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Pharmaceuticals And Personal Care Products: Emerging Contaminants In Aquatic Ecosystems

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PHARMACEUTICALS AND PERSONAL CARE PRODUCTS: EMERGING CONTAMINANTS IN AQUATIC ECOSYSTEMS

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For Luis and Natalie

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ABSTRACT

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In recent years, the presence of pharmaceuticals and personal care products (PPCPs) in aquatic systems has led to research on their fate and effects. PPCPs have been found in mixture in wastewater effluents, surface, ground, and drinking water at low concentrations from areas of intense urbanization. Although adverse effects to human health from the current environmental concentrations are unlikely, the impacts to ecological receptors are not clear. We performed field and laboratory studies to quantify and evaluate effects of PPCPs on fish. First, a field study was conducted at the Baca National Wildlife Refuge, Colorado (2010-2012) because a portion of the Refuge receives discharges of treated water from the Aspen Wastewater Treatment Plant (WWTP). Water and fish samples were used to quantify the presence of PPCPs in surface and wastewater effluents, and to determine the potential impact of PPCPs in fish communities (using histology and gene expression analysis). We focused on fathead minnows (*Pimephales promelas*) since they have been the subject of relevant ecotoxicological research and are a good sentinel species. A total of 120 analytes were quantified using a combination of grab samples and

polar organic chemical integrative samplers (POCIS). Although no PPCPs were detected from the grab samples, POCIS allowed for the detection of PPCPs in all our fish sites. High concentrations of N,N-Diethyl-meta-toluamide (DEET) and thirteen pharmaceuticals (triclocarban, triclosan, gemfibrozil, ibuprofen, progesterone, diphenhydramine, atenolol, caffeine, trimethoprim, levorphanol, cannabidiol, tetrahydrocannabinol (THC), and naproxen) were detected in all fish sites, including the reference site. Cellular changes in gonads and livers and significant changes in gene expression (steroidogenic acute regulatory protein, *star* and androgen receptor, *ar)* were observed from female and male fathead minnows sampled from creeks contaminated with PPCPs. However, because we could not identify a clean reference site, we cannot affirm these changes are due to PPCP exposure. We conclude that POCIS is a sensitive method for the detection and quantification of PPCPs in small streams. Additional studies at the Refuge are needed to better understand the ecological impacts of PPCPs.

Next, we conducted a laboratory study using the same PPCPs found in our field study and exposed adult fathead minnows for 48 hr to the highest environmental concentration of each chemical. Our goal was to evaluate molecular changes of a suite of genes known to respond after exposure to chemicals affecting lipid metabolism, and the endocrine and nervous systems. Fish were exposed to triclocarban (1.4 µg/L), DEET (0.6 µg/L) or to a PPCP mixture consisting of: atenolol (1.5 µg/L), caffeine (0.25 µg/L), diphenhydramine (0.1 µg/L), gemfibrozil (1.5 µg/L), ibuprofen (0.4 µg/L), naproxen (1.6 µg/L), triclosan (2.3 µg/L), progesterone (0.2 µg/L), triclocarban (1.4 µg/L), and DEET

(0.6 µg/L). Vitellogenin (*vtg*) was up-regulated in livers of females and males exposed to triclocarban. Also, an up-regulation of hepatic lipoprotein (*lpl*) and a down-regulation of *ar* and *star* were observed in testes. The group treated with DEET only showed a significant decrease in *ar* in females. In contrast, the PPCP mixture down-regulated *vtg* in females and males, and expression of estrogen receptor alpha (*erα*), *star*, and thyroid hormone receptor alpha 1 (*thra1*) in testes. Our results show the molecular 'estrogenic' effects of triclocarban are eliminated (males) or reversed (females) when dosed in conjunction with several other PPCPs, once again showing that results from single exposures could be vastly different from those observed with mixtures.

CHAPTER 1. INTRODUCTION

1.1 Pharmaceuticals and Personal Care Products in the Environment

Pharmaceuticals and personal care products (PPCPs) include any product used in human and animal health, cosmetic industry or agribusiness for the purposes of enhancing growth, health or reproduction of livestock (Daughton and Ternes 1999). They include a wide range of chemicals from human prescription and over the counter drugs, to veterinary drugs, fragrances, cosmetics, sunscreen, and many compounds used in households such as soaps and detergents (Loraine and Pettigrove 2006). Although water pollution with PPCPs is not a new phenomenon, recent advances in analytical chemistry have allowed their ubiquitous detection in different environmental media and at very low concentrations (Carballa, Omil et al. 2004; Nieto, Borrull et al. 2010).

PPCPs enter the environment from multiple sources, including domestic and industrial wastewater effluents, improper disposal of medication or cosmetics, concentrated animal feeding operations, landfill leachate, and direct release to open waters via washing, bathing or swimming (Nikolaou, Meric et al. 2007). They have been detected in many countries in waste water treatment plant (WWTP) effluents; surface, ground and drinking waters at concentrations ranging from parts per billion (μg/L) to parts per trillion (ng/L) (Ellis 2006). However,

despite their common occurrence in the environment, their effects to aquatic organisms are not fully understood.

1.2 Pharmaceuticals and Personal Care Products Studied in This Thesis

This study focuses on eight classes of PPCPs commonly found in the environment including: bactericides, insect repellents, nonsteroidal anti-inflammatory drugs (NSAIDs), stimulants, beta blockers, antihistamines, cholesterol reducing drugs and steroid hormones. See Figure 1 for diagram showing their chemical structures.

The bactericides triclocarban and triclosan are two of the most frequently antibacterial chemicals detected in surface water at concentrations ranging from 0.0001 to 2.30 μg/L (Brausch and Rand 2011). They are not very soluble in water, are relatively nonvolatile (Ying, Yu et al. 2007) and sorb to sediment/soil organic matter (Reiss, Mackay et al. 2002; Heidler, Sapkota et al. 2006). Their similar chemical structure (Figure 1) suggests that they may have similar fate and mode of action (Halden and Paull 2005; Ying, Yu et al. 2007). They kill bacteria by disrupting cell membranes (triclocarban) or through inhibition of fatty acid synthesis (triclosan). However, and as discussed in more detail in Chapter 3, there is evidence to suggest they can act as weak estrogens and androgens eliciting endocrine disrupting effects in fish (Foran, Bennett et al. 2000; Ishibashi, Matsumura et al. 2004; Chen, Ahn et al. 2008; Raut and Angus 2010).

N,N-Diethyl-meta-toluamide or DEET, is the most common active ingredient used in insect repellents and has been produced for more than fifty years in the

U.S. (Corbel, Stankiewicz et al. 2009). DEET is a volatile and lipophilic substance insoluble in water (Gu, Wang et al. 2005). This chemical can enter surface waters from overspray (direct application or by residents bathing or washing clothing after using it) and via WWTP effluents with concentrations in the range of 0.01 to 0.66 μg/l (Brausch and Rand 2011). Previous studies have reported inhibition of the central nervous system enzyme acetylcholinesterase in rats after exposure to DEET (Corbel, Stankiewicz et al. 2009). While another study suggested that DEET may

interfere with olfactory receptor neurons sensitive to attractants in mosquitoes (Davis 1985). However its mode of action and target sites in vertebrates are still not well understood.

Ibuprofen and naproxen are two commonly NSAIDs used for the reduction of pain, inflammation and fever. They have been found in surface water at concentrations ranging from 0.002 to 1.60 μg/l (Buser, Poiger et al. 1999; Stumpf, Ternes et al. 1999; Kim, Jang et al. 2009; Brozinski, Lahti et al. 2011). Both are poorly water-soluble compounds and based on their adsorption coefficient (K_{oc}) values (ibuprofen 3,400 and naproxen: 330) they are expected to bind to suspended solids and sediments. Volatilization from surface water is not expected to be an important fate process according to their low Henry's Law constants (ibuprofen $1.52x10^{-7}$ atm-m^3/mole and naproxen: 3.39x10-^{10} atm-m^3/mole). NSAIDs main mechanism of action is through inhibition of the enzyme cyclooxygenase COX (Mitchell, Akarasereenont et al. 1993).

Caffeine is a stimulant found in numerous beverages and food products (tea, coffee, soft drinks, chocolate, etc.) and it is used as a brain, cardiac, and respiratory stimulant and diuretic. In the U.S., caffeine consumption averages ~200 mg/person/day (Buerge, Poiger et al. 2003). However, it is extensively metabolized with only 3% of ingested caffeine excreted in the urine (Tangliu, Williams et al. 1983). It is commonly found in surface waters at concentration ranging from 0.01 to 0.25 μg/L (Gomez-Martinez 2011). Caffeine is classified as an adenosine antagonist blocking adenosine receptors in the brain and other organs.

The beta blocker atenolol is used for treating cardiovascular disorders. The solubility of atenolol in water is very low and it has a half-life of 3-10 days. It has been detected in surface waters at concentrations ranging from 0.02 to 1.50 μg/L (Zuccato, Castiglioni et al. 2005; Al-Odaini, Zakaria et al. 2013). Atenolol acts by blocking the β1-adrenergic receptor.

Diphenhydramine is an antihistamine available in numerous over-the-counter preparations. It has been identified in several environmental compartments including streams (0.01 to 0.10 μg/L) (Berninger, Du et al. 2011) and fish tissues (Stanley, Ramirez et al. 2006; Gould, Brooks et al. 2007). This drug is water soluble and acts by blocking histamine H1-receptors.

Gemfibrozil is used to reduce plasma triglycerides and total cholesterol. It is one of the most commonly found pharmaceuticals in the environment occurring at concentrations from 0.08 to 19.4 μg/L in wastewater and from 0.01 to 1.50

μg/L in surface water (Skolness, Durhan et al. 2012). It is a poor water-soluble chemical. The main gene target of gemfibrozil is lipoprotein lipase (*lpl*). However, almost nothing is known regarding the effects of this cholesterol-lowering drug to aquatic organisms.

