture exposure on fish, focusing on molecular targets in the hypothalamic-pituitary-gonadal (HPG) axis.

HPG Axis: Normal Function and Chemical Perturbation

In vertebrate species, the hypothalamus, pituitary, and gonads function together in a multi-faceted feedback system collectively referred to as the HPG axis, which is the endocrine system key to sexual development and reproduction (Kennish and Lutz 1998, Isidori et al. 2008). Regulation of reproduction in teleost fish occurs through interaction of the HPG axis with environmental cues such as photoperiod, temperature, pheromones, and spawning substrate presence (Kennish and Lutz 1998, Kobayashi et al. 2002). Based on these cues, gonadotropin-releasing hormone (GnRH) is released from the hypothalamus in the brain, which in turn stimulates the pituitary gland to release follicle stimulating hormone (FSH) and leutenizing hormone (LH). Together FSH and LH induce gonadal growth, steroidogenesis, and ultimately, egg and sperm maturation and discharge (Kennish and Lutz 1998, Adkins-Regan 2008). In addition to its role in reproduction, the HPG axis is also responsible for reproductive development in early-life stage organisms. The HPG axis controls steroidogenesis, the production of the steroid hormones including testosterone (T), 17β-estradiol (E2), and 11-ketotestosterone (KT), which play an important role in the organizational events that control sex determination in developing fish, which regulate reproduction later in life (Kennish and Lutz 1998). In

theory, normal development and reproduction may be altered by chemicals at any of a number of points within the HPG axis (Liu et al. 2009).

Endocrine-active chemicals have been shown empirically to inhibit and/or stimulate HPG function at various points within the axis (Figure 2) (Ankley et al. 2009). For example, genotypic male fish that are exposed to estrogen receptor (ER) agonists can develop oocytes in the testis (a condition referred to as testis-ova) (Ankley and Johnson 2004), and female fish can develop male secondary sexual characteristics when exposed to androgen receptor (AR) agonists (Ankley et al. 2003). Chemicals also may inhibit or antagonize system function (Gray et al. 2006). For example, exposure of fathead minnows to the model AR antagonist flutamide causes increased plasma E2 in males and increased plasma T concentrations and occurrence of atretic ovarian follicles in females, resulting in overall reduced fecundity (Jensen et al. 2004). Other chemicals, such as ketoconazole, can inhibit steroid synthesis through targeting cytochrome P450 enzymes (CYPs), resulting in decreased T and E2 production by the gonads of exposed fathead minnows (Ankley et al. 2012). The HPG axis is a multifaceted molecular pathway, and EDCs can inhibit and/or stimulate this pathway at various points within the axis (Ankley et al. 2009).

Endocrine-active chemicals in WWTP effluents can include endogenous hormones (e.g., E2) (Desbrow et al. 1998, Snyder et al. 1999), exogenous hormones (e. g., 17α-ethinylestradiol; EE2) (Desbrow et al. 1998, Snyder et al. 1999), and other commonly used pharmaceuticals with endocrine activity (e.g., drugs that inhibit steroid synthesis such as conazole fungicides) (Roberts and Thomas 2006, Kasprzyk-Hordern et al. 2009). There are also other less well studied targets within the HPG axis (e.g.,

neurotransmitters) that could be affected by effluent contaminants (Ankley et al. 2009). Consequently, contaminants in WWTP effluents can theoretically contribute to "endocrine disruption" at several points within the HPG axis.

Research objectives

One objective of my research project was to assess the potential adverse effects of exposure to WWTP effluent on adult fathead minnow (*Pimephales promelas*) reproduction, as described in Chapter 2. An integrative approach utilizing analytical chemistry, *in vitro* bioassays, and measurements of endpoints at multiple biological levels of organization, including whole organism apical outcomes (e.g., fecundity and fertility) was employed to assess the impact of exposure to an environmentally relevant chemical mixture at a local WWTP. The biological results from the reproduction study were compared with analytical chemical measurements with the aim of validating known AOPs and hypothesizing new AOPs for chemicals present in the effluent, with an aim toward a better mechanistic understanding of how the complex mixture was affecting fish reproduction.

A second objective of this project was to implement whole mount in situ hybridization (WISH) assays with fathead minnow embryo toxicity testing, as described in Chapter 3. In instances where chemical MIEs may lead to subsequent effects on early-life stage development, WISH may be particularly useful for further investigation of AOPs. As proof of concept and to determine potential early-developmental effects of a chemical exposure, a short-term time-course exposure with a known ER-agonist, estrone, was conducted using fathead minnow embryos. The endpoints associated with the

embryo estrone exposure included phenotypic developmental staging and HPG-specific gene expression patterns using a combination of quantitative polymerase chain reaction (QPCR) and WISH.

Chapter 2: Pathway-based approaches for assessment of real-time exposure to an estrogenic wastewater treatment plant effluent on fathead minnow reproduction

Introduction

Wastewater treatment plant (WWTP) effluents are a well-established point of convergence through which anthropogenic chemicals enter surface water environments. These mixtures can be comprised of a variety of chemicals of potential concern, including natural substances, pharmaceuticals, and pesticides, that affect endocrine function through their action on the vertebrate hypothalamic-pituitary-gonadal axis, and have the potential to negatively impact exposed biota (e.g., Guillette and Gunderson 2001, Hotchkiss et al. 2008). In particular, many studies have examined the relationship between estrogenic WWTP effluents and biological effects (e.g., Tilton et al. 2002, Aerni et al. 2004, Martinovic et al. 2007).

Chemicals that act as estrogen receptor (ER) agonists are frequently detected in WWTP effluents and receiving waters (e.g., Sumpter and Jobling 1995, Braga et al. 2005, Martinovic et al. 2008b, Sun et al. 2008, Kahl et al. 2014), and typically include endogenous ER agonists (e.g., 17β-estradiol [E2] and estrone) and exogenous estrogens (e.g., 17α-ethinylestradiol [EE2], bisphenol A [BPA]) and plant-derived phytoestrogens (Braga et al. 2005, Sun et al. 2008, Liu et al. 2010). Feminization of male fish has been linked to ER agonist exposure, as indicated by changes in different types of biomarkers. For example, induction of vitellogenin (VTG), an egg yolk precursor protein normally detected at very low concentrations in male fish, is indicative of exposure to ER agonists (Sumpter and Jobling 1995). Additionally, hepatic ER-α (*esr1*) mRNA levels are

generally low in male fish, but these levels can be up-regulated in males exposed to exogenous estrogens (Filby et al. 2007, Wehmas et al. 2011). Laboratory studies have shown decreased reproductive success in fish chronically exposed to low (0.1-100 ng/L) concentrations of a variety of steroidal estrogens commonly found in WWTP effluents (Caldwell et al. 2008, Caldwell et al. 2012). Moreover, long-term exposure to environmentally-relevant concentrations of EE2 in the field negatively impacted fish population sustainability, as indicated in a whole-lake exposure study (Kidd et al. 2007). Although these types of single chemical studies have indicated that ER agonist exposure can adversely affect fish reproduction, to date, few studies have explicitly examined whether similar impairment occurs in WWTP effluents containing mixtures of estrogenic chemicals.

Over the past decade, the US Environmental Protection Agency, Mid-Continent Ecology Division (MED) laboratory has been involved in a number of studies focused on the effects of the discharge from the local WWTP (Western Lake Superior Sanitary District, WLSSD) in Duluth, Minnesota (MN) that has shown estrogenicity via both *in vitro* and *in vivo* systems (Martinovic et al. 2008b, Wehmas et al. 2011). For example, *in vivo* exposure of male fathead minnows (*Pimephales promelas*) to the effluent resulted in significant plasma VTG induction after 7 d; concurrent studies using an *in vitro* rainbow trout ER binding assay estimated that estrogenicity of the effluent ranged from 4 to 33 ng/L E2 equivalents (EEQ) (Martinovic et al. 2008b). Approximately five years later, another study with a transcriptional activation assay estimated estrogenic activity to be approximately 1 to 2 ng/L EEQ, with a concurrent 48 h static-renewal exposure of male fathead minnows to the effluent causing significant up-regulation of hepatic *vtg* and *esr1*

mRNA transcript abundance (Wehmas et al. 2011). Short-term (typically 4 d) field studies with fathead minnows caged at varying distances from the WWTP discharge site have documented co-occurrence of measured exposure to estrogenic chemicals (e.g., estrone, BPA [unpublished data]) and *in vitro* determination of estrogenic activity (Kahl et al. 2014). Taken together, this suite of lab and field studies examining a combination of chemical analyses, *in vitro* assays, and *in vivo* biomarkers of estrogen exposure, have shown that the effluent is a complex chemical mixture containing multiple potential ER agonists, and is consistently estrogenic. However, it remains uncertain whether this activity is sufficient to have direct effects on fish reproduction.

To address this question, adult fathead minnows were exposed to dilutions of the estrogenic WWTP effluent in a 21-d reproduction study in June 2014. This experiment was conducted using an exposure system constructed on-site at the WWTP to provide a continuous flow-through exposure to the final treated effluent under real-time conditions. The study included measures of both apical (e.g., reproduction) and pathway-based endpoints (e.g., gene expression, biomarkers of ER activation, and *in vitro* assays), paired with analytical chemistry, to assess the potential long-term effects of the contaminant mixture on different biological pathways. While estrogenicity of the WWTP effluent was a primary focus of the current study, a novel chemical-gene interaction network modeling approach was also used to generate hypotheses regarding which additional pathways may be impacted by non-estrogenic chemicals also present in the effluent.

Materials and Methods

Organisms

Adult male and female fathead minnows (5-6 months of age) were obtained from an on-site culture facility at the US Environmental Protection Agency, Mid-Continent Ecology Division (MED), and transported in culture water to the WWTP for subsequent experimentation. Laboratory procedures involving the minnows were reviewed and approved by the Animal Care and Use Committee in accordance with the Animal Welfare Act and Interagency Research Animal Committee guidelines.

Exposure conditions

A continuous flow-through test system was constructed in a laboratory space at the WWTP, allowing exposure of fish in real-time to several concentrations of the final treated effluent prior to discharge into the receiving water. Effluent was pumped into the exposure system where it was mixed with control water to achieve the desired treatment concentrations of 0 (control), 5, 20, and 100% effluent. The 5% and 20% effluent treatments were selected based on our previous fieldwork in the system (Kahl et al. 2014), with 20% effluent representing the estimated dilution factor within 5 m of the point of discharge into the receiving water, and 5% effluent representing the approximate dilution factor at a test site approximately 250 m from the discharge point. Effluent from WLSSD is typically diluted by a factor of ca. 5 upon discharge into the receiving water (J. Mayasich, unpublished data). The control and dilution water was sand-filtered (10 µm), UV-treated Lake Superior Water (LSW), transported from MED to the WWTP using a 1,700 L medium-density polyethylene transfer tank (US Plastics), and held in the

WWTP lab in three 1,000 L medium-density polyethylene tanks (US Plastics). Control LSW was transported to the WWTP every day during an acclimation phase of the study and every other day during the effluent exposure phase and subsequently delivered to 13 L stainless steel mixing cells using a centrifugal pump (Grundfos). Target effluent dilutions were achieved using piston pumps (Model QVG50, Fluid Metering, Inc.) to deliver calibrated flows to the mixing cells containing LSW. Each effluent exposure treatment was continually delivered to 20 L, glass aquaria containing 10 L of water, at a flow rate of 39-42 mL/min (about 6 volume additions/d). Aquaria were divided into thirds by transparent, porous, plastic dividers, and each of the three sections held a spawning substrate (a split section of PVC pipe). Aquaria, mixing cells, and tubing were cleaned and siphoned routinely during the effluent exposure. All aquaria were aerated with a constant flow of ambient air using diaphragm pumps and air stones. Water temperature was maintained at 25.0 ± 1.0°C, and photoperiod was set at 16:8 h light:dark throughout the exposure. Fathead minnows were fed thawed adult brine shrimp (San Francisco Bay Brand) to satiation twice daily.

Twenty-one day reproduction study

The 21-d fathead minnow exposure was based on a previously described design (Ankley et al. 2001). In brief, three pairs of male and female fathead minnows were placed in the glass aquaria, with each pair separated by the porous dividers. The pairs of fish were initially acclimated in control LSW at MED for 9 d during which fecundity was monitored daily to identify successful breeding pairs. Following this, successful breeding pairs (as defined by spawning at least once) were transported in plastic bags containing

fresh LSW to the exposure unit at the WWTP. To balance the relative productivity of pairs across treatments, individual pairs were assigned to exposure tank sections in a stratified, random manner, based on relative fecundity of pairs during the initial acclimation period. Specifically, pairs were ranked by total number of eggs produced during the acclimation period; the top four pairs were randomly assigned to each of the four treatments, then the next four most prolific pairs were randomly assigned to each treatment, and so on. There were four replicate tanks per treatment, resulting in a total sample size of n=12 pairs (i.e., 12 individuals of each sex) per treatment. To ensure the fish withstood the potential stress of transport and were able to effectively spawn in the WWTP exposure unit, the pairs were further acclimated in control LSW at the WWTP for 12 d, during which their fecundity was again recorded daily.

Following the second acclimation phase, the 21 d WWTP effluent flow-through exposure commenced. Fecundity, fertility rate, and survival were recorded, along with qualitative observations of behavior and food intake. Water flow rate, temperature, conductivity, and pH were measured daily, and alkalinity and hardness were measured 2-3 times per week throughout the exposure (Table 1). Dissolved oxygen (DO) measurements were also recorded daily (Figure 3). In addition to daily manual measurements, temperature and conductivity were automatically recorded every 30 min using HOBO Data Loggers (Onset Computer Corporation).

After 21 d of effluent exposure, fish in all treatments were anesthetized with buffered tricaine methanesulfonate (MS-222; Finquel; Argent), and wet weights were recorded. Blood was collected in heparinized microhematocrit tubes from the caudal peduncle of each fish and centrifuged to separate the plasma. Plasma samples were stored

at -80°C until used to measure VTG protein and sex steroids. Liver samples were collected and snap frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction and quantitative real-time polyermase chain reaction (QPCR) analyses. Total gonad weights were recorded and used to calculate the gonadosomatic index (GSI; gonad wet weight / whole body wet weight). A ~10-mg subsample of gonad tissue was removed for an ex vivo steroidogenesis assay, and another portion of the gonad was placed in a tissue cassette for histopathological analysis. The remainder of the gonad was snap frozen for RNA extraction and QPCR analyses. Care was taken to prevent RNA degradation and cross-contamination by using RNaseZap on dissection tools between samples. Tubercle size and number, a measure of male secondary sex characteristics in fathead minnows, were scored according to procedures previously described (Jensen et al. 2001).

Gene expression – identification of target genes and primer design

An initial set of genes of interest in the gonad were selected based on known roles in endocrine function, and past experience in our laboratory with a variety of HPG-active chemicals: gonadal androgen receptor (*ar*), follicle stimulating hormone receptor (*fshr*), steroidogenic acute regulatory protein (*star*), cytochrome P450 cholesterol side-chain cleavage (*cyp11a*), cytochrome P450 17α hydroxylase, 17, 20 lyase (*cyp17*), and cytochrome P450 aromatase-A (*cyp19a1a*) (Ankley and Villeneuve 2015). Hepatic mRNA transcript abundance of the estrogen-inducible genes, *vtg* and *esr1*, was also examined.

Additional genes hypothesized as potentially influenced by chemicals detected in the effluent were also examined. These gene targets were identified using a chemical-

gene interaction network analysis described by Schroeder et al. (2015). Briefly, the chemical composition of the 100% effluent (analytical techniques are described below) was used to hypothesize which biological pathways may be influenced by exposure to the measured chemicals using models based on chemical-gene/protein interaction data compiled in the Comparative Toxicogenomics Database (CTD) and STITCH ('search tool for interactions of chemicals,' versions 3.1 and 4.0) (Kuhn et al. 2008, Davis et al. 2013). All chemicals detected in at least one 100% effluent sample were included in the network analysis, but any chemicals flagged as present in laboratory blank samples were not included (see Table 2). In my analysis, the top 20 known gene/protein interactions (based on the sum of the number of references within the databases) for each detected chemical were identified and subsequently used to generate a chemical-gene interaction network, which was visualized and analyzed using Cytoscape v 3.0.2 software as described elsewhere (Schroeder et al. 2015). From this network analysis, a list was generated of genes previously reported to be influenced by three or more chemicals detected in the effluent. The gene candidates were analyzed using DAVID Bioinformatics Resources 6.7 (Huang et al. 2009) to identify enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Enriched KEGG pathways were considered significant at $p < 0.05$. From this analysis, potential interactions with pathways involved in oxidative and conjugative xenobiotic metabolism, xenobiotic efflux transportation, apoptosis, cellular proliferation, and oocyte maturation were inferred. Probability of expression in either liver or gonads (i.e., tissues collected in the current study), and availability of annotated transcript sequences in fathead minnow or other model small fish species (e.g., zebrafish, Danio rerio) were also considered. Based on these analyses, seven additional genes were

selected for QPCR analysis: two inducible cytochrome P450 (CYP) isozymes associated with xenobiotic metabolism (*cyp1a1* and *cyp3a* [Whyte et al. 2000, You 2004]), UDP-glucurononsyltransferase 1, polypeptide a1, isoform 1 (*ugt1a1*), which codes for an enzyme involved in the conjugation of xenobiotics in vertebrates (You 2004), ATP-binding cassette, sub-family C, member 2 (*abcc2*), which is involved in xenobiotic detoxification by coding for transporter transmembrane proteins that transport exogenous compounds from hepatocytes (Borst et al. 1999, Long et al. 2011), progesterone receptor (*pgr*), a membrane receptor involved in the control of oocyte maturation in fish (Patiño and Sullivan 2002), prolactin (*prl*), which is associated with cellular proliferation and oocyte maturation in mammals (Bole-Feysot et al. 1998, Freeman et al. 2000), and caspase 3 apoptosis-related cysteine peptidase (*casp3*), which codes for a caspase protein involved in apoptosis (Porter and Jänicke 1999) and therefore, could play a role in oocyte atresia (Miranda et al. 1999).

Primers and dual-labeled probes for *vtg* and *esr1*, and primers for *pgr* were designed with previously annotated fathead minnow coding sequences using PrimerQuest (Integrated DNA Technologies) (Table 3). Primer and probe design for *ar*, *fshr*, *star*, *cyp11a*, *cyp17*, *cyp19a1a*, *cyp1a1*, and *cyp3a* have been previously described, and their sequences (and associated literature citations) are listed in Table 3. Sequences for *ugt1a1*, *abcc2*, *prl*, and *casp3* have not been annotated for fathead minnow but were available for zebrafish. Fathead minnow transcript sequences were derived from alignments between the annotated zebrafish sequence for each gene and a draft fathead minnow genome assembly (Burns et al. 2015) using BLAST (basic local alignment search tool, National Center for Biotechnology Information). To obtain partial transcript sequences for each

gene (introns removed), the fathead minnow whole genome sequences were arranged based on alignment with the zebrafish transcript sequence. To verify proper sequence construction, the resultant fathead minnow nucleotide sequences were tested for alignment with the corresponding zebrafish (and other default species) gene using BLAST. The derived fathead minnow transcript sequences were used to design primers using PrimerQuest (Table 3).

Gene expression – specific procedures

Relative abundance of mRNA transcripts was quantified using QPCR. Total RNA from liver and gonad tissues was extracted using TRI Reagent® (Sigma), according to the manufacturer's protocol. RNA concentrations and quality were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies). All samples had A260 nm/A280 nm ratios > 2.0 and A260 nm/A230 nm ratios > 1.50. Prior to QPCR analyses, samples were diluted to 10 ng/µL. The QPCRs were performed using either Taqman RNA to Ct 1-step kits (*ar*, *fshr*, *star*, *cyp11a*, *cyp17*, *cyp19a1a*, *vtg*, *esr1*, *cyp1a1*, and *cyp3a* [Applied Biosystems]) or Power SYBR Green RNA-to-Ct 1-step kits (*pgr*, *ugt1a1*, *abcc2*, *prl*, and *casp3* [Applied Biosystems]). Each 20 µL Taqman reaction contained 20 ng total RNA (except female livers where 0.2 ng was used in the vtg analysis), 300 nM forward primer, 300 nM reverse primer, and 150 nM probe. The thermocycling program was set to 48°C for 15 min, 95°C for 10 min, and finally 40 cycles of 95°C for 15 s and 60°C for 1 min. Each 20 µL SYBR Green reaction contained 20 ng total RNA, 215 nM forward primer, and 215 nM reverse primer. The thermocycling program for the SYBR Green assays was set to 48°C for 30 min for the reverse transcription step, followed by

95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min for PCR amplification, and a final dissociation stage of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Relative transcript abundance was quantified relative to gene-specific mRNA (for Taqman) or cDNA (for SYBR Green) standard curves with six concentrations, following a 10-fold dilution series (2 x 10^7 to 200 copies/µL). The standards were prepared through a series of cDNA amplifications and *in vitro* transcription reactions (MEGAscript, Ambion) as described by Villeneuve et al. (2007). Approximately 20% of the samples were run in duplicate on each plate.

Steroid and vitellogenin protein measurements

Ex vivo production of E2 and testosterone (T) by ovaries, and ex vivo production of T by testes, were determined using a radioimmunoassay method described elsewhere (McMaster et al. 1995, Ankley et al. 2007). Male and female plasma T and E2 concentrations were also determined using radioimmunoassay (Jensen et al. 2001). Vitellogenin protein concentrations were measured in plasma of both sexes using enzyme-linked immunosorbent assays (ELISAs) as previously described (Parks et al. 1999, Korte et al. 2000). A purified fathead minnow VTG standard and a polyclonal antibody were used in the ELISAs.

Gonad histopathology

For histopathology, a portion of the left gonad of each fish was fixed in Davidson's fixative for 48 h, and then rinsed twice with deionized water and transferred to 10% neutral buffered formalin for storage until processed in paraffin. Gonad tissues were

processed in a Tissue Tek VIP automated tissue processor and embedded in paraffin. Tissue blocks were trimmed to the widest section of the tissue using a microtome and sectioned longitudinally. A single slide was prepared for each sample in which 3, 4 µm-thick sections were collected at 50 µm intervals, mounted on a glass slide, and stained with hematoxylin and eosin. Gonads were staged and observed histopathologies scored for severity, in an unblinded manner, based on guidelines developed through the Organisation for Economic Co-operation and Development (Crissman et al. 2004, Johnson et al. 2009).

Water Collection and Analyses

Water samples were collected for nutrient determinations, *in vitro* bioactivity measurements, and analysis of almost 200 organic chemicals over the course of the 21-d test. One L grab samples were collected for nutrient measurements from one of the replicate LSW and 100% effluent tanks every other day during the exposure (Figure 4). Nutrient concentrations including total ammonium (NH_4^+), nitrate (NO_3^-), phosphate (PO_4^-), total nitrogen (TN), and total phosphorus (TP) were measured in water samples. Water subsamples for PO_4^-, NO_3^- and NH_4^+ were filtered through a 0.45µm pore membrane and preserved within 24 hours of collection. Both filtered and unfiltered subsamples were stored frozen until analyzed. The unfiltered TN and TP subsamples were digested using the persulfate method ([APHA] 1998). Total nitrogen was analyzed using the cadmium reduction method (QuikChem method 10-107-04-1O; Lachat Instruments 2009, ([APHA] 1998)). Total phosphorus and dissolved PO_4^- were determined by the molybdate-ascorbic acid method (10-115-01-1-B; Lachat Instruments

2009, ([APHA] 1998)). The filtered subsample was analyzed for dissolved NO_3^- using the cadmium reduction method ([APHA] 1998) and NH_4^+ by the salicylate method (QuikChem Method 10-107-06-2-C; Lachat Instruments 2009). Resultant unionized ammonia (NH_3) concentrations were calculated from NH_4^+ based on pH and temperature measured in the tanks (Thurston et al. 1979).

Seven-day composite water samples were collected weekly from each treatment during the 21-d exposure, and also from control water during the last week of the on-site acclimation period. Every 20 min, autosamplers (see Kahl et al. 2014) pumped a 28 mL sample into a 20-L 5-mil Norton R MPTFE Film bag (Welch Fluorocarbon), yielding a 14-L composite sample over 7 d. After each 7-d collection period, the sample bags for each treatment were exchanged to collect a series of three 7-d composite samples over the course of the 21-d test. A 3-L aliquot was removed and shipped on ice overnight to the USGS National Water Quality Laboratory (NWQL, Denver, CO) for analysis of about 200 organic chemicals. Eight 12-mL aliquots for *in vitro* bioassays were also removed and frozen at -20ºC.

In vitro bioassays

Total estrogenic and androgenic activity was estimated in each of the 7-d composite samples using the T47D-KBluc and MDA-kb2 cell lines, which have been stably transfected with human ERα- or AR-luciferase reporter gene constructs, respectively (Wilson et al. 2002, Wilson et al. 2004). Water samples were prepared with powdered media to obtain a dosing media appropriate for the two assays, as previously described (Wehmas et al. 2011, Cavallin et al. 2014). Sample estrogenicity and androgenicity,

respectively, were determined relative to simultaneously-generated EE2 and 17β-trenbolone (TRB) standard curves.

Organic contaminant analysis

Concentrations of 190 organic contaminants were determined in all three of the 100% effluent composite samples (i.e., days 0-7, 7-14, and 14-21) and the control samples from the LSW acclimation and days 7-14 of the effluent exposure phase. Analytes included steroid hormones, sterols, and BPA (schedule 4434 [Foreman et al. 2012]), wastewater indicators (schedule 4433 [Zaugg et al. 2006]), and pharmaceuticals (schedule 2440 [Furlong et al. 2014]) by the USGS NWQL. The complete list of measured analytes, corresponding protocols, and quality assurance/quality control procedures for the steroid hormone, wastewater indicators, and pharmaceutical methods are described elsewhere (Zaugg et al. 2006, Foreman et al. 2012, Furlong et al. 2014).

Statistical analysis

All statistical analyses were conducted using GraphPad Prism v. 5.02 and Statistica 10 (StatSoft). *In vivo* data were tested for normality using the Kolmogorov-Smirnov test, and if data did not fit a normal distribution, they were log-transformed and tested for normality again. When data fit a normal distribution, a one-way analysis of variance followed by Duncan's multiple comparison test was used to determine differences between treatments. Data failing normality tests were analyzed using the non-parametric Kruskal-Wallis test followed by Dunn's post-hoc analysis. When plasma VTG protein concentrations were below the detection limit but above the zero standard, values of one-

half the detection limit were used. Differences were considered significant at $p < 0.05$.

In vitro bioassays assessing total estrogenicity and androgenicity of the water samples were analyzed as previously described (Cavallin et al. 2014). In brief, the maximum and minimum luminescence values for the EE2 or TRB standard curve were determined and used to calculate background adjusted percentage of maximum EE2 or TRB response for the standard curve and samples. The percentage of maximum response data for each sample were interpolated against the standard curve using nonlinear regression (log agonist versus response-variable slope). Using the percent maximum response data for the nonlinear regression constrains the data in order to obtain equal slopes of the standard and sample dilution curves. The resulting EE2-EQs and TRB-EQs were adjusted for sample dilution in the assay. A sample response was considered significant if it exceeded three standard deviations above the mean assay media control.

Results

Water quality measurements

As expected, given the nature of WWTP effluent, conductivity, alkalinity, and hardness measurements varied across effluent treatment dilutions, with the greatest values observed in the 100% effluent. Most water quality parameters remained consistent within each treatment across the 21-d exposure (temporal data not shown; means (±SD) shown in Table 1). Dissolved oxygen concentrations were relatively high and stable over time in the control, 5% and 20% effluent treatment tanks; however, DO concentrations in the 100% effluent tanks were more variable (Figure 3). For example, DO concentrations in 100% effluent were between 5.0 and 6.5 mg/L on exposure days 1-4, less than 5.5

mg/L on days 5-13, and greater than 6.5 mg/L on days 16-21. Mean (±SD) DO concentrations (n = 21 for all treatments) from the 21 d exposure were 6.91 (±0.23), 6.77 (±0.23), 6.49 (±0.37), and 5.80 (±0.96) mg/L in the control, 5%, 20%, and 100% effluent treatments, respectively.

Analyses showed nutrient concentrations in LSW control samples were stable and low throughout the 21-d exposure (Figure 4). As might be expected, nutrient concentrations were higher and more variable in the 100% effluent samples than in LSW controls. The mean (±SD) NH_3 concentration of the 100% effluent grab samples was 0.15 ± 0.11 mg/L.

Analytical chemistry

Of the 190 chemical analytes measured in the 7 d composite samples, 8 chemicals were detected in at least one LSW control sample, and 79 chemicals were detected in at least one 100% effluent sample (Table 2). Chemicals detected in at least one control sample included 3 of the 20 steroid hormones, 2 of the 69 wastewater indicators, and 3 of the 109 pharmaceuticals. However, concentrations of all chemicals detected in the LSW control samples were very low compared to the effluent and many were near the reporting limit. In the 100% effluent, 5 steroid hormones, 25 wastewater indicators, and 54 pharmaceuticals were detected in at least one 7 d composite sample. Forty-eight chemicals were detected in all three of the 100% effluent samples, including BPA, estrone, cholesterol, caffeine, cotinine, and a variety of pharmaceuticals such as atenolol, metformin, fluconazole, lidocaine, methocarbamol, pseudoephedrine, tramadol, and venlafaxine (see Table 2 for the complete list). The majority of these chemicals were

present at similar concentrations in all three 100% effluent samples. Concentrations of only one chemical, estrone, fluctuated by an order-of-magnitude across the three effluent sampling periods, with a notable spike during week 3 of the experiment.

In vitro bioassays

No measurable estrogenic activity was observed in LSW control composite samples during exposure weeks 1 and 2 in the T47D-KBluc assay. During week 3, very low estrogenic activity (0.3 ng/L EE2-EQ) was detected in the LSW control, near the assay's significant response level of 0.2 ng/L EE2-EQ. Substantial estrogenic activity was detected in all three 100% effluent composite samples (1.5 to 18.9 ng/L EE2-EQ; Figure 5). Measurable estrogenicity was also noted in the 5% and 20% dilutions during all three weeks. Estrogenicity measured in the 5% and 20% treatments during weeks 1 and 2 was proportional to the degree of dilution. A spike in estrogenic activity (~18.9 ng/L EE2-EQ) was observed in the 100% effluent sample from week 3 of the exposure. However, a parallel increase in estrogenicity in the diluted effluent treatments was not measured.

No androgenic activity was detected in any of the 7 d composite LSW controls or effluent dilutions, as determined using the MDA-kb2 assay (data not shown).

In vivo exposure

There was no treatment-related mortality, but one female died in a control tank on day 15 of the 21 d exposure. There was no significant difference in body mass at test conclusion among treatments for either sex (Table 4). While there were no significant differences in female GSI among treatments, mean GSI in male fish exposed to 100%

effluent (2.1%) was significantly higher than that in other treatments (1.3 to 1.7%; Table 4). Male tubercle scores were not significantly different among treatments (Table 4), and no tubercles were observed on females.

Overall control reproduction averaged 25 eggs/female/day, which met the minimum 15 eggs/female/day test acceptability criterion for the 21-d fathead minnow test (US Environmental Protection Agency, 2002a). Overall fecundity (total eggs per female) was significantly reduced in fish exposed to 100% effluent and significantly enhanced in fish exposed to 20% effluent, compared to the control (Figure 6A). The most pronounced effects on egg production were expressed during week 1, with complete cessation of spawning from days 2 to 7 in the 100% effluent treatment, and significantly higher production in 20% effluent (Figure 6B). During week 2, fecundity in 20% effluent was not different from control, and egg production increased in 100% effluent, while still significantly lower than all other treatments. By week 3, there were no significant differences in fecundity among the treatments. Consistent with reduced cumulative fecundity in the 100% effluent treatment, there was also a reduction in the mean number of spawns/female/day (Figure 7A). Although there was a significant increase in fecundity in fish exposed to 20% effluent, there was no significant difference in the mean number of spawns/female/day in this treatment (Figure 7A). Neither the mean number of eggs/spawn nor fertilization rate were impacted by any of the effluent treatments (Figures 7B & C).

Histopathological evaluation of male and female gonads did not reveal any major treatment-related changes. Approximately half of the males presented minimal to mild levels of interstitial cell hypertrophy/hyperplasia in their testes, and a low prevalence of

post-ovulatory follicles and oocyte atresia was observed in female ovaries across all treatments, including controls (Tables 5 & 6). The gonadal stage of both males and females was not affected by treatment (Tables 5 & 6).

Exposure to WWTP effluent had no significant effect on circulating T or E2 concentrations in males and females (Figures 8A & B). Additionally, no significant dose-dependent treatment effects on ex vivo steroid production by male and female gonads were observed (Figures 8C & D).

Male VTG protein concentrations and hepatic *vtg* mRNA transcript abundance increased significantly in a dose-dependent manner with exposure to WWTP effluent (Figure 9A). Plasma VTG concentrations varied substantially in males exposed to 100% effluent, with VTG concentrations in some males falling below the minimum detection limit while others approached average plasma VTG levels normally observed in the females. Despite the comparatively high variability, VTG protein concentrations and *vtg* transcript abundance were highly correlated in males from the 100% effluent treatment (Spearman $r = 0.97$, $p < 0.0001$). Exposure to WWTP effluent did not impact female plasma VTG protein concentrations; however, hepatic *vtg* mRNA transcript abundance was significantly down-regulated in 100% effluent-exposed females (Figure 9B). Consistent with male *vtg* up-regulation, there was a concentration-dependent elevation of hepatic *esr1* transcript abundance in males (Figure 10). *Esr1* was not affected in females.

Of the core suite of six endocrine-related gene transcripts examined, there was significant up-regulation of *cyp17* abundance in testes of fish exposed to 100% effluent (Figure 11). In females, the only observed concentration-dependent effect of WWTP effluent exposure on gene expression was significant down-regulation of *ar* transcript

abundance (Figure 11).

Chemical-gene interaction network analyses

A chemical-gene interaction network was constructed from the list of chemicals detected in at least one 100% effluent sample and genes associated with these detected chemicals as curated in CTD and STITCH (Figure 12). The chemical-gene interaction network was used to create a ranked list of genes that might be impacted by exposure to these chemicals (Table 7). Seven targeted genes, with at least three known chemical associations and involvement in various biological pathways, were assessed using QPCR analyses. Consistent with the network predictions, expression of targeted genes involved in oxidative xenobiotic metabolism (*cyp1a1* and *cyp3a*), glucuronidation (*ugt1a1*), and metabolite transport (*abcc2*) was up-regulated in the livers of both sexes, often in a concentration-dependent manner (Figure 13A-D). Relative abundance of targeted transcripts involved in oocyte maturation (*pgr*), cellular proliferation (*prl*), and cellular apoptosis (*casp3*) in gonads of both sexes were not affected in a concentration-dependent manner (Figure 13E-G). However, there was a trend toward decreased ovarian *prl* expression in females, as well as variable effects on *casp3*.

Discussion

Previous studies have established consistent estrogenic activity associated with the effluent from the local WWTP that serves the city of Duluth, MN, and surrounding area. In previous laboratory studies with this effluent, an increase in *vtg* and *esr1* mRNA transcript abundance in exposed male fish has been observed, reflective of estrogenicity

(Wehmas et al. 2011). The effluent and associated water samples also are consistently estrogenic *in vitro* (Wehmas et al. 2011, Kahl et al. 2014). The present study aimed to determine whether the estrogenicity indicated by these molecular and biochemical changes would translate to possible adverse reproductive effects either in the 100% effluent or in the receiving water impacted by the complex effluent. To address this, we conducted a 21-d reproduction study, exposing fathead minnows to dilutions of effluent at the WWTP. Although the most realistic way to examine potential impacts would be through exposure in the field, fish reproduction studies with fecundity as a major endpoint are challenging in caged fish systems due to handling logistics and other confounding environmental variables (e.g., temperature and feeding). Consequently, a real-time, flow-through exposure on-site at the WWTP provided a controlled environment (e.g., fixed temperature, feeding regime, and photoperiod) in which to directly test the potential effects of effluent exposure on reproduction while reducing the variability introduced with caged fish systems. A flow-through system with no holding time also minimizes the likelihood for chemical degradation in the final effluent, which could be problematic for an effluent collected at the WWTP and subsequently tested elsewhere at a later time (e.g., Wehmas et al. 2011).

Observed Reproductive Effects

Overall, while a significant reduction in fecundity in the 100% effluent concentration was observed, there was no evidence of reproductive impairment in 20% and 5% effluent, environmentally relevant concentrations of effluent in this particular watershed. It should be noted, however, that effluent discharge into this watershed (i.e.,

Duluth-Superior harbor) has a relatively large dilution factor compared to effluent-dominated streams in arid and semi-arid watersheds, which can be seasonally dominated by effluent (e.g., Brooks et al. 2006, Carey and Migliaccio 2009). A temporal effect on reproduction was observed, with significant reproductive impairment occurring in the 100% effluent treatment during weeks 1 and 2 and recovery during week 3. Given this temporal pattern of fecundity with recovery during week 3, the possibility of conditional acclimation or compensation by the fish cannot be completely dismissed; however, this study aims to identify possible causative factors that might have contributed to impaired reproduction.

Potential water quality parameters influencing reproduction

When assessing dose-response relationships with an effluent, it is extremely challenging to match all of the water quality characteristics of the continuously fluctuating 100% effluent. Consequently, basic water quality parameters other than chemical contaminants cannot be ignored as they have potential to confound interpretation of the role of chemical effects on apical reproductive outcomes. For example, in the present study, we need to consider at least three factors, DO, NH_3, and conductivity, that potentially could have caused a reduction in fecundity in 100% effluent-exposed fish, irrespective of anthropogenic contaminants in the effluent.

Dissolved oxygen

Given that low DO concentrations are known to influence fathead minnow reproduction (Brungs 1971, Corsi et al. 2011), the varying concentrations in 100%

effluent (e.g., as low as 4.3 mg/L during week 2 of the test) cannot be ignored. However, a previous study conducted by Brungs (1971) examined the effects of hypoxic conditions on fathead minnow reproduction and found no effect on reproduction in a life-cycle test at concentrations of 3.0 mg DO/L and greater. Corsi et al. (2011) reported an effect on fathead minnow spawning success at DO concentrations less than 3.1 mg/L in 21-d exposures in in situ spawning chambers. In the present study, the mean (±SD) DO in the 100% effluent treatment was 5.80 (±0.96) mg/L and the mean DO in each tank was never less than 3.0 mg/L, suggesting that DO was not likely solely responsible for the observed reduction in fecundity, although it cannot be completely eliminated as a confounding factor in the context of interactions with other stressors (e.g., chemicals) in the 100% effluent.

Ammonia

Ammonia can enter the aquatic environment through anthropogenic sources such as WWTP effluents (US Environmental Protection Agency, 2013). In a chronic exposure reported by Thurston et al., the no observed effect concentration (NOEC) for fathead minnow reproduction was 0.37 mg NH_3/L (Thurston et al. 1986). The mean (±SD) NH_3 concentration of the 100% effluent in the present study was 0.15 (± 0.11) mg/L, which is well below this value. Therefore, it is unlikely that NH_3, at least alone, played a role in the reduced fecundity in the 100% effluent.

Conductivity

Conductivity is another measure of water quality that co-varied with effluent

dilution. Conductivity of a water sample reflects a mixture of ions of varying biological activity/toxicity that can vary substantially among surface waters and effluents (Field and Reed 1996, Atekwana et al. 2004, US Environmental Protection Agency, 2011a). While I did not measure ionic composition of the effluent, broad inferences can be made as to possible effects on reproduction. In the present study, the mean (±SD) conductivity measurement in the 100% effluent was 1,605 (±139.7) µS/cm. Kavanagh et al. (2011) observed no significant reproductive impairment in fathead minnows exposed to pond water with conductivity of 2,780 µS/cm in a static-renewal 21-d study examining the effects of oil sands process-affected waters. Based on that, although conductivity cannot be completely eliminated as a potential factor influencing fecundity, it seems unlikely the main cause of reproductive impairment. Furthermore, given that reproduction was initially impacted during weeks 1 and 2 but recovered during week 3, yet conductivity was relatively stable throughout the exposure, conductivity was not likely responsible for reduced fecundity, unless some sort of compensatory response occurred.