Progesterone is an steroid hormone used to prevent preterm labor and to treat menstrual disorders and female infertility. Similar to all steroid hormones, progesterone is a hydrophobic compound with minimal volatilization. It has been detected in surface water at concentrations ranging from 0.01 to 0.20 μg/L (DeQuattro, Peissig et al. 2012). Several studies with mammals have reported female and male reproductive effects subsequent to progesterone exposure (CamachoArroyo, Pasapera et al. 1996; Camacho-Arroyo, Guerra-Araiza et al. 1998; Sheehan and Numan 2002). In female fish decrease in fecundity, fertility, gonadosomatic index and vitellogenin expression was reported (DeQuattro, Peissig et al. 2012).

1.3 Wastewater Treatment Plants

Conventional WWTPs utilize activated sludge processes which were not designed to remove PPCPs. Their efficiency to remove organic chemicals varies greatly from 0-90% depending of multiple factors (Wanner, Kucman et al. 1988; Diez, Castillo et al. 2002; Dorival-Garcia, Zafra-Gomez et al. 2013; Maeng, Choi et al. 2013). One of the most important factors is solid retention time (SRT) which promotes the growth of a diverse biological community significantly reducing PPCP concentrations in waste effluents (Ternes, Stumpf et al. 1999; Kanda,

Griffin et al. 2003; Joss, Andersen et al. 2004; Kreuzinger, Clara et al. 2004; Clara, Kreuzinger et al. 2005; Nakada, Tanishima et al. 2006; Wick, Fink et al. 2009; Snyder, Lue-Hing et al. 2010). PPCPs that are not degraded in the WWTP can contaminate surface waters directly from discharged effluents or indirectly from runoff of agricultural lands treated with digested sludge as a fertilizer.

1.4 Major Concerns

Although the production of PPCPs is increasing yearly, there are no federal regulations limiting PPCPs levels in sewage, surface or drinking waters (Ellis 2006). WWTPs were not designed for PPCP removal and their efficiency to remove these chemicals varies depending on the chemical structure and of the type of treatment used. Because PPCPs and their metabolites are biologically active and potent at low concentrations, there is a potential for effects to non-target organisms. Although there is no evidence that environmental levels of PPCPs affect human health, impacts of PPCPs to aquatic organism have been reported. Several studies have shown reproductive abnormalities in fish exposed to PPCPs including changes in sex ratios, feminization of males and behavioral changes (Painter, Buerkley et al. 2009; Madureira, Rocha et al. 2011; Sanchez, Sremski et al. 2011; Schultz, Painter et al. 2011; Galus, Jeyaranjaan et al. 2013).

1.5 Research Objectives

Chapter 2: The goal of this investigation was to examine the presence and distribution of PPCPs in the Baca Wildlife National Refuge, CO and whether their

presence was associated with adverse health effects in imperiled fish species in the Refuge. Our findings suggest that PPCPs are present in the Refuge's creeks at low concentrations. Although some of these PPCPs are likely being released from the Aspen WWTP, their presence in streams not receiving effluents is puzzling and their source is unknown at this time. We found multiple cellular changes in gonads and liver as well as changes in genes expression in fish from the Refuge's creeks by qPCR and histological analysis, but cannot attribute these changes to PPCP exposure at this time.

Chapter 3: The aim of this chapter was to examine the mechanisms of toxicity and effects of a selected number of PPCPs on fathead minnows. Adult fish were acutely exposed to triclocarban, DEET, or a PPCP mixture and measured a wide range of responses, from molecular to whole organism-level. We hypothesized that triclocarban would elicit estrogenic effects, while DEET would cause effects to the nervous system. We also hypothesized that the PPCP mixture would induce additive effects for those PPCPs sharing common mechanisms of action such as triclocarban and triclosan. Although we observed estrogenic effects from triclocarban, DEET did not elicit effects to the central nervous system measured as changes in expression of one neurotransmitter. Also, contrary to our prediction, the PPCP mixture caused antagonistic effects.

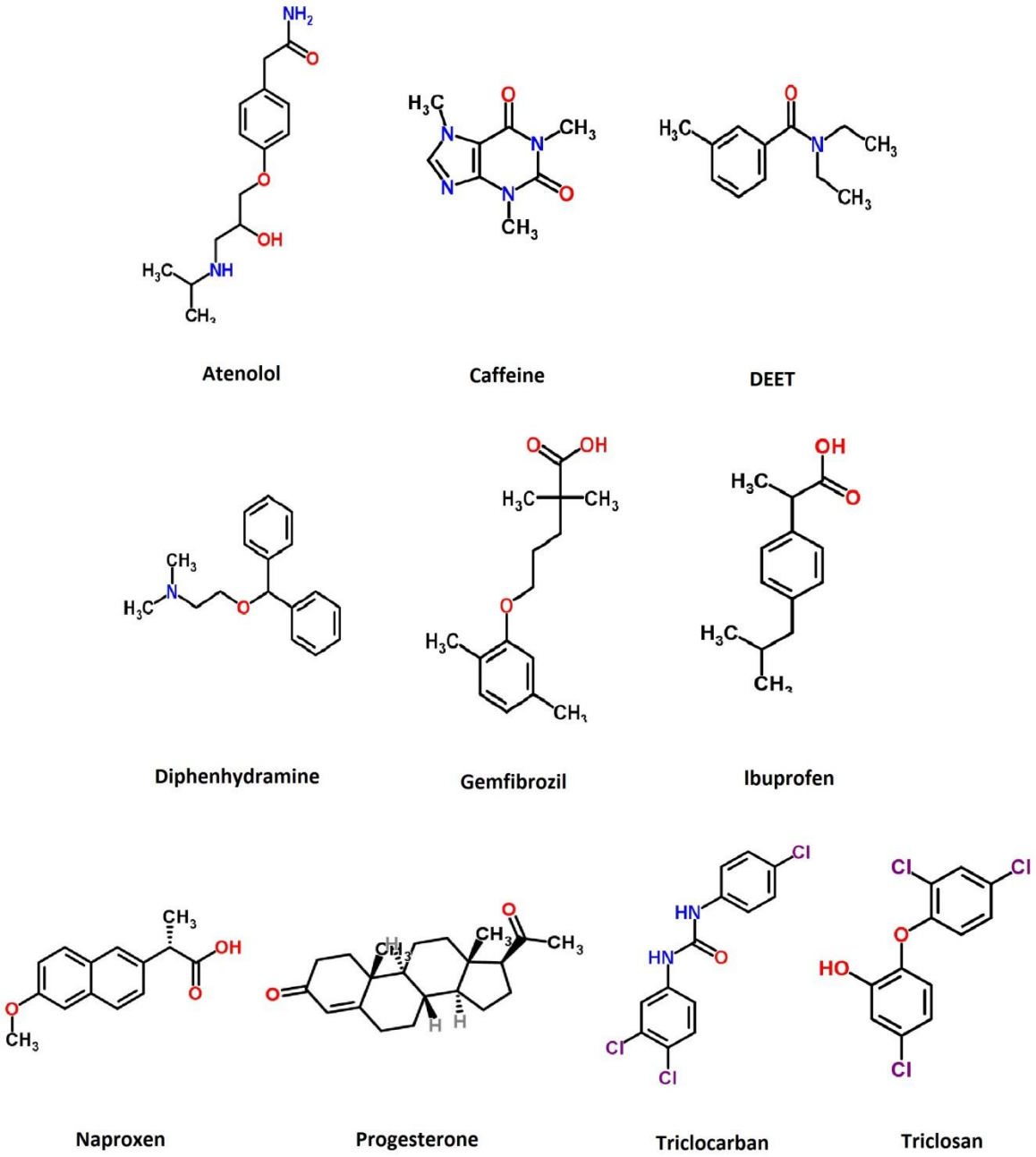

Figure 1.1 Chemical structure of pharmaceutical and personal care products tested in the laboratory.

CHAPTER 2. PHARMACEUTICAL AND PERSONAL CARE PRODUCTS ON THE BACA NATIONAL WILDLIFE REFUGE, COLORADO, AND IMPACTS ON NATIVE FISH

2.1 Abstract

The presence of pharmaceuticals and personal care products (PPCPs) in surface and ground waters has raised substantial concerns due to the difficulty to remove them by conventional water treatments and their potential effects to aquatic organisms. These chemicals, including natural and synthetic hormones, appear in mixtures at very low concentrations. The Baca National Wildlife Refuge (BNWR), Colorado, is home for several endemic fish species, including the Rio Grande chub (*Gila Pandora*) and Rio Grande sucker (*Catostomus plebeius*). Within the last century, these fish species have been extirpated from much of their range and thus are considered vulnerable or threatened. The objective of this study was to evaluate the presence, concentration and distribution of PPCPs in BNWR surface waters and determine whether these chemicals could be impacting reproductive function in native imperiled fish species. We focused on fathead minnows (*Pimephales promelas*) also present at the Refuge, since they have been the subject of relevant ecotoxicological research and are a good sentinel species. During 2011 and 2012, a total of 120 analytes were quantified from water using a combination of grab samples and polar organic chemical

integrative samplers (POCIS). Although no PPCPs were detected from the grab samples, high concentrations of N,N-Diethyl-meta-toluamide (DEET) and thirteen pharmaceuticals (triclocarban, triclosan, gemfibrozil, ibuprofen, progesterone, diphenhydramine, atenolol, caffeine, trimethoprim, levorphanol, cannabidiol, tetrahydrocannabinol (THC), and naproxen) were detected in all fish sites. This data was used to estimate the cumulative and time-weighted average water PPCP concentrations under our study conditions. Changes in liver and ovarian histology and on gene expression (androgen receptor, *ar* and steroidogenic acute regulatory protein, s*tar*) were observed in fish collected from creeks contaminated with PPCPs; however the presence of PPCPs in the reference site did not allow for a proper comparison across sites. We conclude that POCIS is a sensitive method for the detection and quantification of PPCPs in small streams. Additional data are needed to better understand the significance of PPCPs as single compounds and mixture to aquatic organisms.