Potential Chemical Influences on Reproduction

Based on my analysis of the potential effects of basic water quality parameters on reproduction, the basic water quality parameters examined in the present study do not seem to be primarily responsible for the observed reproductive effects. Therefore, it seems plausible that chemical contaminants were responsible for decreases in fecundity in the 100% effluent. Over the course of the 21-d exposure, 79 chemicals were detected in the WWTP effluent, and the effluent consistently exhibited estrogenic activity. Below I consider the role of estrogens versus other chemicals relative to their potential role in

producing the observed reproductive effects.

Potential Chemical Influences on Reproduction: Estrogens

Analytical data

Analytical measurements revealed a number of known natural and synthetic estrogens in the WWTP effluent, most prominently estrone and BPA. There are data for a few of these chemicals relative to potential effects on reproduction in the fathead minnow but, in general, observed effect concentrations for the individual chemicals were higher than what was measured in the effluent. For example, estrone has been shown to cause reproductive impairment at concentrations of 0.32 µg/L and greater (Thorpe et al. 2007) in a 21-d test with fathead minnows, while the mean concentration detected in the 100% effluent in the present study was only 0.02 µg estrone/L. Similarly, BPA adversely affected reproduction in a 21-d test with fathead minnows at a concentration of 344 µg/L (US Environmental Protection Agency, 2005), but the mean concentration of BPA detected in the effluent was only 0.74 µg/L. Additional estrogens measured in the present study included EE2, 17α-estradiol, E2, and 4-nonylphenol; however, concentrations of these chemicals were below the analytical reporting limits. The lowest observed effect concentrations for these estrogens in 21-d fathead minnow reproduction tests were 0.0001 µg EE2/L (Pawlowski et al. 2004), >0.15 µg 17α-estradiol/L (Shappell et al. 2010), >0.044 µg E2/L (Shappell et al. 2010), and 100 µg 4-nonylphenol/L (Harries et al. 2000), most of which are well above the reporting limits of the present study.

In vitro results

The T47D-KBluc assay indicated that the effluent and effluent dilutions showed measurable estrogenic activity in all three weeks of the exposure. *In vitro* estrogenicity measurements in the present study were within the same order of magnitude as previous work with this effluent (e.g., Martinovic et al. 2008b, Wehmas et al. 2011). To assess the degree to which the chemical measurements captured contributors to *in vitro* estrogenicity in the effluent, I compared the EQs derived from the *in vitro* T47D-KBluc assays with estimated EQs for chemicals detected in each sample. For example, if higher EE2-EQs were observed *in vitro* than calculated analytically, this could indicate that some important estrogenic chemicals were not among the measured analytes. The estimated EQs based on the chemistry data were calculated for each effluent composite sample. In brief, the potency factors for estrogenic compounds included in the analytical screening suite (e.g., E2, EE2, BPA, 17α-estradiol, estrone, diethylstilbestrol, and 4-nonylphenol) were determined based on EC50 values in previous studies using the T47D-KBluc assay (Bermudez et al. 2010, Leusch et al. 2010, Bermudez et al. 2012, Cavallin et al. 2014) relative to the potency of EE2 in the same assay. The measured concentrations of the analytes were then adjusted for their respective potency factor and summed within each sample to determine the total estimated EE2-EQ of the sample. Overall, the calculated analytical EE2-EQ estimates aligned well with the *in vitro*-derived EE2-EQs. Results from this comparison showed only a 1.4 mean fold-difference between the chemistry and *in vitro* estimates of the 100% effluent composite samples (n=3), with the analytical chemistry estimates slightly overestimating the observed *in vitro* EE2-EQs (data not shown). These data suggest that the chemicals selected for analytical

measurement could plausibly account for the *in vitro* estrogenicity in the effluent samples, with fluctuating concentrations of estrone (especially during week 3) appearing to be a primary driver of estrogenic activity.

When fecundity data were broken down over time, the most substantial effects on reproduction occurred during the first week of the exposure, with complete cessation of egg production occurring after 2 d in the 100% effluent and a significant increase in egg production in the 20% effluent. The corresponding *in vitro* EE2-EQs of the 20% and 100% effluent were 1.67 and 3.28 ng/L, respectively. Significant estrogenic activity was detected in all effluent treatments again during week 2, the week in which reproduction began to recover in the 100% effluent. Most significantly, during week 3, in which there were no significant differences in fecundity among the treatments, the *in vitro* EE2-EQ for the 100% effluent increased dramatically to 18.9 ng/L, which was correlated with a substantial increase in estrone in the effluent. Overall, the disagreement between the fluctuating *in vitro* estrogenic activity and recovery of fecundity suggests that impaired reproduction was not likely due to ER agonists.

In vivo responses

Given the estrogenic response in the *in vitro* cell assay, and detection of at least two known ER agonists (estrone and BPA) in the effluent, I hypothesized that I might observe *in vivo* effects on endocrine function consistent with fathead minnow studies with known ER agonists. This was clearly the case in males in which plasma VTG concentrations were significantly elevated in the 100% effluent, and slightly increased (albeit not significantly) in the 20% dilution, at the termination of the 21-d exposure (Figure 9A).

Consistent with previous studies (Wehmas et al. 2011), male hepatic *vtg* mRNA transcript abundance was also up-regulated, with plasma VTG and *vtg* mRNA expression highly correlated (Spearman $r = 0.9719$, $p < 0.0001$) in males exposed to 100% effluent. These data, as well as trends in VTG observed in the 20% treatment, imply that modest increases in male VTG concentrations may not necessarily be predictive of egg production in females. Furthermore, although the effluent is clearly estrogenic, there were no significant differences among treatments in fertility rate (which, ostensibly, could be more directly linked to males than egg production) throughout the 21-d exposure. Despite increased GSI in males exposed to 100% effluent, there were no apparent histopathological changes in the testes, which can occur in high-dose studies with estrogens (Ankley and Jensen 2014).

The overall reduction in fecundity observed in the 100% effluent was correlated with a significant reduction in spawning frequency by the females, so it is possible that, despite the occurrence of overt estrogenic responses in males, putative adverse effects of estrogens in the effluent were manifested in the females (Figure 7A). However, various measures of reproductive endocrine function in the females were not consistent with effects of estrogens in single chemical studies with female fathead minnows. For example, no changes in GSI or histopathological alterations characteristic of exposure to estrogens were observed in gonads of females exposed to 100% effluent (Ankley and Jensen 2014). Further, ex vivo steroid production and/or plasma E2 levels in females also were not affected by exposure to the effluent in a manner consistent with exposure to estrogens (Ankley and Jensen 2014).

Potential Influences on Reproduction: Other Chemicals

Based on my analysis, it seems that observed reproductive effects were not solely attributable to either measured water quality parameters or estrogenic chemicals, so it seems probable that other chemicals were responsible for the observed effects. One way to identify what these unknown chemical(s) could be is to consider pathway-based biological responses in the exposed animals. For example, in the females, there was a significant decrease in *vtg* mRNA expression in the 100% effluent-exposed fish. Although this was not translated into effects on plasma VTG levels in the fish (at least at 21 d), in previous studies with chemicals that inhibit vitellogenesis we have observed decreased egg production in fathead minnows. For example, conazole fungicides used as pesticides and pharmaceuticals (e.g., propiconazole and ketoconazole) have been shown to inhibit VTG production and cause reproductive impairment in fish (e.g., Ankley et al. 2007, Skolness et al. 2013). One chemical consistently detected in the effluent was fluconazole, a human pharmaceutical. However, I did not observe changes in other molecular and biochemical endpoints consistent with the effects of conazoles on known adverse outcome pathways associated with alterations in vitellogenesis. For example, steroid synthesis inhibitors depress VTG production through decreases in T and/or E2 production (Ankley and Jensen 2014), a response not observed in this study. However, the design of our study may have precluded making these types of observations as most endpoints were measured at 21 d, rather than when the most substantial impacts on egg production were observed (e.g., week 1 of the assay). Whether the recovery in egg production by week 3 of the assay was due to an uncharacterized change in effluent composition or some sort of compensatory response in the fish is unknowable. However,

these data highlight the desirability for more refined temporal data when assessing endocrine endpoints at lower biological levels of organization (Ankley and Villeneuve 2015).

A variety of chemicals not known to affect reproductive endocrine function also were detected in the effluent and, for most, there is no available information concerning their possible effects on biological pathways in fish. Consequently, in an attempt to "mine" the analytical data, a chemical-gene interaction network based on all chemicals measured in the effluent was created to aid in the identification of those that might be involved in reducing fecundity, through actions on additional pathways. The network analysis identified several hepatic genes that play a role in xenobiotic metabolism or efflux from cells (e.g., *cyp1a1*, *cyp3a*, *ugt1a1*, and *abcc2*) and could be potentially impacted by exposure to chemicals present in the effluent based on chemical-gene/protein interactions previously reported in the literature. Significantly, these four hepatic genes were up-regulated in a concentration-dependent manner in males and/or females exposed to WWTP effluent in the present study (Figure 13A-D). Chemicals that were present in all three effluent samples and associated with the up-regulation of gene transcripts coding for hepatic enzymes associated with xenobiotic metabolism included caffeine (Goasduff et al. 1996), carbamazepine (Giessmann et al. 2004, Oscarson et al. 2006), citalopram (Milkiewicz et al. 2003), cotinine (Joubert et al. 2012), estrone (Basu et al. 2004, Lepine et al. 2004), fluconazole (Sun et al. 2006), oxycodone (Samer et al. 2010), piperonyl butoxide (Ryu et al. 1997, Muguruma et al. 2007), and thiabendazole (Price et al. 2004, Diani-Moore et al. 2006). However, it should be noted that the gene expression measurements were made at the end of the study when there was no longer a

difference in egg production among treatments. While impacts on these xenobiotic metabolic pathways may not directly cause reproductive impairment, they certainly have potential to have other physiological consequences. For example, up-regulation of cytochrome P450s (including *cyp1a1*) are known to generate reactive oxygen species as a metabolic byproduct, which can then induce DNA damage through oxidative stress (Puntarulo and Cederbaum 1998), and may eventually lead to apoptosis through caspase activation (Nebert et al. 2000). Furthermore, increased metabolic activity is also associated with increased energetic demands to maintain homeostasis, which may potentially divert energy required for reproduction.

In contrast to the strong agreement between chemical-gene interaction network predictions and *in vivo* gene expression in liver tissue, the network analysis was not broadly predictive of changes in gene expression patterns in the gonads. The genes examined in the gonads were affiliated with a variety of potentially important pathways (e.g., egg maturation, cellular proliferation, and apoptosis) that have potential to alter reproductive success of fish. These genes, however, were not consistently impacted by effluent exposure (Figure 13E-G). Specifically, based on the chemical-gene interaction network, we might have expected gonadal *pgr*, *prl*, and *casp3* to be impacted by effluent exposure; however, no significant differences were observed among treatments in males. There were, however, variable effects on ovarian *prl* and *casp3* expression. There are several possible explanations for lack of a stronger linkage between the network hypotheses and gonadal gene expression results. For example, the test duration may have facilitated compensatory responses by the fish in which transcriptional activity may have initially been impacted by exposure but recovered by the end of the 21-d test (e.g.,

Ankley and Villeneuve 2015). Another limitation to the chemical-gene interaction network analysis is that it only accounts for the presence/absence of chemicals, but not chemical concentration. For example, some chemicals that were included in the network may have been detected at concentrations lower than those expected to elicit a biological response in the exposed organism. Finally, the chemical-gene interactions are derived from mammalian-focused databases, which may not directly translate into responses by teleost fish.

Conclusion

The present study demonstrates the value of utilizing an integrative approach, encompassing analytical chemistry, *in vitro* bioassays, and *in vivo* apical and pathway-based approaches with endpoints spanning from molecular- (e.g., gene expression) to organismal- (e.g., reproduction) levels of biological organization to help infer causal relationships between chemistry and potential effects on reproduction. Using this approach allowed us to determine that ER agonists were likely not the primary cause of reduced fecundity in the 100% effluent as indicated, for example, by the disconnect between weekly *in vitro* EE2-EQs along with analytical data and the observed fecundity effects. This suggests that *in vitro* detection of estrogenic activity and even *in vivo* indicators of ER activation cannot automatically be associated with adverse apical effects. Of course, there may be a potential "tipping point" of *in vitro* estrogenic activity and/or biomarker induction that may lead to adverse apical responses, which needs to be identified with additional research. Further, this integrated study demonstrates application of the chemical-gene interaction network analysis in providing insights as to additional

chemicals (other than ER agonists, in this case) and biological pathways that may contribute to undesirable biological effects. In addition to providing a platform to identify chemicals that may be driving gene expression changes, the chemical-gene interaction network holds promise as a useful tool to further prioritize testing of environmentally relevant chemicals hypothesized to impact apical endpoints of interest in risk assessment.

Chapter 3: Evaluation of whole mount in situ hybridization as a tool for pathway-based toxicological research in early-life stage fathead minnows

Introduction:

Embryonic and larval fish often can be more sensitive to toxicants than mature, adults (McKim 1977). Consequently, early-life stage tests have become commonly used in regulatory toxicology (Woltering 1984). In addition to direct, immediately quantifiable impacts during development, some chemicals can cause latent effects that are not fully expressed until later life stages. For example, some endocrine-active chemicals have potential to impact early developmental processes via effects on nuclear hormone receptors such as the estrogen or androgen receptors (ERs, ARs) or alterations in sex steroid synthesis (Ankley and Johnson 2004, Leet et al. 2011). Adverse outcome pathways (AOPs), describing perturbations in these types of key events during early fish development, can be used to predict potential adverse outcomes of interest to risk assessment (Ankley et al. 2010, Volz et al. 2011, Villeneuve et al. 2014c). For example, Volz et al. (2011) describe a three-tiered approach, based on AOP knowledge, for testing and prioritizing chemical testing using fish early-life stages, including 1) initial *in vitro* screening, 2) medium-to-high throughput *in vivo* screening with pre-hatch embryos and yolk-sustained larvae, and 3) conventional lower-throughput fish early-life stage testing (Volz et al. 2011). In order to gain insight into chemical effects and/or mechanisms of action in embryonic development, whole mount in situ hybridization (WISH) assays paired with gene expression assays could be of particular use in the proposed tier 2 phase of AOP development associated with fish early-life stages.

Whole mount in situ hybridization is a chromogenic technique that allows

visualization of the spatial and temporal expression of targeted genes in preserved embryos and/or tissue sections at different developmental stages. Consequently, in addition to supporting basic research on early development (e.g., baseline genetic ontogeny), WISH also has potential applications for pathway-based toxicological studies. For example, tissue-specific markers can be identified at given stages of development, and changes in expression linked to phenotypic alterations following chemical exposure (Thisse and Thisse 2008). Thus, molecular-level changes can be anchored to apical endpoints, such as gross morphology and embryonic developmental staging, to support AOP development. Consequently, WISH assays could be a useful complement to the standardized fish early-life stage toxicity tests used by the EPA (US Environmental Protection Agency, 1996b), which focus primarily only on apical endpoints (e.g., survival, growth).

While WISH has been frequently used in zebrafish (*Danio rerio*) early-life stage developmental work (e.g., Puschel et al. 1992, Jowett 2001, Palevitch et al. 2007, Thisse and Thisse 2008, Carlson et al. 2009), this technology has not previously been applied to fathead minnows (*Pimephales promelas*), another well-established laboratory small fish model. The fathead minnow is endemic to North America and has historically been used extensively for regulatory testing, including evaluation of endocrine disrupting effects of chemicals (Ankley et al. 2001, Jensen et al. 2001, Ankley and Villeneuve 2006). Many basic mechanistic techniques used for measuring endpoints associated with reproductive endocrinology in adult fathead minnows have been well-characterized (Ankley et al. 2001); however, comparable work in early developmental stages has been more limited. Like zebrafish, several features of fathead minnows, including rapid development,

embryonic transparency, and large number of offspring, make them amenable to WISH. Information gained from fathead minnow WISH assays with chemicals such as endocrine disruptors could be useful for AOP development in fish early-life stage studies.

As proof of concept to demonstrate the utility of the WISH assay in toxicity testing with fathead minnows, I conducted a study exposing fathead minnow embryos to the endogenous ER agonist, estrone (E1). Because E1 is less potent *in vivo* than other ER agonists (e.g., 17β-estradiol [E2] and 17α-ethinylestradiol [EE2]), it has received relatively little attention, despite being ubiquitously detected in wastewater treatment plant (WWTP) effluents discharge and surrounding surface waters (e.g., Belfroid et al. 1999, Servos et al. 2005, Miege et al. 2009, Chapter 2). In the present study, I (1) optimized a WISH assay developed for zebrafish to fathead minnows, and (2) used samples from an E1 exposure to demonstrate utility of the assay. In the E1 study, I first evaluated whether fathead minnow embryos exposed to E1 would show up-regulation of several gene transcripts known to be induced by estrogens in adult fish (e.g., vitellogenin [*vtg*] (Sumpter and Jobling 1995), estrogen receptor-α [*esr1*] (Filby et al. 2007, Wehmas et al. 2011), and cytochrome P450-aromatase-B [*cyp19b*] (Callard et al. 2001)) using quantitative polymerase chain reaction (QPCR). I then examined up-regulated genes (selected based on the QPCR data) in WISH assays to determine the spatial distribution of the transcripts in whole fathead minnow embryos and larvae.

Methods:

Estrone and 17α-ethinylestradiol range-finding exposure

An initial 48 and 96 h range-finder study with fathead minnow embryos (obtained from an on-site culture unit) was conducted with EE2 (Sigma, purity > 98%) and E1 (Sigma, purity > 99%) in a solvent-free, flow-through test system to determine target concentrations for a subsequent 6 d E1 time-course exposure. Although our chemical of interest was E1, EE2 was included in the initial experiment because several early-life stage exposures to EE2 have been conducted with fathead minnows (e.g., Van Aerle et al. 2002, Johns et al. 2011), but to date, no early life-stage studies had been conducted with E1. Concentrations of EE2 for the range-finding exposure were based on previous studies in which induction of *vtg* mRNA was assessed in the fathead minnow (Van Aerle et al. 2002, Flick et al. 2014), and were 0 (Lake Superior water control), 0.5, 5, and 50 ng/L. According to Van den Belt et al. (2004), EE2 is approximately 36 times more potent than E1 based on VTG induction in adult female zebrafish. Using this *in vivo* potency equivalency factor, nominal E1 concentrations of 0, 18, 180, and 1,800 ng/L were selected.

Two replicate tanks were used per treatment with two mesh-bottomed egg baskets suspended in each tank. At test initiation, 20 fathead minnow embryos (~24 hours post-fertilization [hpf]), pooled from 6 spawning pairs, were placed in each of the two egg baskets per tank. Egg baskets were continually, gently agitated with a mechanical rocker. Dead eggs or those visibly infected with fungus were removed from the baskets daily. After 48 h of exposure, 20 embryos from each tank were pooled, and after 96 h of exposure, 10 larval fish were pooled from each tank. All samples were snap frozen in

liquid nitrogen, and stored at -80°C for subsequent RNA extraction and *vtg* QPCR analysis as described below.

Water samples were collected for analytical measurements from each tank on days 0, 2, and 4 of the exposure. Both EE2 and E1 were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an Agilent 6410 system equipped with an atmospheric pressure photoionization (APPI) source. Separation of 500 µL injections was performed by gradient elution of water and methanol at a flow rate of 300 µL/min on a Kinetex PFP (2.1 x 50 mm, 2.6 µm) column. The gradient program began at 10% methanol increased to 95% from 2-11 min, held for 3 min, then returning to starting conditions for a 20 min total run. Under these conditions, retention times for EE2 and E1 were 11.1 min and 11.5 min, respectively. EE2 and E1 were quantified by selected reaction monitoring using internal standard method with estradiol-d4 as the internal standard. EE2 and E1 were quantified monitoring the transitions 279.3 m/z to 159.2 m/z and 271.2 m/z to 159.1 m/z, respectively. Two ion transitions were monitored for each analyte for compound confirmation. Aliquots of 500 µL tank water from the two higher E1 treatments (180, 1800 ng/L) were directly injected onto the system, while the 18 ng/L E1 treatment and all EE2 treatments were concentrated prior to analysis on Strata-X (200 mg) SPE cartridges. After SPE, samples were evaporated and reconstituted in 550 µL mobile phase and then analyzed by LC-MS/MS.