2.2 Introduction

Pharmaceuticals and personal care products (PCPPs) are emerging environmental contaminants which have attracted much attention for their potential to cause adverse effects in aquatic organisms. They mostly enter aquatic environments from effluents discharged from sewage waste water treatment plants (SWWTPs) as either parent compounds or metabolites. They are now ubiquitous having been detected from SWWTP effluents and sludge,

surface water, ground water, and drinking water world-wide (Ferrari, Mons et al. 2004; Glassmeyer, Furlong et al. 2005; Lee and Rasmussen 2006).

Typical environmental concentrations of high consumption PPCPs have been reported to be in the range of <1,800 – 3,900 ng/L> in surface water and <39,000 – 56,000 ng/L> in WWTPs effluents (Oosterhuis, Sacher et al. 2013). Concentrations of PPCPs in aquatic systems can vary depending on several parameters including the efficiency of the WWTP to remove them; the distance of the WWTP to the site of discharge; type and abundance of microbial communities that degrade them; and abiotic conditions such as water temperature (Benotti and Brownawell 2007; Miege, Choubert et al. 2009). Some of the most common PPCPs detected in surface water are cosmetics, antibiotics, and steroids. Most PPCPs have intermediate lipophilicity ($2.5 < \log K_{ow} < 5$) (Gertz, Kilford et al. 2008) and thus easily cross cell membranes, targeting specific tissues and physiological functions, and may remain active for relatively long times (months) depending on environmental conditions and initial concentrations (Halling-Sorensen, Nielsen et al. 1998). These chemicals have been associated with a myriad of negative effects in non-target ecological receptors at low concentrations as discussed below.

Several studies have demonstrated that fish exposed to wastewater treatment effluents respond with an “estrogenic” signature that includes development of ova-testis, sex ratios skewed toward females, increased vitellogenin (VTG) and estrogen synthesis, and decreased egg production (Nimrod and Benson 1998; Flammarion, Brion et al. 2000; Zha, Wang et al. 2007;

Xu, Yang et al. 2008). Some of these effects have been replicated in the laboratory after exposure to the synthetic estrogen ethynylestradiol (EE2) used for birth control and commonly found in surface waters downstream from SWWTPs.

Non-steroidal anti-inflammatory drugs (NSAID) such as ibuprofen, naproxen, and diclofenac have also been associated with endocrine effects via up-regulation of aromatase (or Cytochrome P45019, CYP19) activity, the enzyme responsible for aromatizing androgens to estrogens. It has also been shown to cause a down-regulation of other genes involved in steroidogenesis such as 3-beta-hydroxysteroid dehydrogenase, 17-beta-hydroxysteroid dehydrogenase, and cytochrome P450 17. In these studies, changes in gene expression were correlated with increased gonadosomatic index (GSI) (Lee, Ji et al. 2011), delayed hatching (Han, Choi et al. 2010; Lee, Ji et al. 2011; Ji, Liu et al. 2013), decrease egg production, increase 17ß-estradiol (E2), and decrease in testosterone levels (Ji, Liu et al. 2013).

Anti-depressant drugs including fluoxetine, venlafaxine, and paroxetine regulate serotonin release in the central nervous system. Fluoxetine has been best studied in fish causing decreased egg production, lower ovarian steroid hormone production, and down-regulation in the expression of *cyp19a* and gonadotropin receptors (*fshr* and *lhr*) (Lister, Regan et al. 2009). It is also known to delay sexual maturity in Western mosquitofish (*Gambusia affinis*) (Henry and Black 2008).

Beta-blockers such as propranolol, atenolol, and metoprolol are some of the most widely prescribed pharmaceuticals found in WWTP effluents and surface waters and are used to treat cardiovascular disorders. Very few studies exist on effects on fish. In one study with Japanese medaka (*Oryzias latipes*) propranolol significantly inhibited egg production (Huggett, Brooks et al. 2002).

Bactericides (triclosan and triclocarban) are also very commonly found in association with WWTP effluents. The fate of triclocarban is unknown, but due to its structural similarity to triclosan, it is expected to have a similar mode of action. Toxic effects in rainbow trout (*Onchorhynchus mykiss*) have been reported at high concentrations ($LC_{50,\ triclosan}$ = 0.35 mg/L (Orvos, Versteeg et al. 2002) and $LC_{50,\ triclocarban}$ = 0.12 mg/L (Palenske 2009)). Previous studies attributed a significant decrease in androgen receptor (*ar*) and steroiodogenic acute regulatory protein (*star*) expression in different organisms to both chemicals (Chen, Ahn et al. 2008; Kumar, Chakraborty et al. 2009). They have also been show to increase hepatic VTG concentrations in males (Ishibashi, Matsumura et al. 2004; Raut and Angus 2010).

However, most of the studies published so far have evaluated effects of single PPCPs when it is well known that real-world exposures involve several dozen PCPPs which could result in additive, synergistic or antagonistic effects. Yet only a handful of studies have examined effects of mixtures of PPCPs (Painter, Buerkley et al. 2009; Parrott and Bennie 2009; Ankley, Jensen et al. 2010; Madureira, Rocha et al. 2011; Sanchez, Sremski et al. 2011; Schultz, Painter et al. 2011; Madureira, Rocha et al. 2012; Thomas, Joshi et al. 2012; Thomas and Klaper 2012; Galus, Jeyaranjaan et al. 2013).

In this Chapter we report on studies we conducted at the Baca National Wildlife Refuge, Colorado, which is currently receiving WWTP discharged into one of its creeks. Between 2000 and 2006, the subdivisions of Crestone and Baca Grande have increased by 68% (total of ~ 2,000 people) and there is concern that PPCPs are/could reach Refuge waters at high enough concentrations to harm fish communities. Therefore the purpose of this study was to determine (1) the types and concentrations of PPCPs in the Refuge, and (2) potential reproductive effects due to PPCP exposure in native fish.

2.3 Materials and methods

2.3.1 Site description

The Baca National Wildlife Refuge is located in Saguache and Alamosa counties in the San Luis Valley of south-central Colorado (Figure 1). The Refuge is habitat for over 200 bird, 51 mammalian, 8 amphibian, 8 reptilian, and 4 fish species. In 2006 at Crestone Creek (CC), fish communities consisted of Rio Grande chub (*Gila pandora*, minimum population estimate of 33,725); fathead minnows (*Pimephales promelas*, 15,994); Rio Grande sucker (*Catostomus plebeius*, 3,545); and longnose dace (*Rhinichthys cataractae*, 1,600). Cutthroat trout (*Oncorhynchus clarkii)* are sometimes observed in the upper reaches of the stream during the summer months which are dried by early fall (The Colorado Division of Wildlife, personal communication).

The San Luis Valley is classified as a semi-desert shrubland or greasewood fan/flat (Colorado GAP Analysis 2000) with only 7 inches of annual

precipitation. The Refuge consists of 92,500 acres that are bordered by Crestone and the Baca Grande subdivisions. Six intermittent creeks that are fed largely by melting mountain snow enter the Refuge from the east. Willow (WC), Spanish (SC), and Cottonwood (CWC) creeks flow through the Baca Grande Subdivision prior to reaching the Refuge, while North (NCC) and South Crestone (SCC) creeks run through Crestone town (Figure 1). Crestone creek born from the convergence of NCC and SCC inside the refuge is used for irrigation. Deadman creek (DM), was used as a reference creek in the present studies because is considered relatively free of anthropogenic influences flowing through the Great Sand Dunes National Park instead of any populated area before entering the Refuge. None of this water exits the Refuge (Figure 2). The communities surrounding the Refuge use the Aspen WWTP to treat their domestic sewage. The Aspen WWTP uses an activated sludge sequence batch reactor system and its sewage sludges are disposed by application to land for use as a soil amendment (50 miles away from the Refuge).The Aspen WWTP discharges treated wastewater into an unnamed ditch (we will call it Aspen outflow, AO) close to SCC.

2.3.2 Water sample collection

A total of 21 sites A total of 21 sites were sampled in August 2010, August 2011, May 2012, and August 2012 from NCC, SCC, CC, WC, SC, CWC, DM, and AO , with SCC and CC representing sites closest and further away from the Aspen WWTP, respectively and DM the reference site (Figure 2). Grab water

samples were collected following the U.S. EPA surface water sampling operating procedure (USEPA 2007). Briefly, samples were collected from each creek facing upstream and without disturbing the sediment by direct dipping of 40 mL glass vials with Teflon® septa into the stream. Vials were filled completely to avoid bubbles or headspace after they were capped. A clean pair of new, non-powdered, disposable gloves was worn for each sampling collection and vials were kept in resealable plastic bags at 4°C until further analyzes.

Since water analyses from 2011 were negative for PPCPs in all of the three creeks chosen for our fish studies, during 2012 we decided to install polar organic chemical integrative sampling (POCIS, Environmental Sampling Technologies, EST Inc, St. Joseph, MO, USA) devices to better estimate the cumulative aqueous exposure to hydrophilic organic chemicals in fish. POCIS contain a solid sequestration media inside a polyethersulfone membrane that are attached together by compressions rings (Figure 4a). One stainless steel POCIS canister with three membranes per canister (Figures 4b and 4c) was deployed at each of the fish sites (SCC-7, CC-1, and DM-3) and directly downstream from the Aspen WWTP effluent (AO-1) during June for 30 days. Creek water flow rates were determined using a float-operated digital water-stage recorded with a Sutron SatLink 2 satellite telemetry (Sutron Co., Sterling, CO, USA).

2.3.3 Water quality and nutrient analyses

Water quality parameters (temperature, pH, dissolved oxygen, and conductivity) were measured using portable meters at the same time water was

sampled. Dissolved oxygen (DO) and temperature were monitored using a YSI ProODO meter (Yellow Springs Instrument Co., Inc., Yellow Springs, OH, USA), while pH and conductivity were measured using an Orion 250A+ pH meter and an Orion model 135A conductivity meter, respectively (Thermo-Orion, Beverly, MA, USA).

Additional water samples from AO-1, SCC-7, CC-1, and DM-3 Creeks were collected and shipped to the Soil, Water & Plant Testing Laboratory at Colorado State University (Fort Collins, CO, USA) for nutrient analysis (total phosphorous and nitrogen). Total phosphorous was determined following the perchloric acid digestion (Method 4500-P B) and ascorbic acid (Method 4500-P E) ((APHA 1992) methods. Briefly, samples were filtered through 0.45 μm membrane filters and 125 mL acidified using concentrated HNO_3. An additional 5 mL of concentrated HNO_3 was added and evaporated on a hot plate to 20 mL. To digest the samples, 10 mL each of concentrated HNO_3 and $HClO_4$ were added and evaporated. After the digested solution was cool, 1 drop of aqueous phenolphthalein solution and 6*N* NaOH solution were added until the solution turned pink. A combined reagent was made (50 mL 5*N* H_2SO_4, 5 mL potassium antimonyl tartrate solution, 15 mL ammonium molybdate solution, and 30 mL ascorbic acid solution) and 8 mL was added to 50 mL of diluted sample. Samples were kept at room temperature for 10 min for color development and then the absorbance was measured at 880 nm in an OI Analytical Flow Solution 3000 (OI Analytical, College Station, TX, USA) using a reagent blank as reference.

Total nitrogen was determined using the organic N (Method 4500-N D), ammonium (Method 4500-NH_3 H) and nitrate (Method 4500-NO_3 F) methods (APHA 1992). Organic nitrogen was determined following the block digestion and flow injection analysis. The digestion procedure converts nitrogen compounds of biological origin to ammonia after addition of 10 mL of digestion solution (134 g K_2SO_4, 7.3 g $CuSO_4$ in 800mL of water and 134 mL of concentrated H_2SO_4) to 25 mL of water sample. Four alundum granules were added and samples were preheated for 1 h at 200°C. Temperature was increased to 380°C for 1 h and then samples left to cool for 10 min. Diluted samples (25 mL) were injected into the carrier system, cleaned onto the manifold and combined with other reagents to produce color. Ammonia reacts with hypochlorite (ClO^-) to form monochloramine (NH_2Cl) which in turn reacts with phenol to form indophenol blue. Ammonia concentration was then quantified using the same spectrophotometer set up to read the resulting indophenol absorption at 630 nm. Determination of ammonium was made by flow injection analysis (this procedure was described previously under organic nitrogen after it is converted to ammonia).

An automated cadmium reduction method was used to determine nitrate concentration. Samples were adjusted to a pH between 5 and 9 with concentrated HCl and NH_4OH, and then passed through a column containing copper-cadmium granules to reduce nitrate to nitrite. A highly colored azo is formed by diazotizing of nitrite with sulfanilamide and coupling with N-(1-

naphthyl)-ethylenediamine dihydrochloride. Finally, this colored azo was measured colorimetrically.

2.3.4 Pharmaceutical and personal care product analyses

All water samples were shipped overnight to the U.S. EPA Region 8 Laboratory (Golden, CO, USA) for PPCP analyzes using an Acquity ultra-performance liquid chromatography (UPLC, Waters, Milford, MA, USA) and liquid chromatography-tandem mass spectrometry (LC/MS/MS), following the U.S. EPA method protocol 1694 (USEPA 2007). Briefly, samples were filtered to remove physical particulates and then separated into two 1000 mL aliquots. The pH of one aliquot was adjusted to 2.0 with HCl and for the second aliquot, to 10 with NH_4OH. Isotopically labeled analogs of the analytes of interest were spiked into their respective acid or base aliquots. A total of 500 mg $Na_4EDTA.2H_2O$ was added to the first aliquot (acid fraction) for stabilization. Solid phase extraction (SPE) was performed using hydrophilic-lipophilic balance (HLB) 20 cm^3 cartridges. Cartridges were pre-conditioned with 20 mL of methanol and 6 mL of reagent water. For the acid aliquot, the 6 mL of reagent water was adjusted to a pH 2.0. Water samples were passed through the SPE cartridges at a rate of 5-10 mL/min. After extraction was completed, only the acid aliquot was washed with 10 mL of reagent water to remove the EDTA. The retained PPCPs were eluted with 12 mL of methanol for the acid aliquot (an extra 6 mL of acetone:methanol (1:1) was used for triclocarban and triclosan). For the base aliquot, 6 mL of methanol followed by 9 mL of 2% formic acid solution was used. The resulting

eluent was condensed to near dryness and the samples were reconstituted by adding 3 mL of methanol to the eluent. Labeled injection acid and base internal standards were added and the final volume was adjusted to 4 mL with 0.1% formic acid solution. Finally 1 mL of each sample was injected for HPL/MS/MS analysis.

For the POCIS analyses, blanks were exposed to airborne contaminants in the field at the same time as the POCIS were exposed during their deployment and retrieval operations and in the laboratory during the extraction procedure. The field blank was then sealed and stored at -4°C during the 30 days the POCIS were left in each site. After retrieval, POCIS were removed from their holders and individually wrapped in aluminum foil (previously rinsed with methanol), packed with wet ice and shipped overnight to the EST for cleaning, extraction and filtration of compounds. These processes were performed by EST according to their standard operating procedures (EST 2010a; EST 2010b) . POCIS were first washed using contaminant-free water and a soft brush to clean bolts and wing nuts avoiding touching the membrane surface and then placed on methanol rinsed foil until the extraction phase. A glass gravity-flow chromatography column method was used for analyte recovery. The columns were fitted with a stopcock on one end, a funnel on the other, and a glass wool plug (1-2 cm) to keep the sorbent from washing through. The column was pre-rinsed with methanol and the eluate discarded. The sequestration medium was washed into the column with extraction solvents to elute the analytes from the sorbent, and placed in a 125 mL flat bottom flask. For extraction of OASIS HLB (pharmaceuticals), 40 mL of

methanol was used, whereas for the extraction of SX-3Ambersorb/Isolute ENV+ (pesticides and hormones) 50 mL of 1:1:8 (v:v:v) methanol:toluene:methylene chloride was used. Extracts from the POCIS were evaporated (over UHP nitrogen) to a volume of 1 to 2 mL and filtered through glass fiber filter paper using methanol as the transfer solvent and then 0.5 mL of transfer solvent added to the filter as the final rinse. After extraction, EST shipped samples in sealed 2.5 mL amber glass ampoules to the U.S. EPA Region 8 Laboratory for chemical analyses and quantification as already described.

We also used the POCIS data to estimate the time weighted average concentration of each PPCP found in the refuge using the equation:

$$C_w = \frac{M_s}{R_s * t}$$

Where C_w is the concentration in the water phase (ng/L), M_s is the mass in the receiving phase (POCIS), R_s is the sampling rate (L/d) and t is the time (days) (Balaam, Grover et al. 2010). As *Rs* depends on the aqueous diffusion coefficient (m^2/s), the thickness of the stagnant film layer (m), and the surface area of the sampler (m^2), *Rs* values are compound-specific. Thus for each of the analytes in our studies, appropriate *Rs* values were either identified from the literature (Table 1) or estimated based on their polar or non-polar properties.

2.3.5 Fish collection and reproductive assessment

With the authorization of the Colorado Division of Wildlife, SCC-7, CC-1, and DM-3 creeks were selected for sampling 12 individuals (mixed genders) of

fathead minnows and Rio Grande chubs. Tables 2a and 2b summarize fish sampling events for August 2011 and May 2012, respectively. Since no Rio Grande chubs were found in the reference site (DM-3), molecular analyzes were not performed in this species. However, because Rio Grande chubs are considered of great conservation need in Colorado, and since we had collected these fish from the additional two sites, we measured all other fish endpoints (see more on this below) including gonadal histology and present that data here for future reference.

Fish were collected with a LR-24 backpack electrofisher (Smith-Root, Inc., Vancouver, WA, USA) using a quick-setup mode in which adjustments are automatically made to produce 30 Hz, 12% duty cycle, and 25 watts average output power based on conductivity and temperature of the stream. All fishes were transported alive in an aerated reservoir for sampling. Fish were euthanized in 300 mg/L of tricaine methanesulfonate solution (MS-222) and weighted and measured (total length) using a digital scale and calipers and this information used to calculate condition factor (CF). Gonads and livers were removed and weighted for determination of gonadosomatic index (GSI) and hepatosomatic index (HSI), respectively. Biological indexes were calculated using the following formulas:

$$CF = \left(\frac{Body\ weight}{Total\ lenght^3}\right) \times 10^5\ ,\ \ GSI = \left(\frac{Gonad\ weight}{Body\ weight}\right) \times 10^2\ ,\ \ HSI = \left(\frac{Liver\ weight}{Body\ weight}\right) \times 10^2$$

A portion of each tissue was placed in a cryovial and flash frozen in liquid nitrogen and stored at -80°C for qPCR analysis. Another section was placed in pre-labeled plastic tissue cassettes and stored in 10% buffered formalin for histological examination.

2.3.6 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Changes in the expression of genes involved in gonadal development and reproduction were quantified in gonads and livers of fish caught from the abovementioned sites. Tissues were placed in 1 mL TriSure (Bioline, Tauton, MA, USA) and homogenized using a pellet pestles cordless motor homogenizer (Sigma-Aldrich, St. Louis, MO, USA). RNA was extracted and quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA quality was determined by the ratio of A260/A280 with samples with a ratio > 1.8 used for qPCR analyses. RNA was treated with DNase I (Fermentas Inc., Glen Burnie, MD, USA) to remove any genomic DNA contamination. cDNA was synthesized by DNase I treated using the high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA, USA) and primed with random primers. All processes were performed following manufacturer's instructions.