Estrone 6 d time-course exposure

Effects on *vtg* mRNA transcript abundance in the range-finder were used to select concentrations for the subsequent time-course experiment with E1. Estrone

concentrations selected were 0 (Lake Superior water control), 18, and 1,800 ng/L. The test system and chemical delivery were the same as used for the range-finder assay. Newly fertilized fathead minnow embryos (<8 hpf) were collected from 11 spawning pairs and pooled prior to chemical exposure. Four replicate tanks were used per E1 concentration, with each tank holding four suspended egg baskets. Twenty-seven embryos were placed in each of the four mesh-bottomed baskets per tank (108 embryos per tank). Egg baskets were continually, gently agitated with a mechanical rocker and held at 25°C with a 16:8 h light:dark photoperiod. Dead or infected embryos were removed from the baskets daily.

Embryos were collected on days 0, 3, and 6 of E1 exposure. Four replicates of 20 control embryos were pooled on day 0 (test initiation), and 10 embryos and 10 larvae were pooled from each tank (n=4 per treatment) on exposure days 3 and 6, respectively. Embryonic developmental stages were phenotypically examined on d 3 and 6 (US Environmental Protection Agency, 1996a). Samples for gene expression analyses were snap frozen with liquid nitrogen, and stored at -80°C until RNA extraction.

At each time point, 96 embryos or larvae were collected from each treatment (24 organisms per tank) for WISH assays. Embryos (d 3), grouped by treatment, were dechorionated with a 5 mg/mL pronase (Sigma) incubation at room temperature for 10 min. The pooled dechorionated embryos (d 3) and larvae (d 6) were rinsed several times with control Lake Superior water and fixed overnight in 4% paraformaldehyde (Sigma) in phosphate buffered saline (PBS). The following day, embryos/larvae were dehydrated in a series of methanol (MeOH) washes. Embryos and larvae were depigmented by incubation in 3% H_2O_2/0.5% KOH for approximately 60 min prior to MeOH dehydration

steps as described by Thisse and Thisse (2008). Dehydrated embryos/larvae were stored in 100% MeOH at -20ºC until used in WISH assays.

Water samples were collected from each tank for analytical verification on d 0, 3, and 6 of the exposure. Estrone concentrations were determined using the LC-MS method as described above with minor modifications. Injection volume was set to 50 µL, and concentrated samples were reconstituted in 100 µL mobile phase. Basic water quality and tank flow rates were assessed throughout the E1 exposure (mean ± SD), including temperature 24.7 ± 0.12°C, dissolved oxygen 7.78 ± 0.15 mg/L, pH 7.92 ± 0.14, and flow rate 44.5 ± 2.7 mL/min.

Gene expression: QPCR

Relative transcript abundance of three estrogen-inducible genes, *vtg* (Sumpter and Jobling 1995), *esr1* (Filby et al. 2007, Wehmas et al. 2011), and *cyp19b* (Callard et al. 2001), were measured by QPCR. Total RNA was extracted from each replicate (n=4 per time point) of pooled embryos using RNeasy Micro kits (Qiagen) according to the manufacturer's protocol. A Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies) was used to determine RNA quality and concentration. Total RNA samples were then diluted to 10 ng/µL prior to QPCR analyses. Relative abundance of *vtg* and *esr1* mRNA transcripts was analyzed by QPCR using Taqman kits (Applied Biosystems), and *cyp19b* mRNA expression was analyzed using a SYBR Green kit (Applied Biosystems) as described in Chapter 2. Primer and probe sequences are listed in Table 3.

Gene expression: WISH

RNA probe development

For each gene of interest, two probes were created: 1) an experimental antisense RNA probe and 2) a control sense RNA probe, to provide information regarding nonspecific binding. Primers were designed based on recommendations by Thisse and Thisse (2008), using a T3 or T7 RNA polymerase promoter. PrimerQuest (Integrated DNA Technologies) was used to generate primer sequences specific to each gene of interest, and a T7 promoter was added to the 5' end of the forward primer, and a T3 promoter was added to the 5' end of the reverse primer (Table 8).

To synthesize the RNA probes, PCR amplification was performed with the gene-specific T7-forward and T3-reverse primers to prepare the template DNA, which would subsequently be used for *in vitro* transcription reactions. The PCR amplification was conducted using JumpStart Taq DNA polymerase kits (Sigma) according to the manufacturer's protocol. Each 50 µL PCR contained 5 µL 10X buffer, 2 µL dNTPs, 2 µL Taq polymerase, 500 nM forward primer, 500 nM reverse primer, and 20 ng template cDNA. The quality and expected base pair size of each amplified PCR product was verified by electrophoresis on a 3% agarose gel (Lonza) in TAE (Tris-acetate-EDTA) buffer. The remaining PCR product was purified using a Wizard® SV Gel and PCR Clean-up system kit (Promega), according to the manufacturer's protocol, except with a final elution volume of 20 µL RNase-free water. The quality and quantity of the purified DNA were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies).

Subsequently, an *in vitro* transcription reaction was conducted using the purified

DNA to obtain a DIG-labeled RNA probe. The transcription reaction was performed using a protocol adapted from the DIG RNA labeling mix (Roche Diagnostics). In brief, the 20 µL reaction for each probe contained up to 200 ng of PCR product, 2 µL DTT, 2 µL 10X DIG RNA labeling mix, 4 µL 5X transcription buffer, 1 µL recombinant RNasin ribonuclease inhibitor (Promega), and 2 µL T7 or T3 RNA polymerase (Promega). The reaction tubes were first incubated at 37ºC for 1 h and 45 min, following which 2 µL of Turbo DNase (Megascript T7 kit, Ambion) was added to remove excess template DNA. The products were incubated for an additional 15 min at 37ºC, and the reaction was stopped by adding 2 µL of 0.2 M EDTA. The RNA was purified using the ethanol precipitation procedure described with the DIG RNA labeling mix according to the manufacturer's protocol (Roche Diagnostics). The RNA concentrations were determined using a Nanodrop ND-1000 Spectrophotometer, and the quality of the RNA was assessed using a RNA 6000 Nano kit (Agilent Technologies) and an Agilent 2100 Bioanalyzer (2100 Expert Software). To inhibit degradation, 1 µL RNasin (Promega) was added to the stock DIG-labeled RNA probes. RNA probes were stored at -80ºC until used in WISH assays.

Because there have been no previously reported studies that have utilized WISH assays with fathead minnows, a positive control using the paired box 2a (*pax2*) gene was assessed in zebrafish embryos concurrent with fathead minnow WISH assays. The zebrafish *pax2* RNA probe was generously provided by Dr. Kerri Carlson (University of St. Thomas, St. Paul, MN).

WISH assay

To determine spatial distribution of gene expression, WISH assays were conducted on a subset of samples from the E1 time-course study using QPCR results indicating which genes were affected at a given treatment/time. Specifically, the expression pattern of *esr1* was examined in the control and high (1,800 ng/L) E1 treatments at both 3 and 6 d, while spatial distribution of *vtg* expression was examined in control and 1,800 ng E1/L-exposed larvae at the 6 d time point. For each gene, 10 embryos were used in the experimental antisense (T3) probe hybridization and 6 embryos were used in the control, sense (T7) probe hybridization at each time point and treatment.

The WISH assay was conducted with slight modifications to the zebrafish protocol of Thisse and Thisse (2008). In short, dehydrated embryos (stored in MeOH) were rehydrated in a series of MeOH to PBS washes. Embryos were then incubated in 10 µg/mL proteinase K (Sigma) for 30 min at room temperature to open cell membranes for RNA probe penetration. After one 20 min rinse in 4% paraformaldehyde and five rinses in PBS Tween-20 (PBT), the embryos were prehybridized for 2-5 h at 70ºC with hybridization mix (HM, contents described by Thisse and Thisse [2008]). Subsequently, the prehybridized embryos were hybridized with the synthesized RNA probe (0.25 ng/µL probe in HM) overnight at 70ºC.

In a series of stringency washes to prevent nonspecific probe hybridization, the HM containing probe was gradually changed to 0.2x saline-sodium citrate (SSC) buffer as described elsewhere (Thisse and Thisse 2008). The SSC was then gradually replaced with PBT. Embryos were incubated at room temperature for 3-4 h in blocking buffer (contents previously described by Thisse and Thisse [2008]), and then incubated

overnight at 4ºC with an anti-DIG-alkaline phosphatase antibody (1:10,000 in blocking buffer) (Roche). Finally, the embryos were rinsed six times with PBT and developed using the NBT/BCIP system (Promega). Once the desired staining intensity was reached, the reaction was stopped with PBT containing 1 mM EDTA, and incubated in glycerol overnight at room temperature with gentle agitation (Thisse and Thisse, 2008). The following day, images were captured using a Nikon SMZ-U microscope and Nikon DXM1200 digital camera with Nikon ACT-1 (version 2.7) imaging software. Samples were placed in glycerol for long-term storage at 4°C.

Statistical analyses

Data normality for the QPCR results were assessed using a Kolmogorov-Smirnov test. Data were log-transformed if they did not fit a normal distribution. Parametric data were analyzed using a one-way analysis of variance followed by Duncan's multiple comparison test to determine significant differences among treatments within each time point. Nonparametric data were assessed using a Kruskal-Wallis test followed by Dunn's post-hoc analysis. Differences among treatments were considered significant at $p < 0.05$. Statistical analyses were conducted using Statistica 10 (StatSoft, Inc.) and GraphPad Prism v. 5.02 (GraphPad Software).

Results

During the ER agonist range-finder experiment, mean (SD, n=4-6) measured concentrations of EE2 were 0.9 (0.4), 4.4 (0.5), and 42.1 (7.0) ng/L, and E1 concentrations were 9.3 (2.4), 176 (13), and 1,910 (91.8) ng/L. Control water samples

contained no detectable EE2 (<0.2 ng/L) or E1 (< 0.2 ng/L). During the 6 d E1 time-course study, mean (SD, n=16) measured concentrations of E1 were 17.3 (3.00) and 2,120 (248) ng/L. Matrix spikes for 18 ng/L (n = 6) and 1800 ng/L (n = 5) treatments were 121% (19.8%) and 113% (8.1%) of spiked concentrations, respectively. No E1 (<1.5 ng/L) was detected in the control water for the time-course study.

In the range-finder study, the overall mortality rate was less than 1%. The overall mean (± SD) mortality rate was 30.1 (±8.6) % in the 6 d E1 time-course study, but the mortality was not treatment related, with 31.9 (±7.6), 30.3 (±8.4), and 28.0 (±8.6) % occurring in the control, low, and high treatment groups, respectively. Most of the mortality occurred during the first day, with 26.2 (±8.1) % mortality occurring during the first 24 h. There were no visible differences in phenotypic developmental stages among treatments at d 3 and d 6. All embryos had hatched by d 6 of the exposure.

QPCR

In the initial range-finding study, *vtg* was up-regulated with exposure to 50 ng EE2/L for 48 h (2.4-fold) and 96 h (108-fold). No significant differences in *vtg* expression were observed in fathead minnow embryos exposed to E1 for 48 h. After 96 h of exposure to 1,800 ng E1/L, *vtg* transcript abundance was up-regulated (183-fold) in larval fathead minnows. Consequently, 1,800 ng E1/L was chosen as the high treatment in the subsequent E1 time-course exposure.

In the time-course study, relative mRNA transcript abundance of *esr1* and *cyp19b* was significantly up-regulated in fathead minnow embryos/larvae exposed to 1,800 ng E1/L for 3 and 6 days, with comparatively higher levels of *esr1* observed on day 6 than

day 3 (Figure 14A & C). Larval *vtg* mRNA transcript abundance was also up-regulated after 6 d of exposure to 1,800 ng E1/L, but was not significantly affected after 3 d of exposure (Figure 14B). There were no significant effects on *vtg*, *esr1*, or *cyp19b* mRNA transcript abundance with exposure to the low E1 treatment (18 ng/L).

WISH

Staining of the zebrafish positive control *pax2a* gene was apparent, predominantly in the optic stalk, midbrain-hindbrain boundary, hindbrain neurons, thyroid primordium, and proctodeum regions, as expected based on previous studies (e.g., (Thisse et al. 2001)), suggesting that the assay was effectively working (Figure 15). Compared to the zebrafish mounts, all of the fathead minnow preparations had a slight pink background staining; however, this did not seem to interfere with detection of the probe genes. In the fathead minnow WISH assays, prominent *vtg* staining was observed in the abdomen area (presumably liver) and pectoral fins in larvae exposed to 1,800 ng E1/L for 6 d (Figure 16). No staining was apparent for *esr1* in embryos exposed to 1,800 ng E1/L for 3 d. Faint *esr1* staining was observed in the abdominal region of fathead minnow larvae exposed to 1,800 ng E1/L for 6 d (Figure 17) despite slight pink background staining.

Discussion

Mortality and developmental staging

The relatively high mortality rate in the definitive E1 time-course study (~30%) compared to the range-finder study (<1%) was probably due to initiation of the study with younger embryos. The definitive E1 study was started with < 8 hpf embryos, while

the range-finder study was initiated with embryos that were approximately 24 hpf. It is likely that the younger embryos were more sensitive to handling during test initiation. For future work, it may be worthwhile to begin studies with slightly more developed embryos (>24 hpf), especially if events occurring during the first 24 h of development are not the primary focus of the particular study.

There were no apparent differences in embryonic phenotypic developmental stages among the treatments, suggesting that E1 exposure did not substantially impact morphological development of the exposed organisms. Further, E1 exposure did not affect hatching success, as 100% of the viable organisms hatched by d 6.

Gene expression: QPCR

Transcripts for three estrogen-inducible genes, *vtg*, *esr1*, and *cyp19b*, were up-regulated after the 6 d exposure to 1,800 ng E1/L. After 3 d of exposure to E1, relative transcript abundance of *esr1* and *cyp19b* was significantly up-regulated, while *vtg* mRNA expression was not significantly affected. The comparative delay in *vtg* induction is perhaps not surprising given that a functional ER(s) is required for *vtg* to be induced (Sumpter and Jobling 1995). In this study, significant up-regulation of *esr1* mRNA transcripts was observed at d 3, before significant up-regulation of *vtg* could be established at d 6. While there were significant effects on gene expression in fathead minnow embryos exposed to 1,800 ng E1/L, there were no significant effects on *esr1*, *vtg*, and *cyp19b* transcript abundance in those exposed to the more environmentally relevant E1 concentration of 18 ng/L (Belfroid et al. 1999, Servos et al. 2005, Miege et al. 2009).

Gene expression: WISH

For genes with relatively low levels of expression in control fathead minnow embryos and larvae (e.g., *esr1*, *vtg*), the degree of up-regulation with chemical exposure, not surprisingly, appears to influence the intensity of staining in WISH. For example, based on QPCR data, *vtg* mRNA transcript abundance was 3-4 orders of magnitude higher in the high E1 (1,800 ng/L) treatment compared to controls at d 6, and prominent staining was subsequently observed in WISH. For *esr1*, there was a 1-2 orders of magnitude difference in expression between the high E1 treatment and controls on d 6, as indicated by QPCR results, and *esr1* staining in the WISH assay was much less pronounced compared to *vtg*. Additionally, while *esr1* stained at d 6, no staining was observed at d 3 despite significant up-regulation of *esr1* transcript abundance as indicated by QPCR. While the lack of *esr1* staining for the d 3 embryos is likely due to relatively low expression levels, there was also slight pink background staining in the embryos, which could potentially hinder visualization of faint gene-specific staining. Future work with the WISH assay in fathead minnows should focus on reducing background staining.

While *vtg* and *esr1* were chosen for further examination in WISH based on the robust QPCR results from d 6, it would also be worthwhile to examine *cyp19b* in WISH. Expression of *cyp19b* mRNA transcripts was significantly increased at both d 3 and 6 with exposure to 1,800 ng E1/L, but by less than an order of magnitude. Further examination of *cyp19b* in the WISH assay could potentially allow for determination of the required degree of expression in order for a gene to be easily visualized with staining in WISH.

Implications

This study with the known, and environmentally highly-relevant, ER agonist estrone that featured examination of expression of three estrogen-inducible genes showed that: 1) the estrogen-responsive gene transcripts *esr1*, *vtg*, and *cyp19b* can be up-regulated at very early-life stages in the fathead minnow, 2) WISH methods developed for zebrafish can also be applied successfully to fathead minnows, and 3) WISH has potential to be a useful tool for toxicological studies pertaining to early-life stage development in the fathead minnow. While QPCR data are informative regarding relative quantification of gene transcript abundance, inferences relative to spatial distribution of gene expression are unattainable when examining pooled whole embryos with QPCR. Whole mount in situ hybridization assays allow for visualization of spatial gene expression patterns in individual whole embryos. Using WISH (as a complement to QPCR) to determine which specific tissues are targeted during chemical exposure has potential to lend insight relative to those biological pathways perturbed by chemicals of interest. Consequently, WISH may be particularly useful for further investigation of AOP development in fathead minnows, notably identification of tissue-specific alterations in key molecular initiating events (in this study ER activation in the liver) that may lead to subsequent effects on early-life stage development.

Chapter 4: Conclusions

Chemicals of emerging concern (CECs) are routinely detected in wastewater treatment plant (WWTP) effluent discharges and surrounding surface waters. Of particular concern are endocrine-disrupting compounds (EDCs) that can affect various points within the hypothalamic-pituitary-gonadal axis in exposed organisms. While some of these chemicals have been tested individually in the laboratory, many untested EDCs exist in the environment in the form of complex mixtures, such as WWTP effluents. While traditional whole effluent toxicity tests are informative relative to apical adverse effects (e.g., growth, survival, reproduction) (US Environmental Protection Agency, 2002b), these tests generally do not examine the potential molecular-level effects WWTP effluents might have on exposed organisms. Information pertaining to molecular-level effects of chemical exposure can aid in predictive approaches for chemical risk assessment. Specifically, the adverse outcome pathway (AOP) framework is increasingly being used to decipher causal linkages between chemical molecular initiating events (MIEs) and observed adverse outcomes at the organismal level (Ankley et al. 2010).

For my thesis research, a variety of pathway-based approaches were utilized to examine the potential effects of a complex WWTP effluent, known to be estrogenic, on reproductive endocrine function in fish. In Chapter 2, I describe results of a 21-d reproduction test with fathead minnows (*Pimephales promelas*) exposed to a "typical" effluent, that examined a number of apical and mechanistic endpoints in an attempt to understand the potential adverse effects of estrogens. In Chapter 2, I also used a predictive chemical-gene interaction modeling approach designed to identify unknown pathways that may be perturbed by chemicals in the effluent. Finally, in Chapter 3, I

describe the development of a whole-mount in situ hybridization (WISH) assay for embryonic/larval fathead minnows that could support both pathway-based monitoring of complex environmental samples such as effluents, and AOP development.

The effluent study (Chapter 2) featured a historically estrogenic WWTP effluent, that was tested in a novel on-site system with reproductively mature fathead minnows for 21 d. Cumulative fecundity was significantly impaired in fish exposed to 100% effluent compared to controls. Potential causes of the adverse effect on reproduction were examined using an integrative approach including analytical chemistry, *in vitro* bioassays, and *in vivo* pathway-based approaches (e.g., gene expression). By using this integrative approach, conclusions could be drawn suggesting that, despite the estrogenic nature of the effluent, estrogen receptor agonists were unlikely the primary driver of impaired reproduction. Furthermore, using the list of chemicals detected in the effluent, construction of a chemical-gene interaction network provided insights as to possible other chemicals that may have played a role in the observed biological effects. Overall, results from this study increase the understanding of the significance of pathway-based effects with regard to predicting adverse reproductive outcomes.

In some instances, early-life stage fish can be more sensitive to chemical exposure than adult fish (McKim 1977). Therefore, determining AOPs for early-life stages is crucial. To determine chemical effects and/or mechanisms of action in exposed fish embryos and larvae, WISH assays paired with quantitative polymerase chain reaction (QPCR) assays hold excellent promise. Whole mount in situ hybridization has been commonly used in zebrafish (*Danio rerio*) developmental research, and this technique was adapted to fathead minnows in the present study (Chapter 3) as a tool for AOP

development and monitoring. As a proof of concept, fathead minnow embryos were exposed to the known estrogen-receptor agonist, estrone, which is commonly found in WWTP effluents, including the one tested in Chapter 2. Estrone significantly up-regulated whole embryonic estrogen-receptor-α (*esr1*) transcript abundance as determined by QPCR. Subsequently, WISH assays revealed spatial distribution of *esr1* in the liver region, suggesting that estrone is activating hepatic estrogen receptors in exposed embryos. This type of information relative to spatial distribution of gene expression is important in determining the biological pathways that may be impacted by targeted chemicals and development of associated AOPs.