Five genes: gonadal aromatase (*cyp19a*), estrogen receptor alpha (*er*α), androgen receptor (*ar*), doublesex and mab-3 related transcription factor (*dmrt1*), and vitellogenin (*vtg*) were quantified with ribosomal protein L8 (*rpl8*) used as a housekeeping gene (Filby and Tyler 2007). Gene specific primers for qPCR were selected from the literature or developed using Primer 3 v.0.4.0 (Table 3). Primer

sequences for fathead minnows were retrieved from the National Center for Biotechnology Information (NCBI) database and purchased through Integrated DNA Technologies (Coralville, IA, USA). Gene expression analysis was performed using SYBR® green supermix on a Bio-Rad CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA). Reactions (20 μl total volume) contained 7 μL 2x qPCR mix (Bio-Rad Laboratories, Hercules, CA, USA), 0.5 μl each of gene specific primers (forward and reverse), 10 μL of nuclease-free water, and 2 μl of cDNA template (15 ng/μL). All samples were carried out in duplicate with no-template negative controls. Conditions used to amplify samples consisted of: Initial template denaturation at 95°C for 3 min; 40 cycles of 95 °C for 10 sec; annealing of primers at 58 °C for 30 sec; extension of product at 72 °C for 30 sec; and final extension at 65 °C to 95 °C in increments of 0.5 °C for 5 sec. Expression of the target genes was normalized relative to the expression of the housekeeping gene (*rpl8*). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008).

2.3.7 Histological analyses

Midsection fish gonads and livers were processed for histological evaluation at the Purdue University Histology and Phenotyping Laboratory (West Lafayette, IN, USA). Tissues were embedded in paraffin, sectioned (every 5 μm for a total of three sections), and subsequently stained with hematoxylin and eosin (H&E). Histological samples were analyzed using a light microscope (10 – 1000X) to determine gender, reproductive stage, and to identify any cellular

changes (USEPA 2006; Johnson, Wolf et al. 2009). The severity of cellular changes were qualitatively graded as absent, minimal, mild, moderate, or severe according to published guidelines (Johnson, Wolf et al. 2009).

2.3.8 Statistical analysis

Data was checked for normality using Shapiro-Wilk test and non-normal data was log-transformed prior to analyses. One-way analysis of variance (ANOVA) was used to compare differences in analyte concentration, water quality parameters, CF, GSI, HSI, and qPCR among sampled creeks ($P < 0.05$) only for male fathead minnows from 2011, and male and female fathead minnows from 2012. No statistics were made for female fathead minnow from 2011 (only 2 samples), and females and males Rio grande chubs in any of these years (no fish reference was found). Non-parametric tests (Chi-Square) were used to determine differences in the frequency of cellular changes across sites. No statistical analysis was performed for nutrient concentrations since only one sample was collected per site. Data is expressed as the mean ± standard deviation (S.D.) or standard error (S.E.).

2.4 Results

2.4.1 Water quality and nutrient analyses

Water flow into the creeks inside and outside the refuge begins to increase in early May with flow peaking the last week of May (Figure 3). Mean values ± S.E. of water quality parameters collected in August 2010 (pilot

sampling), August 2011, May 2012 and August 2012 from SCC-7, CC-1, and DM-3 are shown in Figures 5 and 6. The only parameter that differed across sites was pH which reached ~9 at CC-1 and was significantly higher compared to DM-3 (P =0.002) and SCC-7 (P = 0.001).

Water samples were collected only in May 2012 for quantification of nutrients (total nitrogen, N and phosphorus, P). Results are shown in Table 4. As expected, the WWTP effluent (AO-1) had the highest levels of nutrients, while SCC-7 had the lowest.

2.4.2 Water concentrations of PPCPs

A pilot sampling event was conducted in August 2010 (10 water samples were collected) in order to evaluate feasibility of the study and confirm the presence of PPCPs in the area. Thirty three PPCPs were detected in the WWT effluent (AO-1) and sulfamethoxazole (antibiotic) was detected at the highest concentration (705 ng/L). Only theophylline (bronchodilator) and triclocarban (antibacterial) were found in CC-1, and warfarin (anti-coagulant) was identified in CC-3 with concentrations < 25 ng/L (see Table A1). Furthermore, E2-glucuronide (metabolite of E2) and theophylline (bronchodilator) were detected in CD-1. Importantly, no PPCPs were detected in SCC-7 and DM-3 creeks, the latter chosen as the 'control' site in these studies.

In August 2011, 39 PPCPs were detected only in the sewage effluent from the Aspen WWTP, with gabapentin (anti-convulsive) being detected at the

highest concentration (13,700 ng/L). However, no PPCPs were detected in any of the sites from which fish were sampled (CC-1, DM-3, and SCC-7) (Table A1).

In 2012, 45 and 32 PPCPs were identified in the WWT effluent sampled in May and August, respectively. In both sampling events, opiate analgesics were the most common category detected (Table A1). In May, DEET was detected at concentrations ranging between 12 and 24 ng/L from samples collected at CC-2, CWC-2, WC-2, SCC-1, and SCC-6. In addition, concentrations of caffeine were detected in the range of 11 - 25 ng/L in most sites sampled in August (CC-1, CC-2, CD-1, CD-3, CWC-2, CWC-4, DM-1, DM-2, DM-3, NCC-1, SCC-6, SCC-7, SC-2 and SC3).

2.4.3 Polar Organic Chemical Integrative Sampler (POCIS)

Sixty three analytes were detected at site AO-1 downstream from the Aspen WWTP (Table A2) with DEET found at the highest concentration. Five analytes were detected in CC-1 (caffeine, cannabidiol, DEET, progesterone, and triclosan); twelve in DM-3 (atenolol, caffeine, DEET, diphenhydramine, gemfibrozil, ibuprofen, levorphanol, naproxen, progesterone, THC, triclocarban, and triclosan); and 5 in SCC-7 (caffeine, DEET, triclocarban, triclosan, and trimethoprim) with DEET detected at the highest concentration (Figure 7 and Table A2). No significant differences were seen in cumulative water concentration between fish sites (SCC-7, CC-1, and DM-3). However, cumulative water concentration of the WWTP effluent was significantly higher compared to the three fish sites (SCC-7: $P = 0.014$, CC-1: $P = 0.024$, and DM-3: $P = 0.030$).

2.4.4 Grab and POCIS water samples

Grab and POCIS water samples were only collected during June 2012 from some of the sites. Fish sites (SCC-7, CC-1, and DM-3) were chosen to estimate PCPP uptake by fish and other aquatic organisms. The WWTP outflow (AO-1) was used to estimate the capacity of the Aspen WWTP to remove PPCPs. Compared to grab samples, POCIS samplers allowed for the detection of an additional 18 analytes (Figure 8 and Table A2). Equally important, POCIS allowed for the detection of PPCPs in all our fish sites. Although troublesome, because they were found also in our 'control' site (Figure 7).

2.4.5 Biomarker analyses

In August 2011 and May 2012, 59 and 60 fishes respectively were collected from SCC-7, CC-1 and DM-3 (12 from each site) and several biological endpoints measured. Overall, CFs were ~ 1.0 regardless of location, indicating fish were in good general condition. In fathead minnows, CF differed between sites only for males. In 2011, males from CC-1 had a higher CF (P = 0.036) whereas in 2012, there was a decrease in CF in males from SCC-7 (P = 0.009) compared to the reference site (DM-3) (Tables 5 and 7). In Rio Grande chubs, CFs were higher from CC-1 compared to SSC-7 in males and females from both years (Tables 6 and 8).

Gonadosomatic indexes were higher in 2012 regardless of species and gender. This strongly suggests that fish were sampled past the spawning season during 2011 (August) compared to 2012 (May). During both years of study, no

differences in GSIs were observed between sites for male and female fathead minnows (Tables 5 and 7). However, during both years of study, female Rio Grande chubs from SCC-7 had over twice the GSI compared to females from CC-1 (Tables 6 and 8).

Similarly to what was observed with GSIs, HSIs were much higher in 2012 for all fish examined (Tables 5 to 8). This makes sense, since livers increase in weight during the spawning season and fish sampled in 2011 were likely collected past the peak of the spawning season. In 2011, female fathead minnows and Rio Grande chubs from CC-1 had a lower HSI compared to females from SCC-7 and DM-3. In 2012, this was the case only for female fathead minnows from SCC-7 and CC-1 compared to DM-3 (P = 0.008, P = 0.0007, respectively).

2.4.6 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

In 2011 only 2 fathead minnow females from the 'control' site were collected thus no gene expression analyses were conducted for females this year. For the remaining fathead minnows, there were no significant differences in the expression of *cyp19a*, *er*α, *dmrt1*, and *vtg* genes compared to the 'control' in either of the two years. However, in 2011 significant increases were detected in males from SCC-7 and CC-1 for *ar* (P = 0.035 and P = 0.048, respectively) and for s*tar* (P = 0.035 and P = 0.0006). In contrast, in 2012 a down-regulation of *ar* and *star* was observed in females from CC-1 (P = 0.00013) and males from SCC-7 (P = 0.0008) compared to the 'control' site DM-3 (Figure 9).

2.4.7 Gonad and hepatic histology

During 2011, ovaries from fathead minnows and Rio Grande chubs contained only immature or early stage oocytes (Figure 10a). This is in contrast to what was observed during 2012, in which ovaries contained mostly late vitellogenic with mature and spawned follicles (Figure 10b). In addition, six types of histological changes were observed in ovaries from both species sampled from three of the creeks (SSC-7, CC-1, and DM-3). These included presence of cytoplasmic retraction, atretic oocytes, karyoplasmic retraction, sinusoidal chorionic shape, empty follicles, and anucleated oocytes (Figure 11). This data is summarized for both species in Table 9 for 2011 and in Table 10 for 2012.

Similarly to what was observed with females, during 2011 testicular stages ranged from juvenile (undeveloped) to early spermatogenesis for both species (Figure 12a). In contrast, during 2012 testes from both species were in mid to late spermatogenic stages (Figure 12b). Cellular changes included presence of melanomacrophage centers (MMCs), and accumulation of proteinaceous fluid in seminiferous tubules (Figure 13). Data on cellular changes are summarized in Tables 9 and 10. Male fathead minnows and Rio Grande chubs from 2012 exhibited an increase of proteinaceous fluid accumulations; and MMCs were only present in male fathead minnows.

Livers from females and males sampled during both years of study exhibited proteinaceous fluid accumulation, MMCs and no cytoplasmic vacuolization (Figure 14). In 2011 fathead minnows from SCC-7 only presented a loss of cytoplasmic vacuolization, while fathead minnows from CC-1 and DM-3

exhibited proteinaceous fluid accumulation, loss of cytoplasmic vacuolization, and presence of MMCs. During 2011, frequency of cellular changes significantly differed only between SCC-7 and DM-3 (X^2 = 6, *P* = 0.0498). In 2012, significant differences in the frequency of liver alterations were observed between SCC-7 in relation to CC-1 (X^2 = 9.1, *P* = 0.01) and DM-3 (X^2 = 8.1, *P* = 0.02). All three cellular alterations were observed in all three sites, except for fish from DM-3 which showed no MMCs. The highest frequency of hepatic alterations was observed in fishes from SCC-7.

2.5 Discussion

Results from this three-year field study indicate that PPCPs are being released from the Aspen WWTP and entering BNWR surface waters. The Aspen WWTP utilizes an activated sludge sequence batch reactor system which is not designed to remove organic chemicals. However, studies have demonstrated that a critical minimum solid retention time (SRT) can achieve a significant reduction in many of the PPCPs present in wastewater treatment effluents (Ternes, Stumpf et al. 1999; Kanda, Griffin et al. 2003; Joss, Andersen et al. 2004; Kreuzinger, Clara et al. 2004; Clara, Kreuzinger et al. 2005; Nakada, Tanishima et al. 2006; Wick, Fink et al. 2009; Snyder, Lue-Hing et al. 2010). SRT is one of the most important control parameters for the activated sludge process that promotes the growth of a diverse biological community able to degrade xenobiotic compounds. Excellent removal (>80%) by activated sludge has been reported for caffeine, ibuprofen, thymol, morphine, aspirin, and many others

(Kreuzinger, Clara et al. 2004; Clara, Kreuzinger et al. 2005; Nakada, Tanishima et al. 2006; Oppenheimer, Stephenson et al. 2007; Snyder, Lue-Hing et al. 2010). On the other hand, some compounds with poor removal efficiency (<50%) include DEET, gemfibrozil, tramadol, metoprolol, naproxen, atenolol, sotalol, propranolol, and carbamazepine (Kreuzinger, Clara et al. 2004; Nakada, Tanishima et al. 2006; Oppenheimer, Stephenson et al. 2007; Radjenovic, Petrovic et al. 2007; Wick, Fink et al. 2009; Snyder, Lue-Hing et al. 2010). Therefore, the presence of high concentration of DEET, gemfibrozil, and other beta blockers may be due to their inefficient removal by the activated sludge process.

Two water sampling techniques (grab and POCIS) were used to determine PPCPs in the field. Grab samples detected an average of 37 PPCPs in 2010 and 2012 from the WWTP effluent samples (AO-1). In 2010, low concentrations of theophylline (19.7 ng/L) and triclocarban (20.2 ng/L) were detected in only one of the fish sites (CC-1). Both compounds were also detected from AO-1 at concentrations of 93.6 and 146 ng//L, respectively. In 2011, no PPCPs were detected in any of the refuge creeks. And, in August 2012 caffeine was detected in all the fish sites (CC-1, SCC-7, and DM-3). However, caffeine was also detected in other creeks outside the refuge boundary. This finding indicates that caffeine could be entering the creeks from other sources such as from leakage of private septic systems from Crestone and the Baca Subdivision, from caffeine-producing plants, or from foreign campers visiting the mountains area.

On the other hand, POCIS detected PPCPs in all sites with 18 additional PPCPs being identified from AO-1 compared to grab samples. This is because POCIS measure cumulative levels of PPCPs from large volumes of water over several days, mimicking the bioconcentration process occurring in aquatic animals. This allows for the detection of episodic events in environmental contaminant concentrations. Therefore, and as shown previously by other studies (Jones-Lepp, Alvarez et al. 2004; Alvarez, Stackelberg et al. 2005; Macleod, McClure et al. 2007; Togola and Budzinski 2007; Mazzella, Debenest et al. 2008; Zhang, Hibberd et al. 2008; Rujiralai, Bull et al. 2011; Miege, Budzinski et al. 2012), POCIS sampling should be considered over traditional grab sampling methods for these types of compounds.

Most of the analytes detected in POCIS were prescription drugs (83%) with 32 different drug classes representing opiate analgesics (11%), antidepressants (9%), benzodiazepines (6%), and calcium-channel blockers (6%). Sixty-three PPCPs were detected in AO-1 with calculated time-weighted average water concentrations ranging from 0.01 to 191 ng/L. Ten of the analytes with the highest concentration at this site were DEET, gemfibrozil, diphenhydramine, temazepam, morphine, venlafaxine, ibuprofen, tramadol, metoprolol, and levorphanol. As already discussed, POCIS detected more PPCPs in the fish sites (SCC-7, CC-1, and DM-3) compared to the grab samples. Five analytes were detected in SCC-7 ranging from 0.003 to 16 ng/L (caffeine, DEET, triclocarban, triclosan, and trimethoprim), five in CC-1 ranging from 0.01 to 48 ng/L (caffeine, cannabidiol, DEET, triclosan and progesterone) and twelve

in DM-3 ranging from 0.01 to 62 ng/L (atenolol, caffeine, DEET, diphenhydramine, gemfibrozil, ibuprofen, levorphanol, naproxen, progesterone, THC, triclocarban, triclosan). Three analytes were common in all four sampling sites: caffeine, triclosan, and DEET. DEET was found at the highest concentration in all fish sites with cumulative water concentration ranging from <36,410 ng/L – 143,000 ng/L> and calculated time-weight average water concentrations from <15 ng/L – 63 ng/L>. Although no significant difference was found across fish sites, they all contained lower PPCPs than AO-1. The absence of PPCPs in the 'control' site during 2011 could have been due to having analyzed only grab samples that year.

Previous studies have reported low concentrations of PPCPs in surface water. Some of the most commonly detected PPCPs are ibuprofen, diphenhydramine, gemfibrozil, triclosan, triclocarban, DEET, and caffeine. Ranges of reported concentrations are: ibuprofen <200 ng/L – 1000 ng/L> (Kolpin, Furlong et al. 2002), diphenhydramine <10 ng/L – 100 ng/L> (Berninger, Du et al. 2011), gemfibrozil <9 ng/L – 1500 ng/L> (Skolness, Durhan et al. 2012), triclosan <0.1 ng/L – 2300 ng/L>, triclocarban <19 ng/L – 1425 ng/L>, DEET <13 ng/L – 660 ng/L> (Brausch and Rand 2011), and caffeine <6 ng/L – 250 ng/L> (Gomez-Martinez 2011). All the calculated time-weight average water concentrations of the PPCPs found in the fish sites were in the range of reported surface water concentrations.

Total N and total P concentrations exceeded maximum levels (0.12 - 2.2 mg/L and 0.01 - 0.075 mg/L, respectively) set by the USEPA only at the WWTP

effluent site (3.26 mg/L and 0.308 mg/L respectively) (USEPA 2002). At the fish sites, all other water quality parameters fell within ranges adequate for supporting the fish species studied with a range of pH 7 - 9, DO 6 - 8 mg/L, temperature 14 - 17°C, and conductivity 150 - 200 uS/cm.

Our second goal was to evaluate potential reproductive effects on native fish due to PPCP contamination. During the first year of study, fish were sampled in late August which resulted in all fish having lower GSIs and mostly immature gamete stages compared to fish sampled in May during the second year. Because all of our fish measurements are impacted by reproductive stage, comparisons between years are not possible. Overall CFs were ~1 indicating a good proportion between weight and length of the fish. In addition, gonad and liver cellular changes were observed in fishes from the three sites (SCC-7, CC-1, and DM-3). During both years of study, the highest incidence of liver changes was observed in SCC-7. The incidence of melanomacrophage centers (MMCs) was highest in fathead minnows sampled in 2012 from SCC-7. Increases in MMCs have been attributed to enhanced phagocytic activity by macrophages suggesting it as a biomarker for assessment of chemical pollution in water (Kranz 1989). However, their use for evaluating exposure to PPCPs is not clear. Herraez and Zapata (Herraez and Zapata 1986) reported an increase in MMCs in the kidney and spleen of goldfish (*Carassius auratus*) when erythrocytes were exposed to phenylhydrazin, a chemical intermediate of many pharmaceuticals mainly of tryptamine drugs (used in the treatment of migraine).

Finally, we found significant differences in the expression of *ar* and *star* across sites. In 2011 an up-regulation of *ar* and *star* was observed in males from SCC-7 and CC-1. Also, a down-regulation of *ar* in females from CC-1 and *star* in males from SSC-7 was observed in 2012. Since fish were sampled at different reproductive stages in 2011 compared to 2012, these changes could be normal and related to timing of sampling.

In conclusion, the present study demonstrated that POCIS is a suitable sampling device for PPCPs. Because of the lack of a proper reference site, molecular alterations (*star* and *ar*) and cellular changes in gonads and livers cannot be attributed to PPCP exposure at this time. Further controlled research is necessary to provide a better picture of the environmental behavior and toxicity of the PPCPs identified in this study, the focus of Chapter 3.

Acknowledgements -- Ron Garcia, Refuge Manager, Baca National Wildlife Refuge, provided information on fish diversity and abundance.