In the past, *in vivo* chemical testing strategies have primarily focused on assessing largely apical endpoints. While this information is important relative to risk assessment, having knowledge of the MIEs and key events impacted among several levels of biological organization that lead to those apical endpoints is critical in prioritizing chemical testing. Specifically, by gaining molecular-level information of chemical impacts, chemicals can start to be grouped/classified by MIEs in order to better predict how these chemicals might elicit adverse apical outcomes. Molecular initiating events are not chemical specific, and therefore, several chemicals can have the same MIE(s) (Villeneuve et al. 2014a). Additionally, gaining molecular-level/mechanistic understanding of chemical effects is important in predicting chemical impacts across species (Garcia-Reyero 2015). Overall, there is need for additional mechanistic information in current and future toxicological research, both with single chemicals and complex mixtures.

Table 1. Mean (±SD, n=4 tanks per treatment) flow rates and water quality parameters of wastewater treatment plant effluent from treatment tanks over a 21 d exposure period.

Treatment	Flow rate (mL/min)	Temperature (°C)	pH	Conductivity (µmhos)	Alkalinity[a] (mg/L $CaCO_3$)	Hardness[a] (mg/L $CaCO_3$)
Control	42 (1.9)	25.3 (0.25)	7.39 (0.22)	105.2 (0.95)	42.3 (4.86)	45.0 (3.83)
5% Effluent	41 (4.5)	25.4 (0.24)	7.44 (0.19)	200.2 (16.25)	62.3 (2.35)	62.6 (9.19)
20% Effluent	41 (6.7)	25.3 (0.28)	7.63 (0.18)	511.8 (101.17)	99.7 (21.09)	101.9 (22.08)
100% Effluent	39 (4.6)	25.2 (0.26)	8.02 (0.29)	1605 (129.7)	299 (56.7)	NA[b]

[a] Measurements taken from each treatment mixing chamber on 7 days throughout the exposure (n=7 measurements per treatment).

[b] No observable color change during titration due to sample coloration. Hardness measurement could not be obtained for 100% effluent.

Table 2. Concentrations (µg/L) of steroid hormones, wastewater indicators, and pharmaceuticals measured in dilution Lake Superior water (LSW) and 100% wastewater treatment plant effluent 7 d composite samples. All chemical analyses were conducted at the U.S. Geological Survey National Water Quality Laboratory (Denver, CO). "E" indicates concentration estimate. * Indicates detection in corresponding laboratory blank sample. "NA"= not analyzed. Green text indicates analytes that were detected in LSW samples. Red text denotes the 48 analytes detected in all three effluent samples (samples with detection in laboratory blanks (*) excluded).

Chemical (µg/L)	USGS Analytical Schedule	Reporting Limit	LSW Control Acclimation 5/27/2014	LSW Control Week 2 6/10/2014	100% Effluent Week 1 6/3/2014	100% Effluent Week 2 6/10/2014	100% Effluent Week 3 6/17/2014
1,4-dichlorobenzene	4433	0.08	<0.08	<0.08	<0.08	E 0.007	0.015
1,7-dimethylxanthine	2440	0.0877	< 0.0877	< 0.0877	E 0.949*	E 0.745	0.82
10-hydroxy-amitriptyline	2440	0.0083	<0.0083	<0.0083	E 0.0125	E 0.0127	0.012
11-ketotestosterone	4434	0.002	<0.002	<0.002	<0.002	<0.002	<0.002
17-alpha-estradiol	4434	0.0008	0.00143	<0.0008	<0.0008	<0.0008	<0.0008
17-alpha-ethynylestradiol	4434	0.0008	<0.00195*	<0.0008	<0.0008	<0.0008	<0.0008
17-beta-estradiol	4434	0.0008	0.00137	<0.0008	<0.0008	<0.0008	<0.0008
1-methylnaphthalene	4433	0.04	<0.04	<0.04	<0.04	<0.04	<0.04
2,2',4,4'-tetrabromodiphenyl ether	4433	0.04	<0.04	<0.04	<0.04	<0.04	<0.04
2,6-dimethylnaphthalene	4433	0.04	<0.04	<0.04	<0.04	<0.04	<0.04
2-methylnaphthalene	4433	0.04	<0.04	<0.04	<0.04	<0.04	<0.050
3,4-dichlorophenyl isocyanate	4433	0.32	<0.32	<0.32	E 0.068	<0.32	<0.32
3-beta-coprostanol	4434	0.2	<0.200	<0.200	8.58	9.26	6.99
3-methyl-1h-indole	4433	0.04	<0.04	<0.04	<0.04	<0.04	<0.04
3-tert-butyl-4-hydroxyanisole	4433	0.16	<0.16	<0.16	<0.336	<0.309	<0.462
4-androstene-3,17-dione	4434	0.0008	<0.0008	<0.0008	<0.0008	0.00134	0.00168
4-cumylphenol	4433	0.04	<0.04	<0.04	<0.04	<0.04	<0.04
4-nonylphenol diethoxylate-total (NP2EO)	4433	1.6	<1.60	<1.60	E 0.82	<1.60	<1.60

Chemical (µg/L)	USGS Analytical Schedule	Reporting Limit	LSW Control Acclimation 5/27/2014	LSW Control Week 2 6/10/2014	100% Effluent Week 1 6/3/2014	100% Effluent Week 2 6/10/2014	100% Effluent Week 3 6/17/2014
4-nonylphenol monoethoxylate - total (NP1EO)	4433	1.6	<1.6	<1.6	<1.6	<1.6	<1.6
4-nonylphenol total (NP)	4433	1.6	E 0.17*	E 0.16*	<1.6	<1.6	<1.6
4-n-octylphenol (OP)	4433	0.02	<0.02	<0.02	<0.02	<0.02	<0.02
4-octylphenol diethoxylate (OP2EO)	4433	0.2	<0.20	<0.20	E 0.14	<0.20	<0.20
4-octylphenol monoethoxylate (OP1EO)	4433	0.6	<0.60	<0.60	E 0.12*	<0.60	<0.60
4-tert-octylphenol (TOP)	4433	0.4	<0.4	<0.4	<0.4	<0.4	<0.4
Abacavir	2440	0.00821	< 0.00821	< 0.00821	< 0.00821	< 0.00821	< 0.00821
Acetaminophen	2440	0.00713	<0.080	<0.080	0.0247	<0.080	<0.0116
Acetophenone	4433	0.4	<0.4	<0.4	0.151*	<0.4	0.343*
Acetyl-hexamethyl-tetrahydro-naphthalene (AHTN)	4433	0.04	<0.04	<0.04	0.022	<0.04	<0.04
Acyclovir	2440	0.0222	<0.0222	<0.0222	0.427	0.141	0.319
Albuterol	2440	0.0067	<0.0067	<0.0067	0.0094	0.0104	0.0103
Alprazolam	2440	0.0213	<0.0213	<0.0213	<0.0213	<0.0213	<0.0213
Amitriptyline	2440	0.0372	<0.080	<0.0372	<0.0372	<0.0372	<0.0372
Amphetamine	2440	0.00814	<0.080	<0.010	E 0.00844	<0.010	<0.00814
Anthracene	4433	0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Anthraquinone	4433	0.04	<0.04	<0.04	E 0.191	E 0.099	E 0.189
Antipyrine	2440	0.116	<0.116	<0.116	<0.116	<0.116	<0.116
Atenolol	2440	0.0133	<0.080	<0.080	0.918	E 1.38	1.13
Atrazine	2440	0.0194	<0.0194	0.00428	<0.0194	0.0167	0.00975*
Benzo[a]pyrene	4433	0.02	<0.02	<0.02	<0.02	<0.02	<0.02

Chemical (µg/L)	USGS Analytical Schedule	Reporting Limit	LSW Control Acclimation 5/27/2014	LSW Control Week 2 6/10/2014	100% Effluent Week 1 6/3/2014	100% Effluent Week 2 6/10/2014	100% Effluent Week 3 6/17/2014
Benzophenone	4433	0.08	<0.08	0.03*	0.54	E 0.46	1.27
Benztropine	2440	0.024	<0.024	<0.024	<0.024	<0.024	<0.024
Betamethasone	2440	0.114	<0.114	<0.114	<0.114	<0.114	<0.114
Beta-sitosterol	4433	4.8	<4.8	<4.8	E 0.81	E 0.55*	E 0.87
Beta-stigmastanol	4433	3.4	<3.4	<3.4	E 0.28	<3.4	<3.4
Bis-(2-ethylhexyl) phthalate	4433	2	<34.3*	<2.27*	<2	<2.88*	<2
Bisphenol a (BPA)	4434	0.04	0.063*	<0.045*	1.23	E 0.207	0.61
Bromacil	4433	0.16	<0.16	<0.16	<0.16	<0.16	<0.16
Bromoform	4433	0.16	<0.16	<0.16	0.015	<0.16	0.013
Buproprion	2440	0.0178	<0.0178	<0.0178	0.0896	0.0874	0.0913
Caffeine	4433	0.08	<0.08	<0.08	2.00	E 0.863	1.14
Camphor	4433	0.08	<0.08	<0.08	<1.89	<1.30	<1.69
Carbamazepine	2440	0.011	<0.011	<0.011	0.135	0.243	0.199
Carbaryl	4433	0.06	<0.06	<0.06	<0.06	<0.06	<0.06
Carbazole	4433	0.02	<0.020	<0.020	<0.020	<0.020	<0.020
Carisoprodol	2440	0.0125	<0.080	<0.0125	<0.0125	<0.0125	0.00322
Chlorpheniramine	2440	0.00468	<0.00468	<0.00468	<0.00468	<0.00468	0.00219
Chlorpyrifos	4433	0.12	<0.12	<0.12	<0.12	<0.12	<0.12
Cholesterol	4434	0.2	<0.200	<0.200	7.09	7.89	28.5
Cimetidine	2440	0.0278	<0.080	<0.080	E 0.0403	E 0.0574	E 0.0739
Cis-androsterone	4434	0.0008	<0.0008	<0.0008	<0.0008	<0.0008	<0.0008
Citalopram	2440	0.00658	<0.00658	<0.00658	0.0279	E 0.0309	0.0779
Clonidine	2440	0.0608	<0.080	<0.0608	<0.080	<0.0608	<0.080
Codeine	2440	0.0883	<0.0883	<0.0883	0.0452	E 0.0415	0.0528

Chemical (µg/L)	USGS Analytical Schedule	Reporting Limit	LSW Control Acclimation 5/27/2014	LSW Control Week 2 6/10/2014	100% Effluent Week 1 6/3/2014	100% Effluent Week 2 6/10/2014	100% Effluent Week 3 6/17/2014
Cotinine	2440	0.00637	<0.080	<0.00637	1.05	0.814	0.616
Dehydronifedipine	2440	0.0245	<0.0245	<0.0245	<0.0245	<0.0245	<0.0245
Desvenlafaxine	2440	0.00749	<0.00749	<0.00749	0.788	1.05	0.869
Dextromethorphan	2440	0.0082	<0.0082	<0.0082	0.0145	0.0164	0.0142
Diazepam	2440	0.00224	<0.00224	<0.004	<0.00224	<0.004	<0.004
Diazinon	4433	0.32	<0.32	<0.32	<0.32	<0.32	<0.32
Dichlorvos	4433	0.08	<0.08	<0.08	<0.08	<0.08	<0.08
Diethyl phthalate	4433	0.4	<0.4	<0.4	<0.4	<0.4	<0.4
Dihydrotestosterone	4434	0.004	<0.004	<0.004	<0.004	<0.004	<0.004
Diltiazem	2440	0.0102	<0.0102	<0.0102	0.0319	0.0411	0.0378
Diphenhydramine	2440	0.00579	<0.00579	<0.00579	0.358	0.486	0.334
d-limonene	4433	0.16	<0.16	<0.16	<0.16	<0.16	<0.238
Duloxetine	2440	0.0366	<0.080	<0.080	<0.0366	<0.080	<0.0366
Epitestosterone	4434	0.002	<0.002	<0.002	<0.002	<0.002	<0.002
Equilenin	4434	0.002	<0.002	<0.002	<0.002	<0.002	<0.002
Equilin	4434	0.008	<0.008	<0.008	<0.008	<0.008	<0.008
Erythromycin	2440	0.0531	<0.080	<0.0531	0.0214	0.029	0.023
Estriol	4434	0.002	<0.002	<0.002	<0.002	<0.002	<0.002
Estrone	4434	0.0008	<0.0008	<0.0008	0.00496	0.00337	0.0448
Ezetimibe	2440	0.0635	<0.080	<0.080	<0.200	<0.080	<0.200
Fadrozole	2440	0.00732	<0.00732	<0.00732	<0.00732	<0.00732	<0.00732
Famotidine	2440	0.0107	<0.080	<0.080	E 0.0177	<0.080	<0.0147
Fenofibrate	2440	0.00628	<0.00628	<0.080	<0.080	<0.080	<0.00628
Fexofenadine	2440	0.0199	<0.0199	<0.0199	0.917	1.29	0.992

Chemical (µg/L)	USGS Analytical Schedule	Reporting Limit	LSW Control Acclimation 5/27/2014	LSW Control Week 2 6/10/2014	100% Effluent Week 1 6/3/2014	100% Effluent Week 2 6/10/2014	100% Effluent Week 3 6/17/2014
Fluconazole	2440	0.071	<0.080	<0.080	0.0857	E 0.131	0.107
Fluoranthene	2440	0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Fluoxetine	2440	0.0269	<0.080	<0.0269	<0.080	<0.0269	<0.0269
Fluticasone	2440	0.00462	<0.080	<0.020	<0.080	<0.020	<0.00462
Fluvoxamine	2440	0.0538	<0.080	<0.080	<0.0538	<0.080	<0.0538
Glipizide	2440	0.148	<0.148	<0.148	<0.148	<0.148	<0.148
Glyburide	2440	0.00395	<0.00395	<0.004	<0.00395	0.00666*	0.0037
Hexahydrohexamethyl cyclopentabenzopyran (HHCB)	4433	0.04	<0.04	<0.04	0.14	E 0.042	0.085
Hydrocodone	2440	0.0105	<0.080	<0.0105	0.0354	E 0.0549	0.0224
Hydrocortisone	2440	0.147	<0.147	<0.147	<0.147	<0.147	<0.147
Hydroxyzine	2440	0.00743	<0.00743	<0.00743	<0.00743	<0.00743	<0.00743
Iminostilbene	2440	0.145	<0.200	<0.145	<0.145	<0.145	<0.145
Indole	4433	0.04	<0.04	<0.04	<0.04	<0.04	<0.04
Isoborneol	4433	0.09	<0.090	<0.090	<0.171	<0.090	<0.090
Isophorone	4433	0.05	0.015	0.004	0.21	E 0.050	<0.091
Isopropylbenzene (cumene)	4433	0.04	<0.04	<0.04	<0.04	<0.04	<0.04
Isoquinoline	4433	0.8	<0.8	<0.8	<0.8	<0.8	<0.8
Ketoconazole	2440	0.113	<0.113	<0.113	<0.113	<0.113	<0.113
Lamivudine	2440	0.0161	<0.080	<0.0161	0.0377	E 0.020	<0.0161
Lidocaine	2440	0.0152	<0.0152	<0.0152	0.153	0.187	0.12
Loperamide	2440	0.0115	<0.0115	<0.0115	<0.0115	<0.0115	<0.0115
Loratadine	2440	0.00695	< 0.00695	< 0.00695	< 0.00695	0.00118*	< 0.00695
Lorazepam	2440	0.116	<0.200	<0.200	<0.116	<0.200	<0.116

Chemical (µg/L)	USGS Analytical Schedule	Reporting Limit	LSW Control Acclimation 5/27/2014	LSW Control Week 2 6/10/2014	100% Effluent Week 1 6/3/2014	100% Effluent Week 2 6/10/2014	100% Effluent Week 3 6/17/2014
Menthol	4433	0.32	<0.32	<0.32	0.395	<0.32	<0.372
Meprobamate	2440	0.086	<0.086	<0.086	0.0226	<0.086	<0.086
Mestranol	4434	0.0008	<0.0008	<0.0008	<0.0008	<0.0008	<0.0008
Metalaxyl	4433	0.16	<0.16	<0.16	<0.16	<0.16	<0.16
Metaxalone	2440	0.0156	<0.080	<0.080	<0.0185	<0.080	<0.0185
Metformin	2440	0.0131	0.00438	0.00343	7.94	7.87	6.4
Methadone	2440	0.00761	<0.00761	<0.00761	0.0229	E 0.027	0.0196
Methocarbamol	2440	0.00872	<0.00872	<0.00872	0.578	0.94	0.614
Methotrexate	2440	0.0524	<0.080	<0.080	<0.080	<0.080	<0.0524
Methyl salicylate	4433	0.08	<0.08	<0.08	<0.08	<0.08	<0.08
Methyl-1h benzotriazole	4433	0.141	<0.141	0.0655	0.832	0.944	0.634
Metolachlor	4433	0.04	<0.04	<0.04	<0.04	<0.04	<0.04
Metoprolol	2440	0.0275	<0.0275	<0.0275	0.19	0.273	0.223
Morphine	2440	0.014	<0.014	<0.020	<0.0141	<0.020	<0.080
n,n-diethyl-meta-toluamide (DEET)	4433	0.04	<0.04	0.01*	<0.164	<0.116	0.337
Nadolol	2440	0.0808	<0.0808	<0.0808	<0.0808	<0.0808	<0.0808
Naphthalene	4433	0.02	<0.02	<0.02	<0.02	<0.02	<0.02
n-desmethyldiltiazem	2440	0.0124	<0.0124	<0.0124	E 0.00553*	E 0.00912*	E 0.00657*
Nevirapine	2440	0.0151	<0.0151	<0.080	<0.0151	<0.080	<0.0151
Nicotine	2440	0.0578	<0.080	<0.0578	<0.080	<0.0578	<0.0578
Nizatidine	2440	0.019	<0.019	<0.080	<0.020	<0.080	<0.019
Nordiazepam	2440	0.0414	<0.0414	<0.0414	<0.0414	<0.0414	<0.080
Norethindrone	4434	0.0008	<0.0008	<0.0008	<0.0008	<0.0008	<0.0008

Chemical (µg/L)	USGS Analytical Schedule	Reporting Limit	LSW Control Acclimation 5/27/2014	LSW Control Week 2 6/10/2014	100% Effluent Week 1 6/3/2014	100% Effluent Week 2 6/10/2014	100% Effluent Week 3 6/17/2014
Norfluoxetine	2440	0.199	<0.199	<0.199	<0.199	<0.199	<0.199
Norsertraline	2440	0.192	<0.200	<0.192	<0.192	<0.192	<0.192
Norverapamil	2440	0.00858	<0.00858	<0.00858	E 0.00089	<0.00858	E 0.00113
Omeprazole	2440	0.00562	<0.00562	<0.00562	<0.080	<0.00562	<0.00562
Oseltamivir	2440	0.0146	<0.0146	<0.0146	<0.0146	<0.0146	<0.0146
Oxazepam	2440	0.14	<0.200	<0.140	<0.140	<0.140	<0.140
Oxycodone	2440	0.0249	<0.080	<0.0249	0.0331	0.0335	0.0332
Paroxetine	2440	0.0206	<0.0206	<0.0206	<0.0206	<0.0206	<0.0206
p-cresol	4433	0.08	<0.08	<0.08	<0.084	<0.08	<0.08
Penciclovir	2440	0.0402	<0.080	<0.080	<0.124	<0.080	<0.0864
Pentachlorophenol	4433	1.6	<1.60	<1.60	<1.60	<1.60	<1.60
Pentoxifylline	2440	0.00935	<0.00935	<0.00935	<0.0113*	E 0.0109	<0.00935
Phenanthrene	2440	0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Phenazopyridine	2440	0.0133	<0.0133	<0.0133	<0.0133	<0.0133	<0.0133
Phendimetrazine	2440	0.0311	<0.080	<0.080	<0.080	<0.080	<0.080
Phenol	4433	0.16	<0.16	<0.16	<0.16	<0.16	0.118*
Phenytoin	2440	0.188	<0.188	<0.188	0.0969	E 0.105	<0.188
Piperonyl butoxide	2440	0.00307	<0.00307	<0.080	E 0.0579	E 0.102	E 0.0268
Prednisolone	2440	0.15	<0.150	<0.150	<0.150	<0.150	<0.379
Prednisone	2440	0.168	<0.200	< 0.168	< 0.168	< 0.168	< 0.168
Progesterone	4434	0.008	<0.008	<0.008	<0.008	<0.008	<0.008
Promethazine	2440	0.05	<0.080	<0.080	<0.050	<0.080	<0.080
Prometon	4433	0.16	<0.16	<0.16	<0.16	<0.16	<0.16
Propoxyphene	2440	0.0172	<0.0172	<0.0172	<0.080	<0.0172	<0.080

Chemical (µg/L)	USGS Analytical Schedule	Reporting Limit	LSW Control Acclimation 5/27/2014	LSW Control Week 2 6/10/2014	100% Effluent Week 1 6/3/2014	100% Effluent Week 2 6/10/2014	100% Effluent Week 3 6/17/2014
Propranolol	2440	0.0263	<0.0263	<0.0263	0.0384	0.0458	0.0523
Pseudoephedrine	2440	0.0111	<0.0111	<0.0111	0.601	0.85	0.702
Pyrene	4433	0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Quinine	2440	0.0799	<0.080	<0.0799	E 0.0199	<0.0799	E 0.0294
Raloxifene	2440	0.00972	<0.00972	<0.00972	<0.00972	<0.00972	<0.080
Ranitidine	2440	0.192	<0.192	<0.192	E 0.0916	E 0.0318	E 0.0504
Sertraline	2440	0.0162	<0.080	<0.0162	<0.0162	<0.0162	<0.0162
Sitagliptin	2440	0.0973	<0.0973	<0.0973	E 0.144	E 0.196	E 0.163
Sulfadimethoxine	2440	0.0655	<0.0655	<0.0655	<0.0655	<0.0655	<0.0655
Sulfamethizole	2440	0.104	<0.104	<0.104	<0.104	<0.104	<0.104
Sulfamethoxazole	2440	0.0261	<0.080	<0.080	E 0.0277	E 0.037	E 0.0423
Tamoxifen	2440	0.08	<0.080	NA	<0.080	NA	<0.080
Temazepam	2440	0.0184	<0.080	<0.080	<0.080	E 0.0571*	0.045
Testosterone	4434	0.0016	<0.0016	<0.0016	<0.0016	<0.0016	<0.0016
Tetrachloroethylene	4433	0.16	<0.16	<0.16	<0.16	<0.16	<0.16
Theophylline	2440	0.0415	<0.200	<0.200	0.297	<0.238	<0.205
Thiabendazole	2440	0.0041	<0.0041	<0.0041	E 0.00953	0.0124	0.0095
Tiotropium	2440	0.0431	<0.080	<0.080	<0.0431	<0.080	<0.200
Tramadol	2440	0.0151	<0.0151	<0.0151	0.44	0.581	0.523
Trans-diethylstilbestrol	4434	0.0008	0.00103	<0.0008	<0.0008	<0.0008	<0.0008
Tri(2-butoxyethyl) phosphate	4433	0.64	<0.64	<0.64	E 2.16	E 0.778	E 0.786
Tri(2-chloroethyl) phosphate	4433	0.16	<0.16	<0.16	<0.16	<0.16	<0.16
Triamterene	2440	0.00525	<0.080	<0.00525	E 0.0447	E 0.0624	E 0.0633
Tributyl phosphate	4433	0.064	<0.064	<0.064	0.108	<0.064	<0.092

Chemical (µg/L)	USGS Analytical Schedule	Reporting Limit	LSW Control Acclimation 5/27/2014	LSW Control Week 2 6/10/2014	100% Effluent Week 1 6/3/2014	100% Effluent Week 2 6/10/2014	100% Effluent Week 3 6/17/2014
Triclosan	4433	0.32	<0.32	<0.32	0.336	<0.32	<0.32
Triethyl citrate (ethyl citrate)	4433	0.04	<0.04	<0.04	0.336	E 0.202	0.337
Trimethoprim	2440	0.019	<0.080	<0.080	0.15	E 0.185	0.173
Triphenyl phosphate	4433	0.08	0.04	0.03	0.018	E 0.029	0.024
Tris(dichloroisopropyl) phosphate	4433	0.32	<0.32	<0.32	0.123	E 0.112	0.157
Valacyclovir	2440	0.163	<0.163	<0.163	<0.163	<0.163	<0.163
Venlafaxine	2440	0.00448	<0.00448	<0.00448	0.343	0.508	0.374
Verapamil	2440	0.0155	<0.0155	<0.0155	<0.080	<0.0155	0.00292
Warfarin	2440	0.00603	<0.00603	<0.00603	<0.00603	<0.00603	<0.00603

Table 3. Fathead minnow gene specific primers and dual-labeled oligonucleotide probes used for quantitative real-time polymerase chain reaction (QPCR) analyses.

Gene	Accession #	Amplicon size (bp)	Primer/probe[a]	Sequence (5′ → 3′)	Reference
vtg	AF130354	69	vtg-Q-FW	TCACCACATACGCCAAAAAGC	Initial preparation
			vtg-Q-RV	CAAGTCTAAAGCCCGTCTGGTT	
			vtg-Q-PB[b]	TCTAAGCACATTCCTATGGCGGCT	
esr1	AY775183	112	esr1-Q-FW	GGTGTTGATGATCGGCCTCATA	Initial preparation
			esr1-Q-RV	AGCCATCCCCTCGACACAT	
			esr1-Q-PB[b]	TGGAGGTCCATTCATTCACCTGGA	
ar	AY727529	65	ar-Q-FW	ACGCGAGTGTGGCGAGTT	Martinovic et al., 2008a
			ar-Q-RV	TCGCGCTGTCTCCGAAA	
			ar-Q-PB	CGCATGCAGTAGCGGCATCACC	
fshr	EF219401	74	fshr-Q-FW	CCCCATCGTTCTGGACATCT	Villeneuve et al., 2007
			fshr-Q-RV	GAAGCTTAAGGGTCCACAGCAT	
			fshr-Q-PB	ACGTACTGCTGTCCAGACGCTACCAGAAA	
star	DQ60497	63	star-Q-FW	CTTGAACAGCAAACAGATGACCTT	Villeneuve et al., 2007
			star-Q-RV	CTCCCCCATTTGTTCCATGT	
			star-Q-PB	ATGAGGAGCTGGTGGAT	
cyp11a	DQ60498	72	cyp11a-Q-FW	CGACACCCGGACTTGCA	Villeneuve et al., 2007
			cyp11a-Q-RV	CACGTCTCCTTTAGAGGTGATACG	
			cyp11a-Q-PB	CCGTGCGGAAATCTCGGCTGC	

cyp19a1a	AF288755	77	cyp19a1a-Q-FW	TGCTGACACATGCAGAAAAACTC	Villeneuve et al.,
			cyp19a1a-Q-RV	CAGCTCTCCGTGGCTCTGA	2006
			cyp19a1a-Q-PB	CCAGCTCGACTTCACAGCAGAGTTGATATTC	
cyp17	AJ277867	69	cyp17-Q-FW	ACACAAGGTGGATTACAGTGATAACGT	Ankley et al.,
			cyp17-Q-RV	CTGCGTTTGGCCCTCAGA	2007
			cyp17-Q-PB	CAGCGGGACCTCTTGGACGCAC	
cyp1a1	AF232749	64	cyp1a1-Q-FW	TGCAGGGAGAACTGAGAGAGAAG	Ankley et al.,
			cyp1a1-Q-RV	TCCGTTCGGTCCGACAAG	2007
			cyp1a1-Q-PB	CGGAATGGATCGTATGCCGC	
cyp3a	DT142443	70	cyp3a-Q-FW	CCAGCGGGAGGTTAAAGGA	Ankley et al.,
			cyp3a -Q-RV	CCCAGATTCTGAACTAGAATTTGAGA	2007
			cyp3a -Q-PB	TGTTTGGCATCATGAAGGCACA	
pgr	JX012230.1	323	pgr-Q-FW	GGATCAACTCTCTCTGCTTGAC	Initial preparation
			pgr-Q-RV	CAACTTCCAGTCGGTCATCTT	
ugt1a1		273	ugt1a1-Q-FW	GCCTAAGAGAGACGGGATTTG	Initial preparation
			ugt1a1-Q-RV	CGCTGGCAAACAGTTTATGG	
abcc2		263	abcc2-Q-FW	GTGTCTCGATCTCCCATTTACTC	Initial preparation
			abcc2-Q-RV	GACAGTCCAACCAATCCACTAT	
prl		319	prl-Q-FW	GACAAAGACCAAGCCCTGAA	Initial preparation
			prl-Q-RV	GGAAGCAGGACAACAGGAAA	
casp3		261	casp3-Q-FW	CTTCAGATACAGCCTGAACTACC	Initial preparation
			casp3-Q-RV	TGACTGAGCATCACACATACAA	
cyp19b	AJ2778566	127	cyp19b-Q-FW	GCCGTGGAAGAGCAGATAGTACA	Villeneuve et al.,
			cyp19b-Q-RV	CCGGATGGAACCTCAGAGACT	2006

[a]FW = forward primer, RV = reverse primer, PB = probe

[b]Dual-labeled DNA probe with 5' 6-FAM™/ZEN/3' Iowa Black® FQ Quencher (Integrated DNA Technologies)

Table 4. Effects of a 21 d wastewater treatment plant effluent exposure on male (M) and female (F) fathead minnow body weight, gonadosomatic index (GSI), and secondary sex characteristics (mean ± SD, n = 12, except control females n = 11).

		Treatment			
	Sex	Control	5% Effluent	20% Effluent	100% Effluent
Mass (g)	M	2.6 ± 0.4	2.5 ± 0.3	2.5 ± 0.4	2.4 ± 0.3
	F	1.1± 0.2	1.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.1
GSI (%)	M	1.56 ± 0.27	1.29 ± 0.34	1.67 ± 0.43	2.07 ± 0.57*
	F	13.24 ± 2.53	12.88 ± 2.20	13.22 ± 2.91	13.59 ± 3.71
Tubercle score	M	23 ± 5	23 ± 3	23 ± 3	21 ± 2
	F	0	0	0	0

* Significantly different from control ($p < 0.05$).

Table 5. Gonad stage and prevalence and severity of histopathological findings in male fathead minnows exposed to wastewater treatment plant effluent for 21 d.

Treatment (% wastewater effluent)		**0%**	**5%**	**20%**	**100%**
Number of testes examined		**11**	**11**	**12**	**11**
Interstitial cell hypertrophy/hyperplasia		4	6	3	7
(Minimal)	Grade 1	3	5	3	4
(Mild)	Grade 2	1	1	-	2
(Moderate)	Grade 3	-	-	-	1
(Severe)	Grade 4	-	-	-	-
Gonad stage	Stage 1	4	-	-	-
	Stage 2	7	11	12	11
	Stage 3	-	-	-	-
	Stage 4	-	-	-	-

Table 6. Gonad stage and prevalence and severity of histopathological findings in female fathead minnows exposed to wastewater treatment plant effluent for 21 d.

Treatment (% wastewater effluent)		**0%**	**5%**	**20%**	**100%**
Number of ovaries examined		**11**	**12**	**12**	**11**
Increased post-ovulatory follicles		3	2	3	3
(Minimal)	Grade 1	2	-	2	2
(Mild)	Grade 2	1	1	1	1
(Moderate)	Grade 3	-	1	-	-
(Severe)	Grade 4	-	-	-	-
Increased oocyte atresia		3	0	1	1
(Minimal)	Grade 1	1	-	1	-
(Mild)	Grade 2	2	-	-	-
(Moderate)	Grade 3	-	-	-	1
(Severe)	Grade 4	-	-	-	-
Gonad stage	Stage 1	-	-	-	-
	Stage 2	6	3	4	5
	Stage 3	5	9	8	6
	Stage 4	-	-	-	-

Table 7. Gene list with the corresponding number of chemicals detected in the effluent that are known to interact with each gene. Output was generated from the chemical-gene interaction network created using Cytoscape v 3.2.0. Bolded genes were selected for QPCR based on the chemical-gene interaction network analysis.

Gene	# of Chemical Interactions
CYP3A4	32
ABCB1	21
CYP1A2	20
CYP2D6	18
ESR1	16
CYP2B6	15
CYP3A5	15
CASP3	15
CYP2C19	14
CYP2C9	12
CAT	11
TNF	11
AR	10
CYP1A1	10
INS	9
CYP2E1	8
SLC6A4	8
PGR	8
FOS	8
CYP17A1	8
CYP2C8	7
SLC6A2	7
ESR2	7
CYP11A1	7
IL6	7
SLC22A1	7
REN	6
OPRM1	6
CYP19A1	6
PRL	6
NR1I2	6
AKT1	6
ADRB1	6
HRH1	6

SLC22A8	6
NR1I3	6
GABRG2	6
SLC22A2	6
ACHE	5
SLC6A3	5
ABCG5	5
OPRD1	5
MAPK1	5
MAPK3	5
STAR	5
NOS2	5
HRH2	5
CYP2D7P1	5
UGT1A1	5
CYP2B1	5
MYC	5
GABRA1	5
IL1B	5
HTR2C	5
ABCG2	5
ADRB2	5
CASP9	5
ALB	5
MAPK8	5
OPRK1	5
BCL2	5
ABCC2	4
BAX	4
ABCG8	4
LHB	4
PPIG	4
ABCA1	4
KCNH2	4
UGT2B7	4
CYP1B1	4
ADRA1A	4
TRPA1	4
GABRB2	4
MPO	4
IL8	4
CYP2B2	4
AHR	4

CCND1	4
ICAM1	4
CALCA	4
NQO1	4
CDKN1A	3
DRD2	3
BCHE	3
CYP2A6	3
TP53	3
CYP27A1	3
MAOA	3
APOB	3
CCL2	3
CES2	3
PPARG	3
MMP9	3
PPARA	3
CYP3A7	3
ABCC1	3
CYP3A	3
PCNA	3
TRPV1	3
COL1A1	3
PTGS2	3
HTR2A	3
HTR1B	3
CYP2C11	3
SLC22A6	3
SLC47A1	3
SIGMAR1	3
FASN	3
PTGS1	3
DECR1	3
CYP2B10	3
IFNG	3
SLC22A3	3
HMOX1	3
NPY	3
ABCC3	3
PLA2G4A	3

Table 8. Primer sequences used for RNA probe synthesis used in whole-mount in situ hybridization assays.

Gene	Accession #	Amplicon size (bp)	Primer name	Sequence (5′ → 3′)
vtg	AF130354	598	vtg-T7-FW[a]	**[TAATACGACTCACTATAGGG]**CTCTGTCTTGCTGTCCCATAC
			vtg-T3-RV[b]	**[CATTAACCCTCACTAAAGGGAA]**AGCTTGTGGTCAACTCTCAC
esr1	AY775183	703	esr1-T7-FW[a]	**[TAATACGACTCACTATAGGGAGG]**CGAGGTGGTCAGTGCTTTAT
			esr1-T3-RV[b]	**[CATTAACCCTCACTAAAGGGAA]**CAGCGGGACTCGATTCTTAC

[a] Forward primer with T7 promoter sequence added to 5' end.

[b] Reverse primer with T3 promoter sequence added to 5' end.

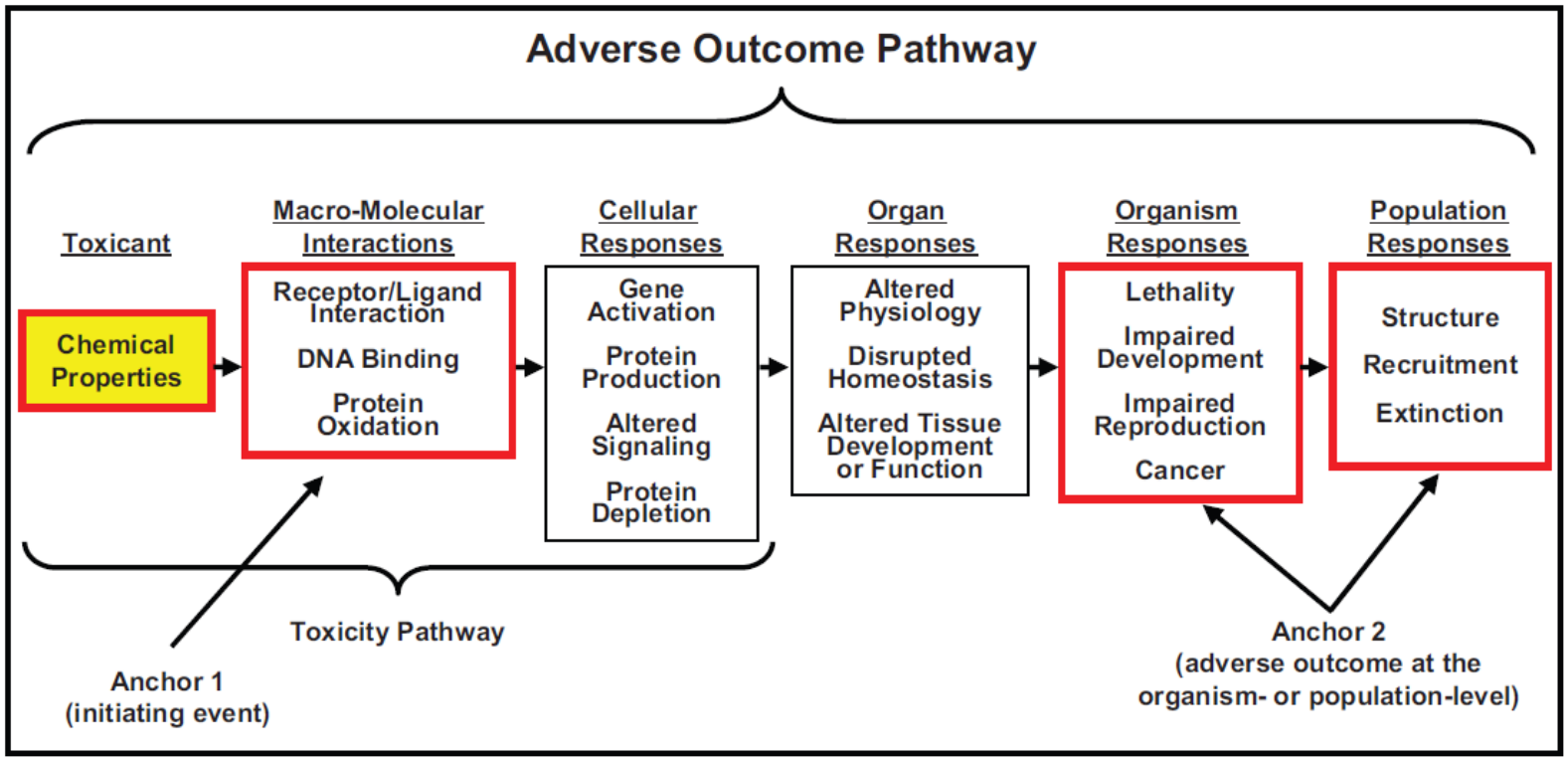

Figure 1. An adverse outcome pathway (AOP) as described by Ankley et al. (2010).