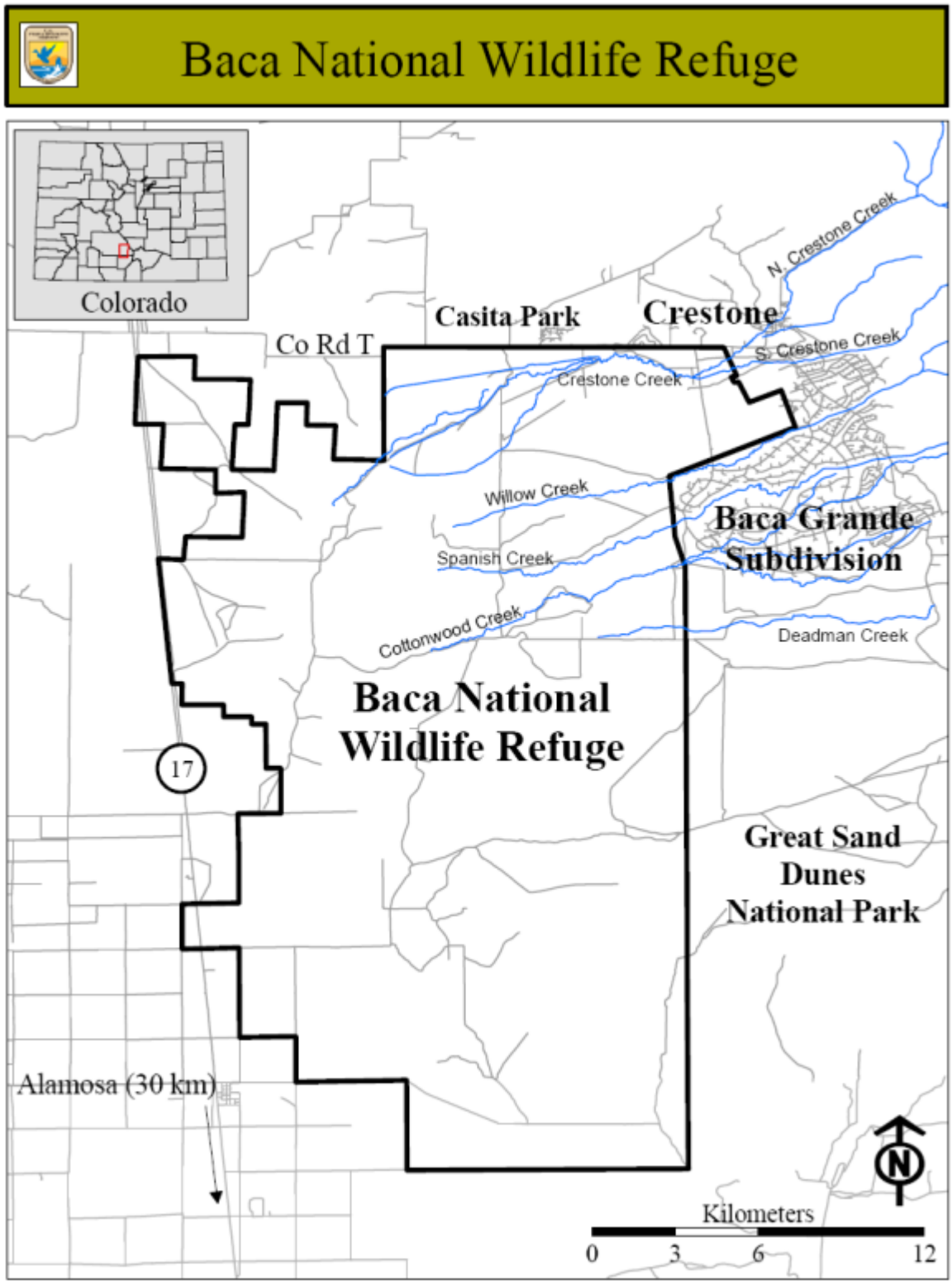

Figure 2.1 Baca National Wildlife Refuge.

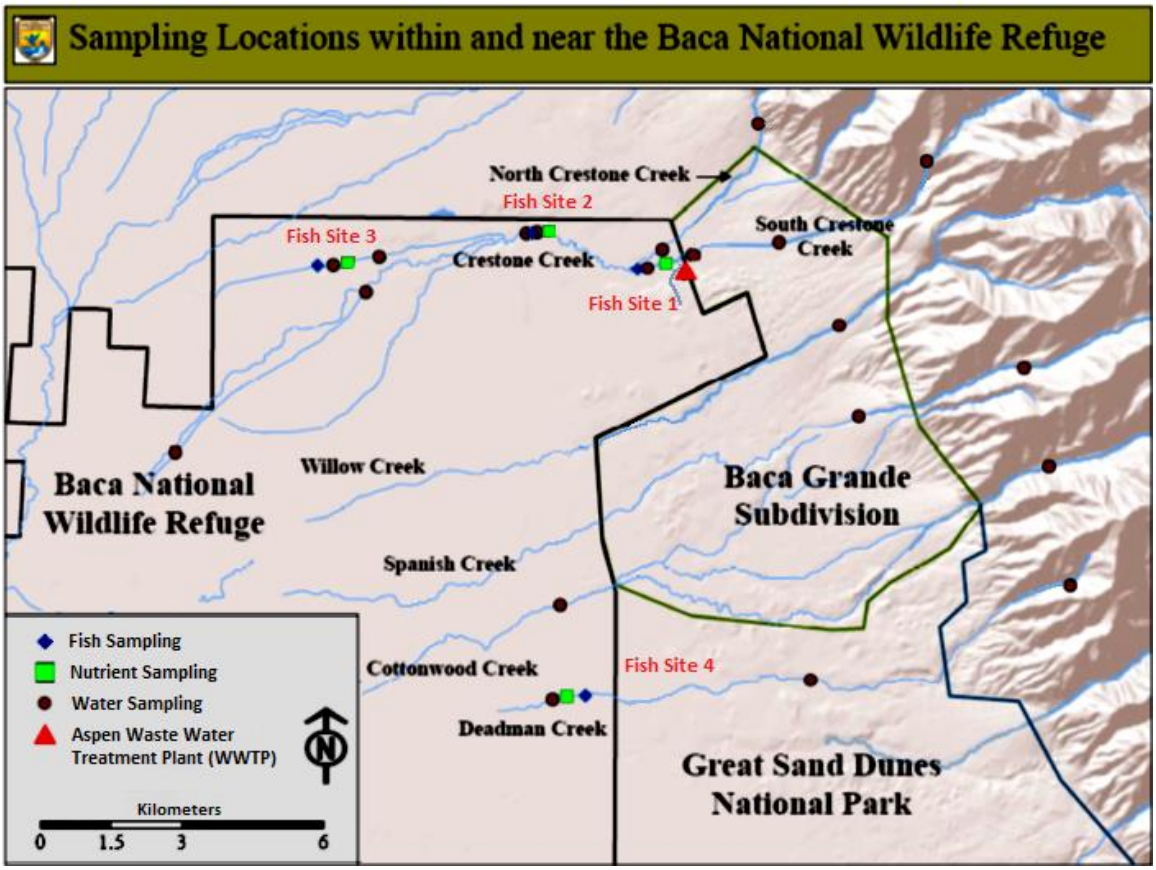

Figure 2.2 Fish, nutrient, and water sampling locations in relation to the Aspen waste water treatment plant (WWTP).

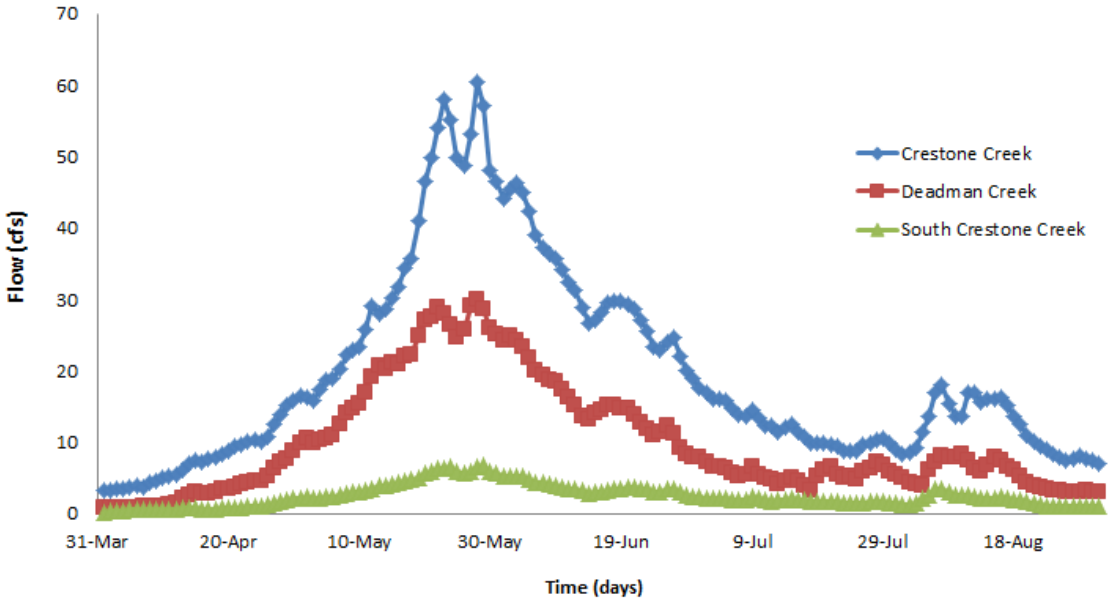

Figure 2.3 Average water flow from each of the creeks sampled in this study.

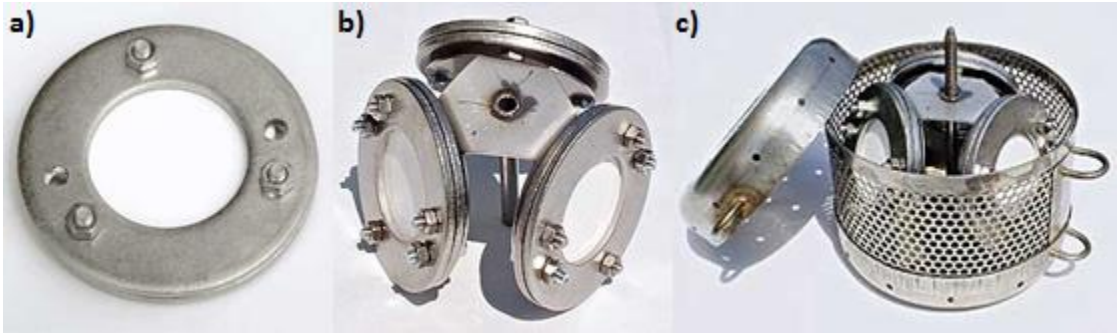

Figure 2.4 Polar Organic Chemical Integrative Sampler (POCIS): a) POCIS membrane, b) POCIS carrier, and c) POCIS holder.