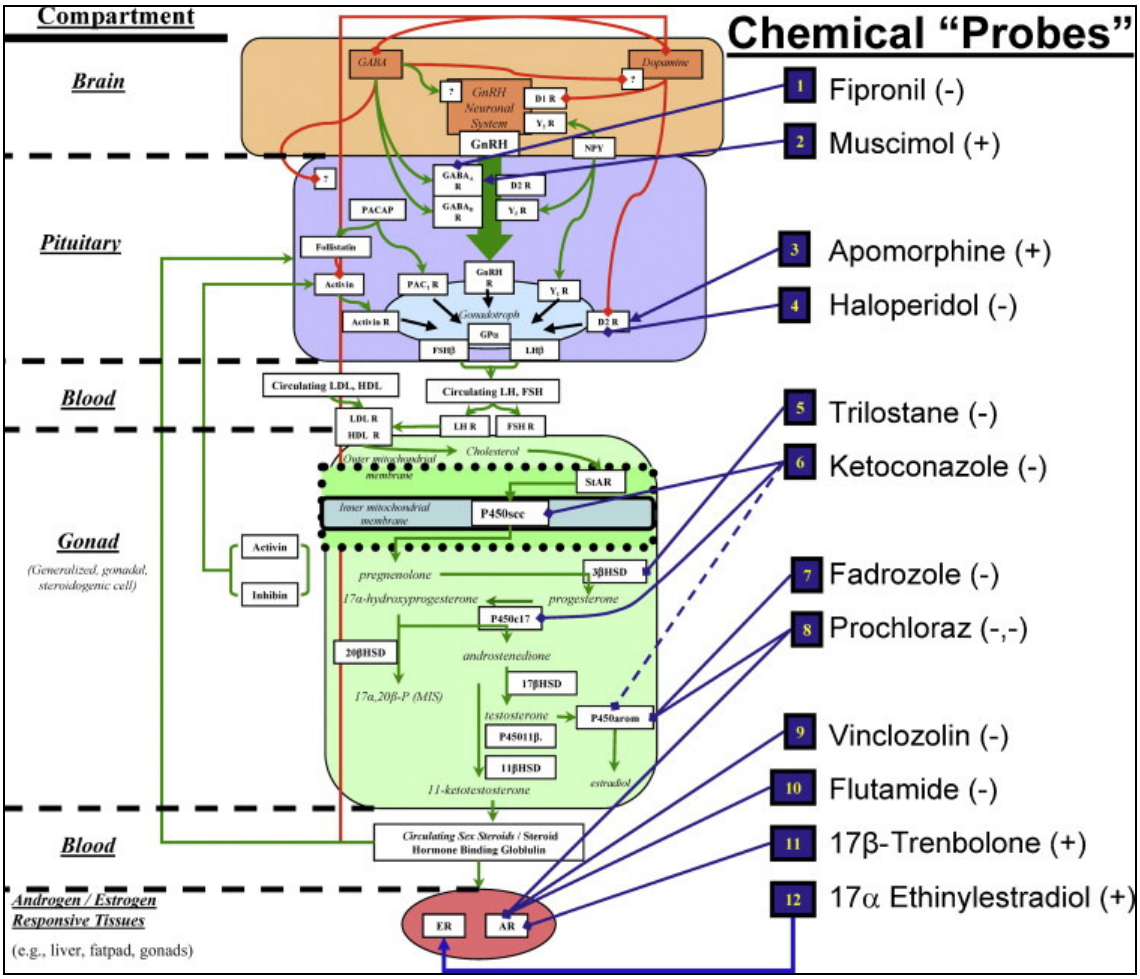

Figure 2. The fathead minnow (*Pimephales promelas*) hypothalamic-pituitary-gonadal (HPG) axis, including examples of experimental chemicals that have differing mechanisms of action (Ankley et al. 2009). The "+" indicates activation and "-" indicates inhibition of the targeted endpoints (enzymes or receptors) of the chemical.

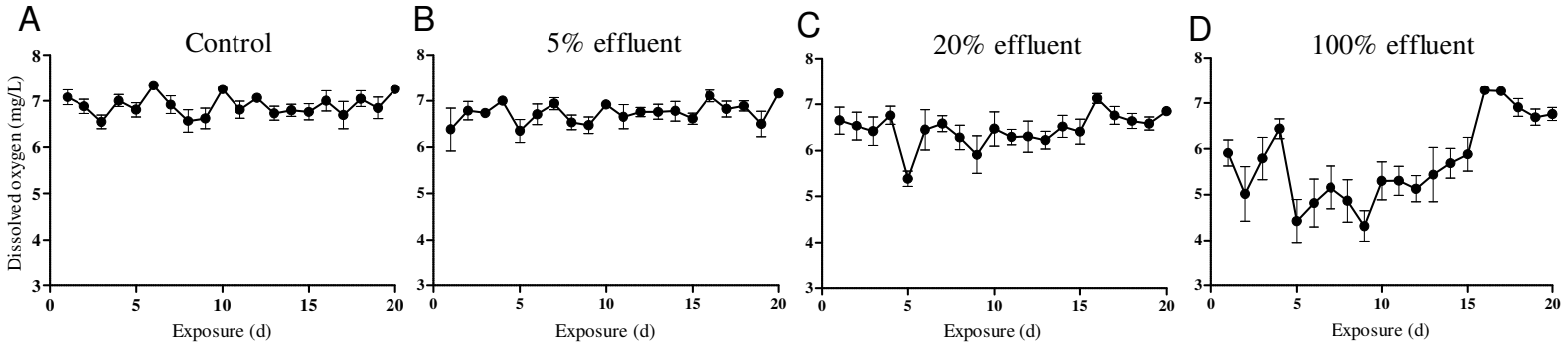

Figure 3. Mean (±SE) dissolved oxygen concentrations over the 21 d wastewater treatment plant effluent exposure measured in treatment tanks: A) Control, B) 5% effluent, C) 20% effluent, and D) 100% effluent. n = 4 tanks per treatment per day.

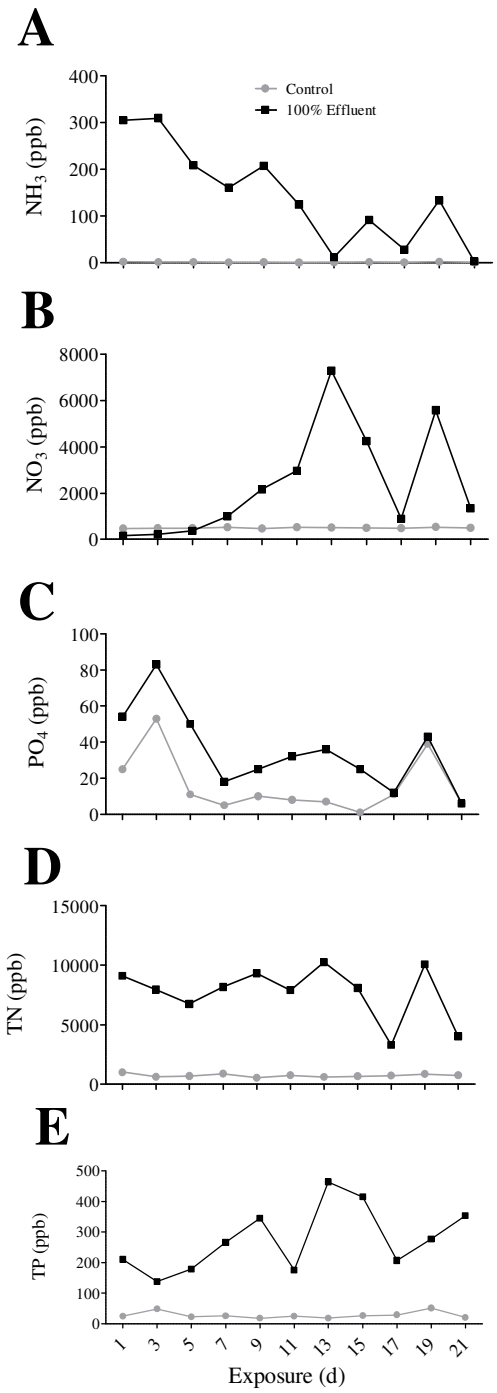

Figure 4. Nutrient concentrations (µg/L) in control water and 100% wastewater treatment plant effluent collected from treatment tanks every other day during a 21 d exposure. (A) Unionized ammonia (NH_3); (B) nitrate (NO_3); (C) phosphate (PO_4); (D) total nitrogen (TN); (E) total phosphorus (TP) expressed as parts per billion (ppb).

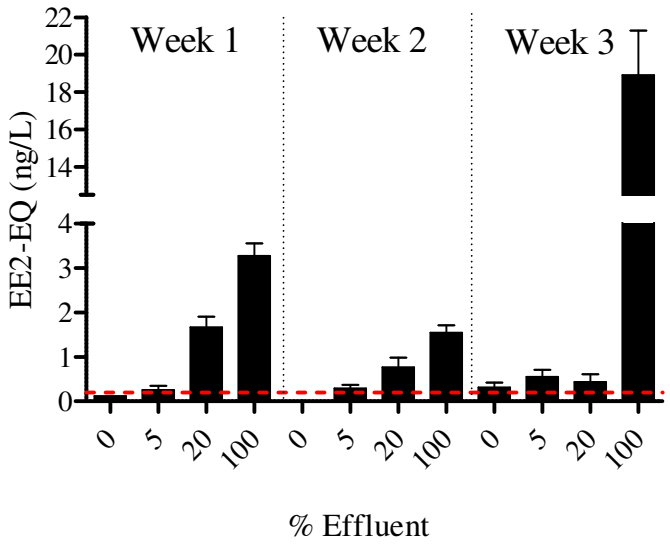

Figure 5. *In vitro* 17α-ethinylestradiol equivalents (EE2-EQs) of 7 d composite samples of control water and dilutions of wastewater treatment plant effluent over a 21 d period determined using the T47D-KBluc assay. Horizontal dotted line indicates the significant response level of three standard deviations above the assay media control mean. Bars represent mean (±SE), with n = 3 assay replicates per sample.

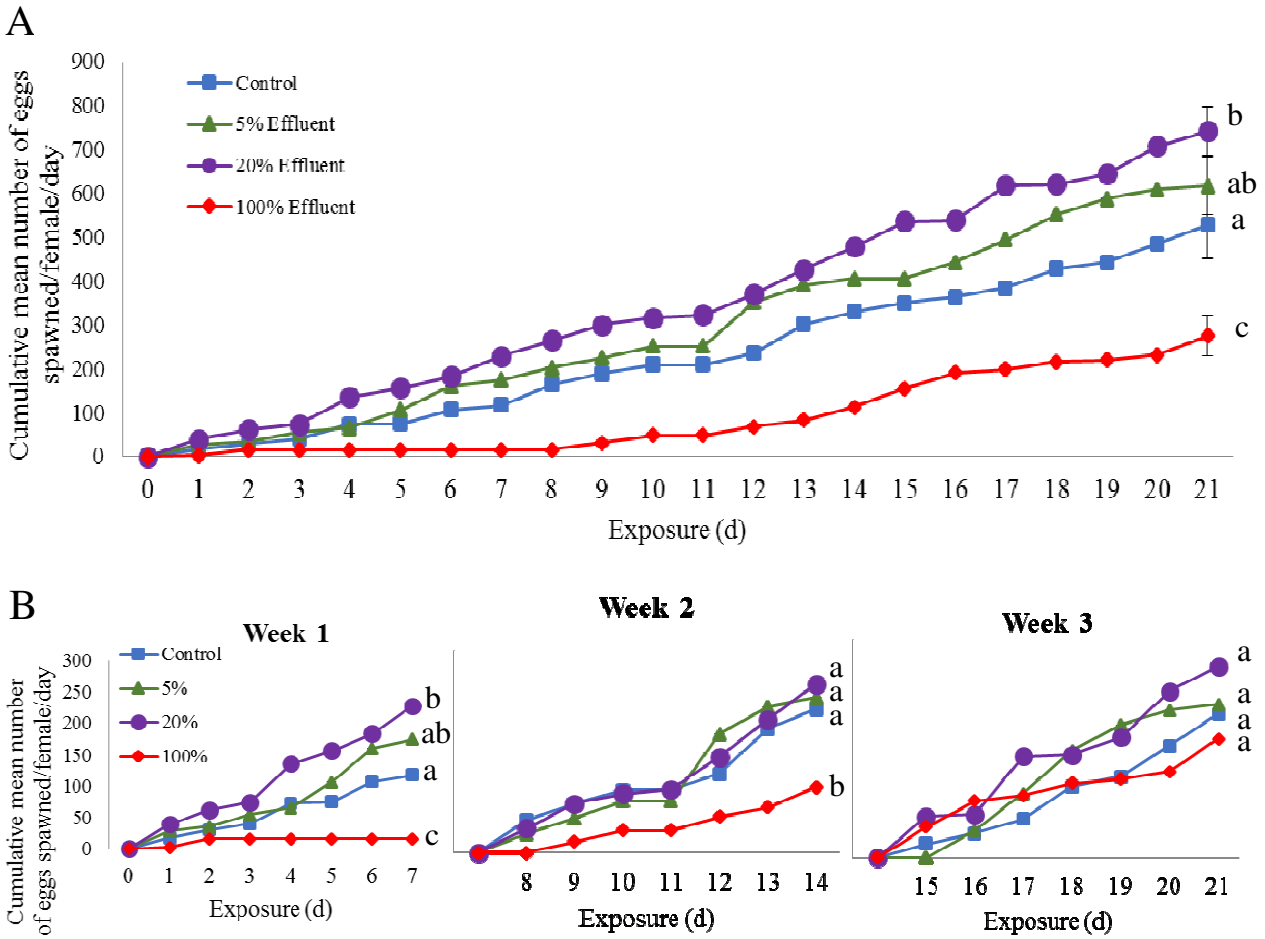

Figure 6. Effects of a 21 d wastewater treatment plant effluent exposure on adult fathead minnow (A) cumulative mean egg production per female per day and (B) weekly cumulative mean egg production per female per day. There were n = 11-12 spawning pairs per treatment over the 21 d exposure. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

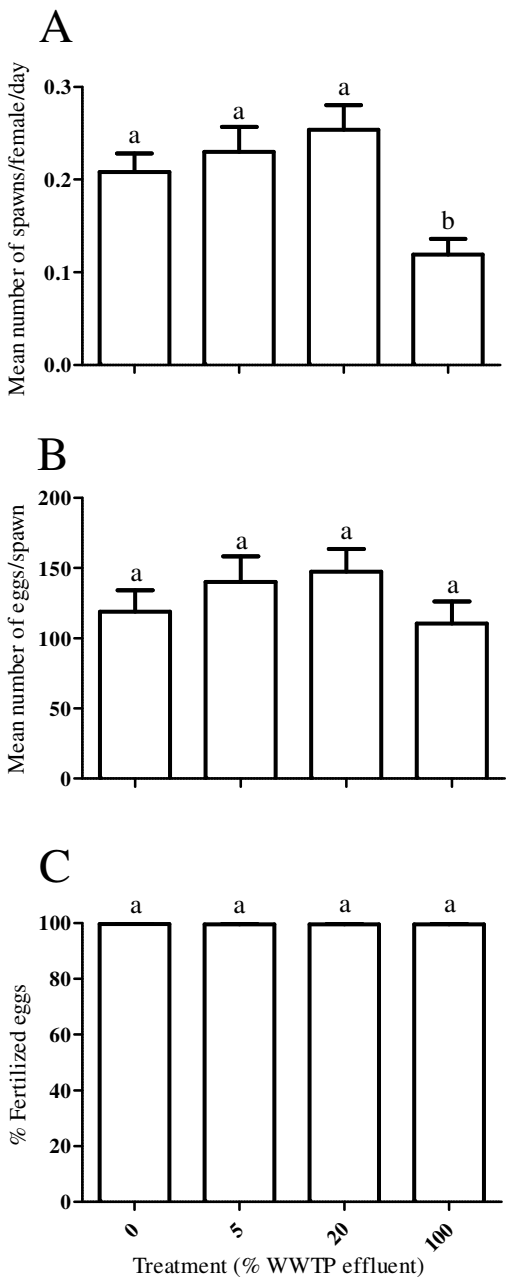

Figure 7. Effects of a 21 d wastewater treatment plant effluent exposure on adult fathead minnow (A) mean number of spawns/female/day, (B) mean number of eggs/spawn, and (C) fertility rate. Bars represent mean (±SE). Letters indicate significant differences between treatments ($p < 0.05$).

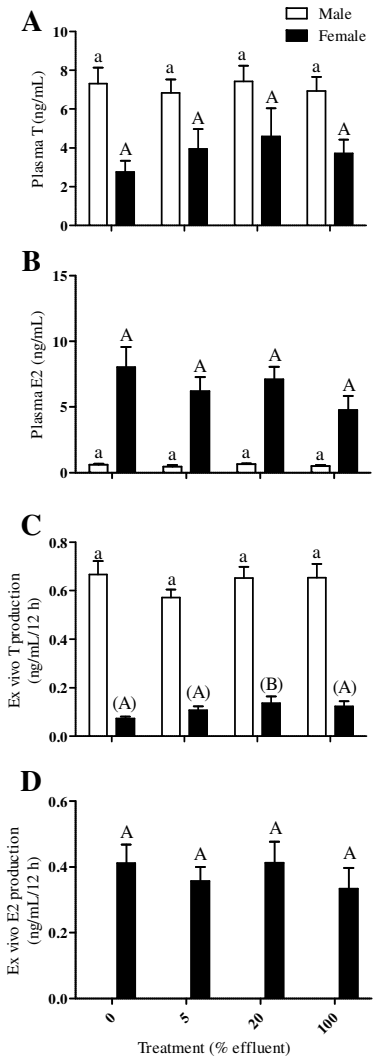

Figure 8. Effects of a 21 d wastewater treatment plant effluent exposure on male (white bars) and female (black bars) fathead minnow (A) plasma testosterone (T) concentrations, (B) plasma 17β-estradiol (E2) concentrations, (C) ex vivo T production by gonads, and (D) ex vivo E2 production by ovaries. Ex vivo E2 production by testes was not measured. Bars represent mean (±SE). Significant differences between treatments are indicated with lower case letters for males and upper case letters for females ($p < 0.05$). Parentheses indicate no significant difference between treatments by ANOVA, but significant treatment effect was observed with Duncan's post-hoc test.

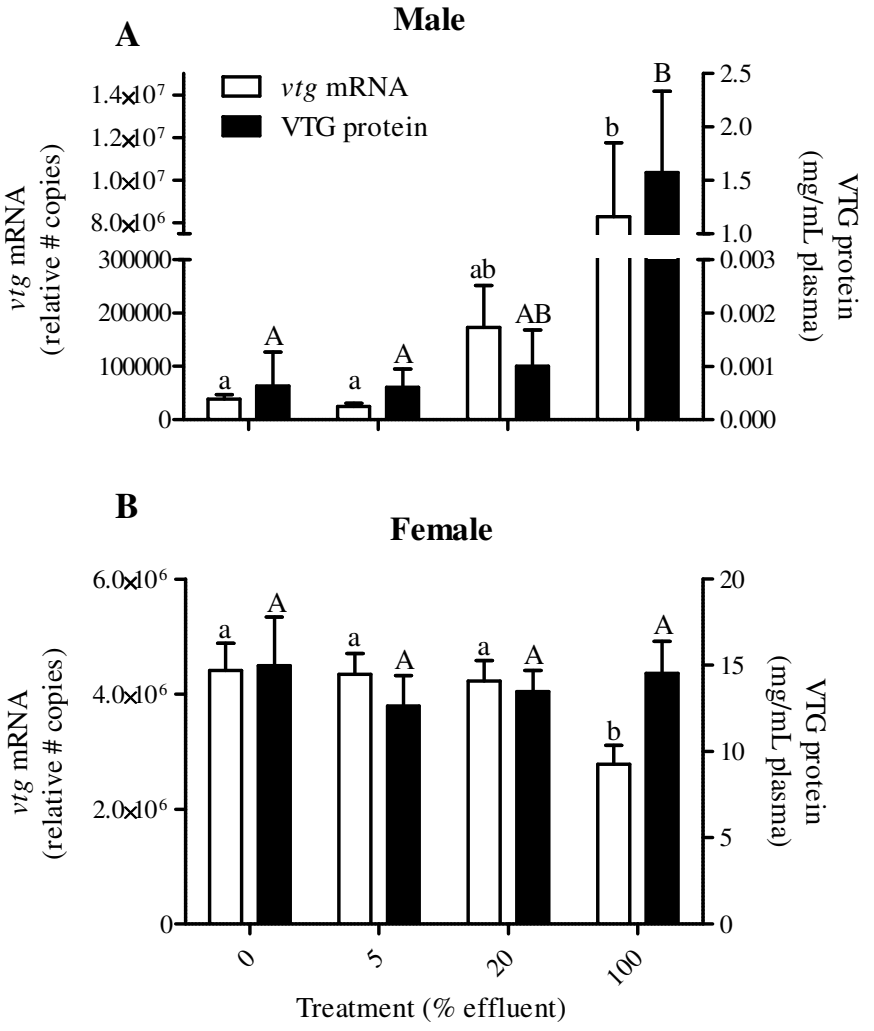

Figure 9. Effects of a 21 d wastewater treatment plant effluent exposure on (A) male and (B) female fathead minnow hepatic vitellogenin (*vtg*) mRNA transcript abundance (white bars) and plasma VTG protein concentrations (black bars). Bars represent mean (±SE). Significant ($p < 0.05$) differences between treatments are indicated with lower case letters for *vtg* transcript abundance and upper case letters for VTG protein concentrations. Different letters indicate statistically significant differences between treatments.

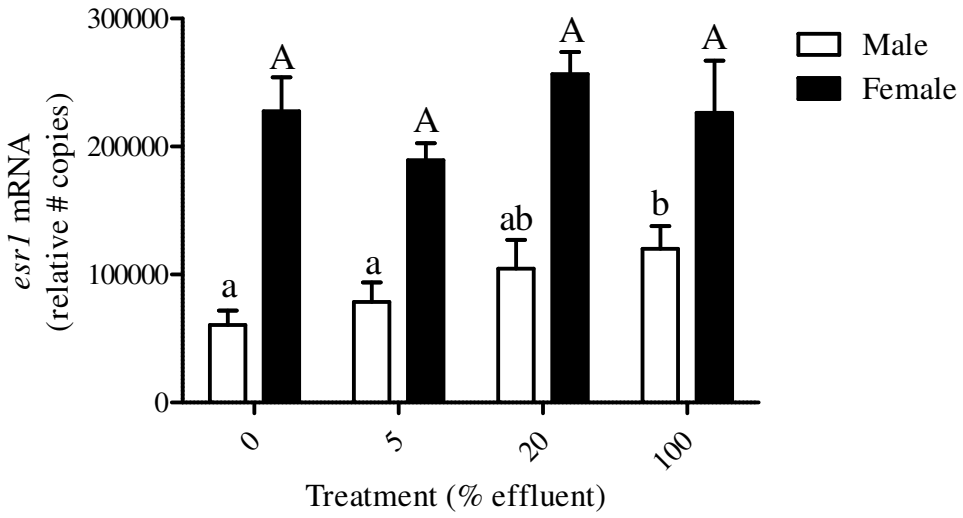

Figure 10. Effects of a 21 d wastewater treatment plant effluent exposure on male (white bars) and female (black bars) fathead minnow hepatic estrogen receptor-α (*esr1*) transcript abundance. Bars represent mean (±SE). Significant ($p < 0.05$) differences between treatments are indicated with lower case letters for males and upper case letters for females. Different letters indicate statistically significant differences between treatments.

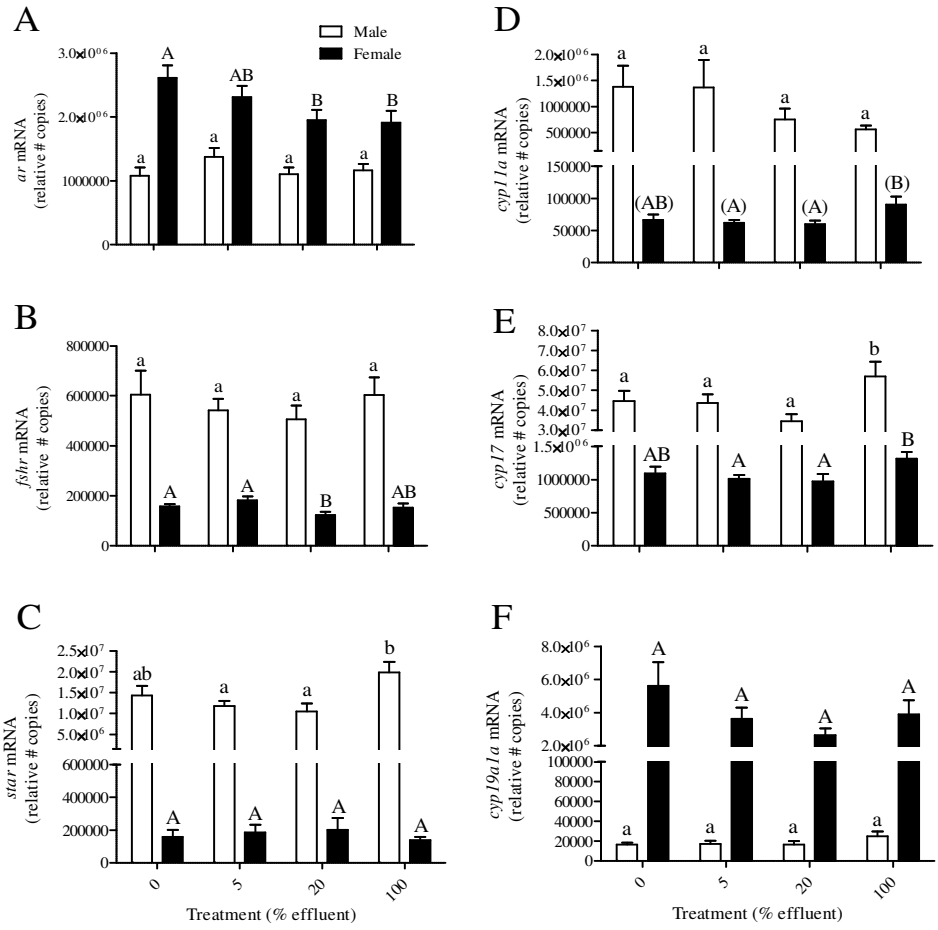

Figure 11. Relative transcript abundance of six gene products associated with endocrine function in gonads from adult male (white bars) and female (black bars) fathead minnows exposed to wastewater treatment plant effluent for 21 d. Bars represent mean (±SE). Significant ($p < 0.05$) differences between treatments are indicated with lower case letters for males and upper case letters for females for each gene. Parentheses indicate no significant difference between treatments by ANOVA, but significant treatment effect with Duncan's post-hoc test. (A) Androgen receptor (*ar*); (B) follicle stimulating hormone receptor (*fshr*); (C) steroidogenic acute regulatory protein (*star*); (D) cytochrome P450 cholesterol side-chain cleavage (*cyp11a*); (E) cytochrome P450 17α hydroxylase, 17, 20 lyase (*cyp17*); (F) cytochrome P450 aromatase-A (*cyp19a1a*).

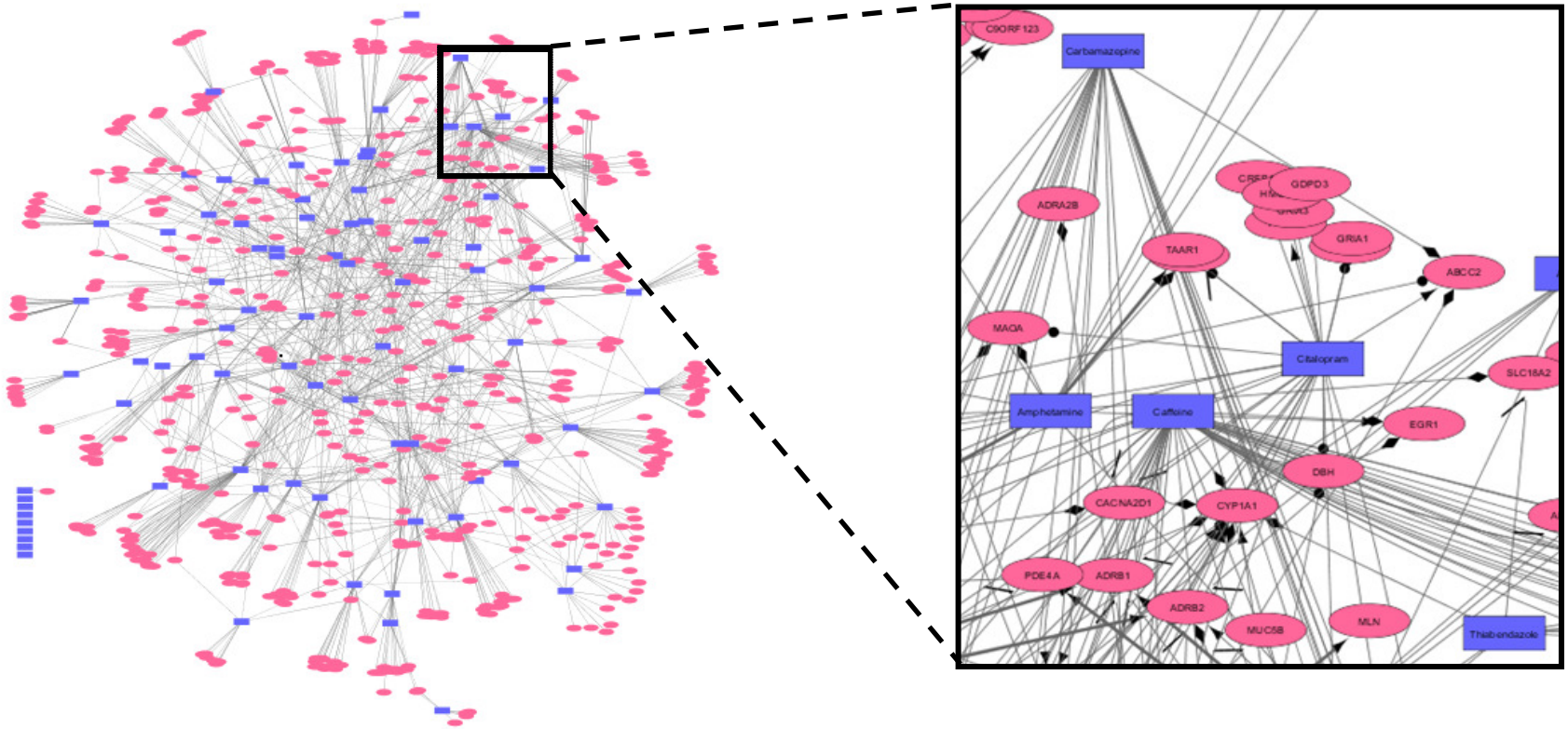

Figure 12. Chemical-gene interaction network of steroid hormones, wastewater indicators, and pharmaceuticals detected in at least one 7 d composite 100% wastewater treatment plant effluent sample. Known chemical-gene/protein interactions were determined using Comparative Toxicogenomics Database and STITCH ('search tool for interactions of chemicals') and were plotted using Cytoscape v 3.2.0 software. Chemicals are represented by rectangles, and associated genes are represented by ovals. The magnified square displays a representation of select chemicals and their known gene/protein interactions.

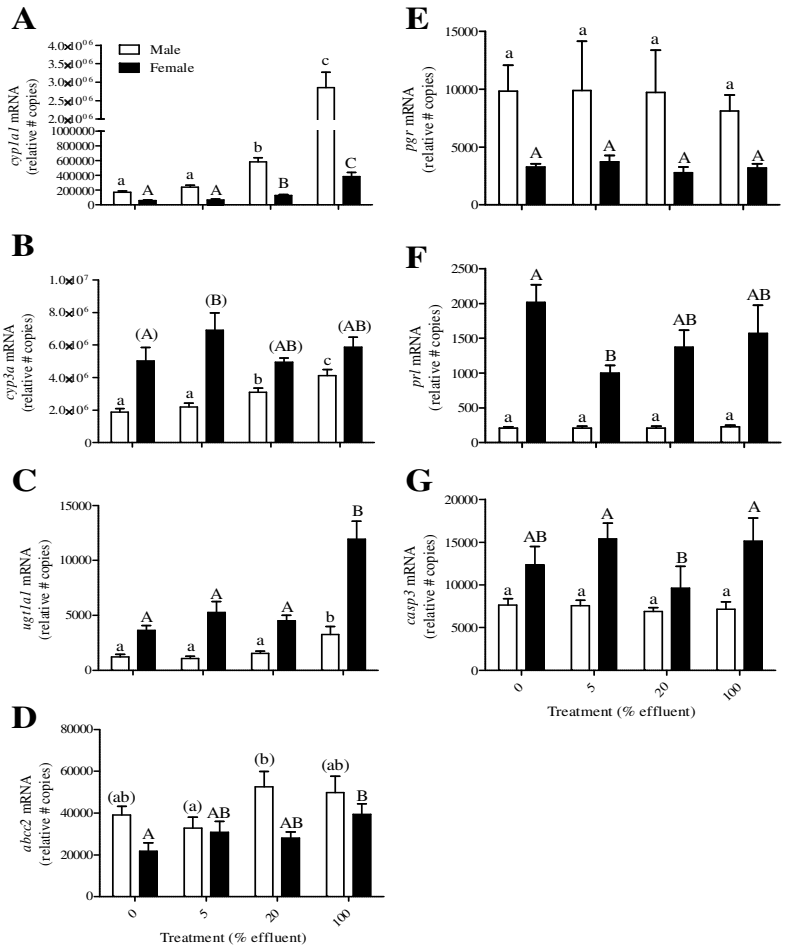

Figure 13. Relative transcript abundance of seven gene products selected based on the chemical–gene interaction network analysis from male (white bars) and female (black bars) fathead minnows exposed to wastewater treatment plant effluent for 21 d. Hepatic genes (A-D) are associated with xenobiotic metabolism or transport or glucuronidation. Gene expression examined in gonads (E-G) is associated with steroid synthesis, osmoregulation, or apoptosis. Bars represent mean (±SE). Statistically significant differences between treatments are indicated with lower case letters for males and upper case letters for females for each gene ($p < 0.05$). Parentheses indicate no significant difference between treatments by ANOVA, but significant treatment effect with Duncan's post-hoc test. (A) Cytochrome P450 isozyme, *cyp1a1*; (B) cytochrome P450 isozyme, *cyp3a*; (C) glucurononsltransferase-1 *ugt1a1*; (D) ATP-binding cassette, *abcc2*; (E) progesterone receptor, *pgr*; (F) prolactin, *prl*; (G) caspase 3 apoptosis-related cysteine peptidase, *casp3*.

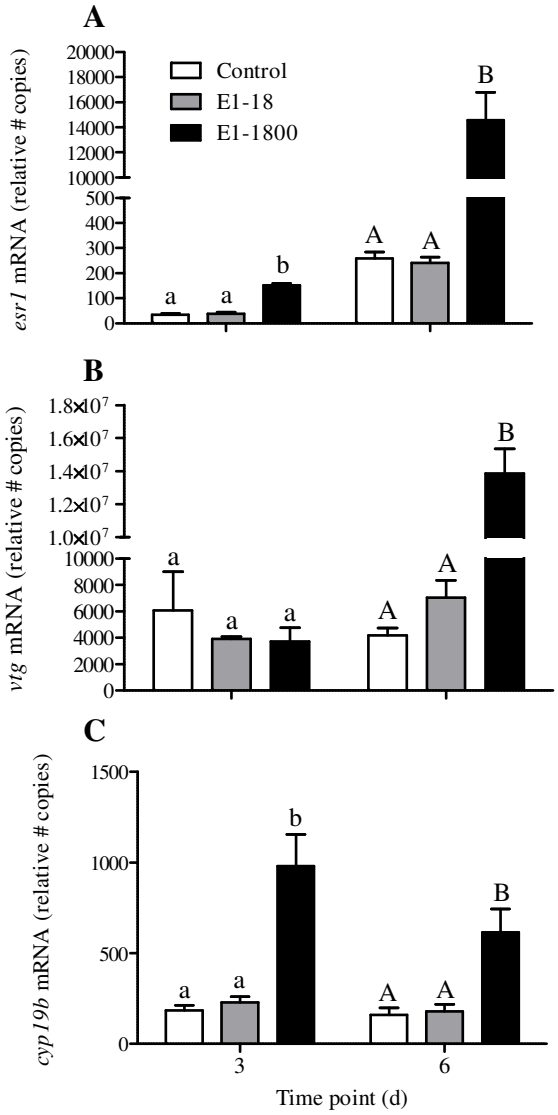

Figure 14. Relative transcript abundance of three gene products in fathead minnow embryos and larvae exposed to estrone (E1) for three and six days with experiment initiation at ~8 hours post-fertilization. Estrone concentrations were 0 (white bars), 18 (gray bars), and 1,800 ng/L (black bars). Bars represent mean (±SE) of n = 4 replicate pools of 10 embryos (d 3) or 10 larvae (d 6). Significant ($p < 0.05$) differences among treatments are indicated by different letters within each time point (lower case letters for d 3 and upper case letters for d 6). (A) Estrogen receptor-α (*esr1*); (B) vitellogenin (*vtg*); (C) cytochrome P450 aromatase-B (*cyp19b*).

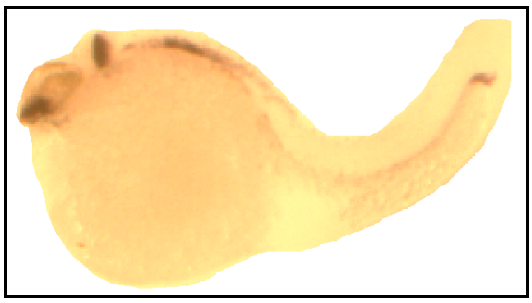

Figure 15. Expression pattern of paired box-2a (*pax2a*) mRNA in a control zebrafish at 30 hours post-fertilization.

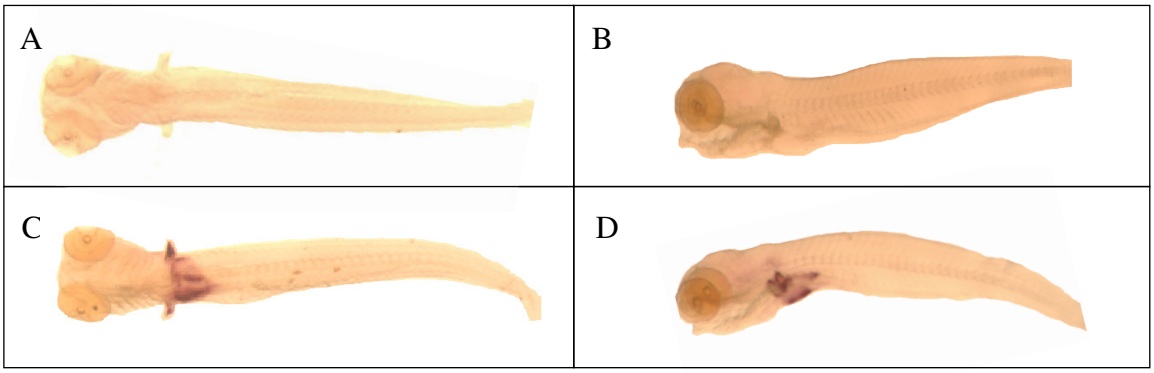

Figure 16. Expression pattern of vitellogenin mRNA in fathead minnow larvae (6 days post-fertilization) exposed to estrone for 6 d using whole-mount in situ hybridization. (A) Control larva, top view; (B) control larva, side view; (C) 1,800 ng estrone/L-exposed larva, top view; (D) 1,800 ng estrone/L-exposed larva, side view.

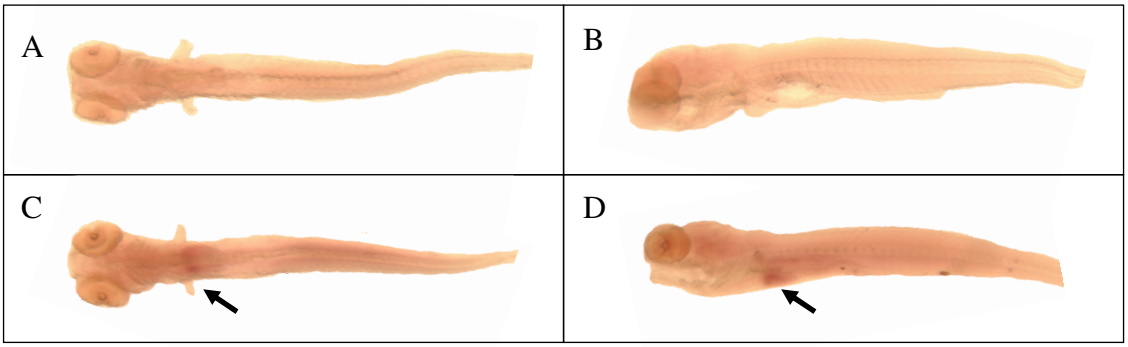

Figure 17. Expression pattern of estrogen receptor-α (*esr1*) mRNA in fathead minnow larvae (6 days post-fertilization) exposed to estrone for 6 d using whole-mount in situ hybridization. (A) Control larva, top view; (B) control larva, side view; (C) 1,800 ng estrone/L-exposed larva, top view; (D) 1,800 ng estrone/L-exposed larva, side view. Arrows point to location of *esr1* expression.

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