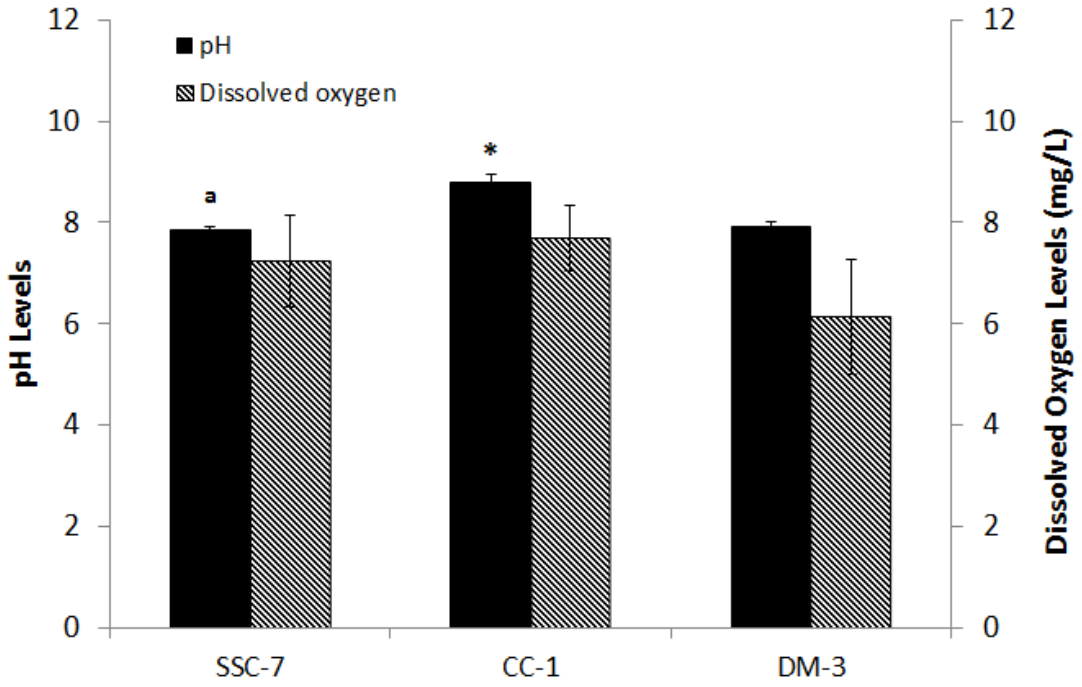

Figure 2.5 pH and dissolved oxygen levels from South Crestone (SCC-7), Crestone (CC-1) and Deadman creek (DM-3) collected in August 2011 and 2012 (n=3). Asterisk indicates significant differences compared to the control (DM-3) and letter significant differences among sites (SCC-7 and CC-1). Data are mean ± SD.

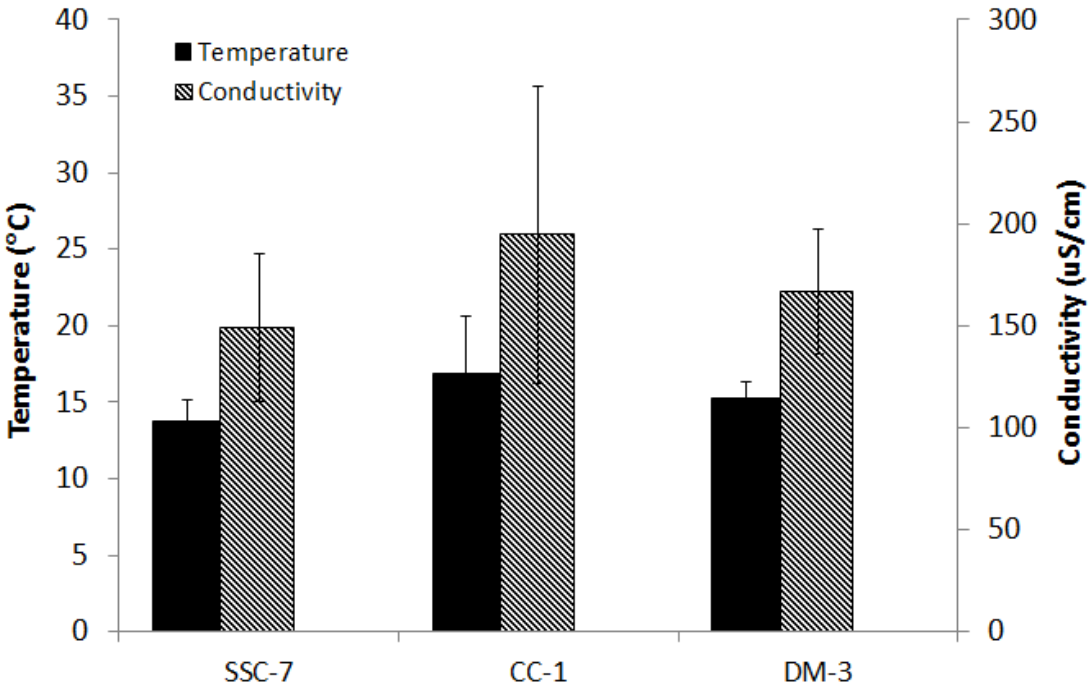

Figure 2.6 Temperature and conductivity levels from South Crestone (SCC-7), Crestone (CC-1) and Deadman creek (DM-3) collected in August 2011 and 2012 (n=3). Data are mean ± SD.

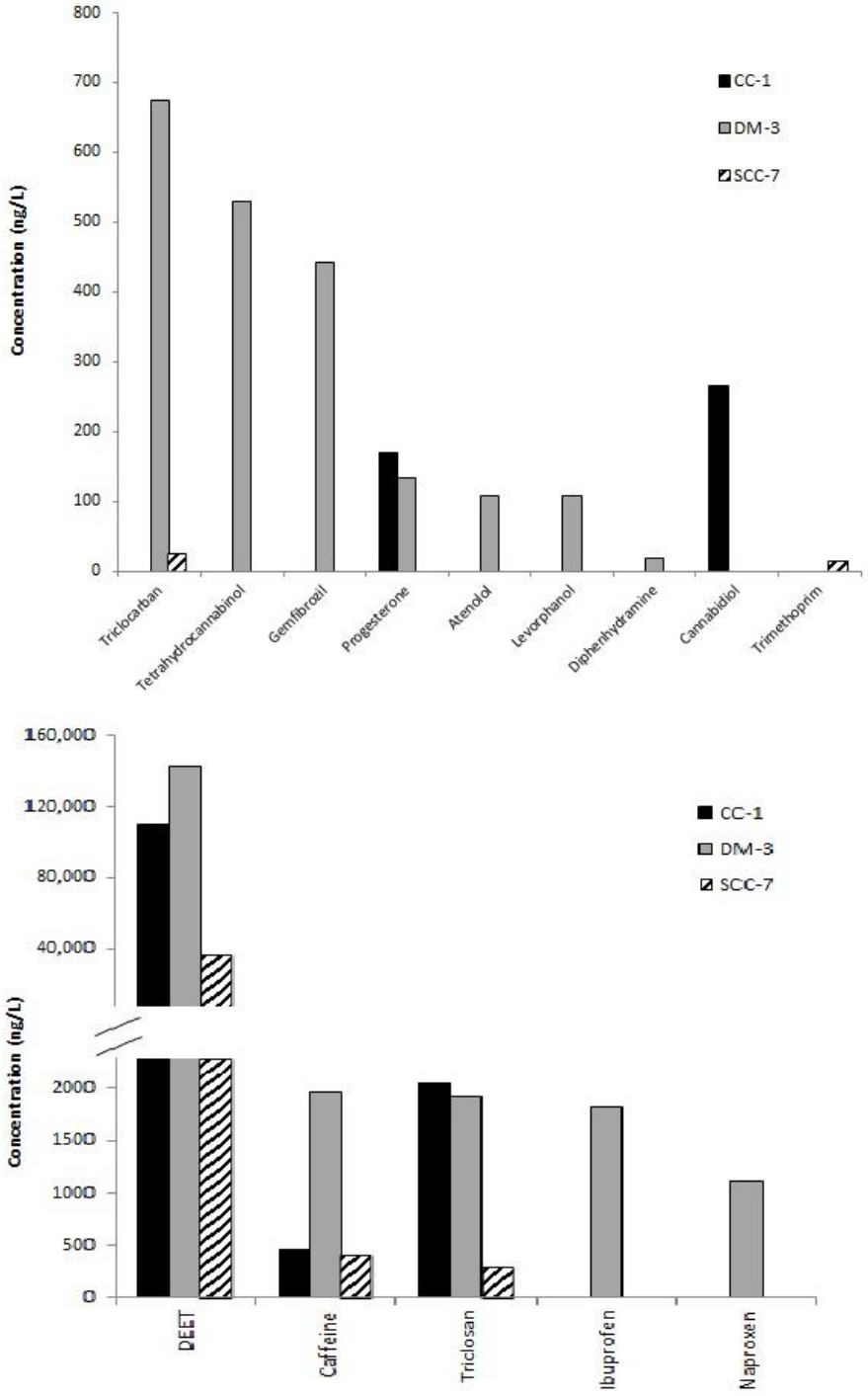

Figure 2.7 Cumulative water concentration of different pharmaceuticals and personal care products (ng/L) quantified by Polar Organic Chemical Integrative Sampler (POCIS) after a 30-d deployment from South Crestone (SCC-7), Crestone (CC-1) and Deadman creek (DM-3).Samples were collected in May 2012.
*DEET: N,N-Diethyl-meta-toluamide.

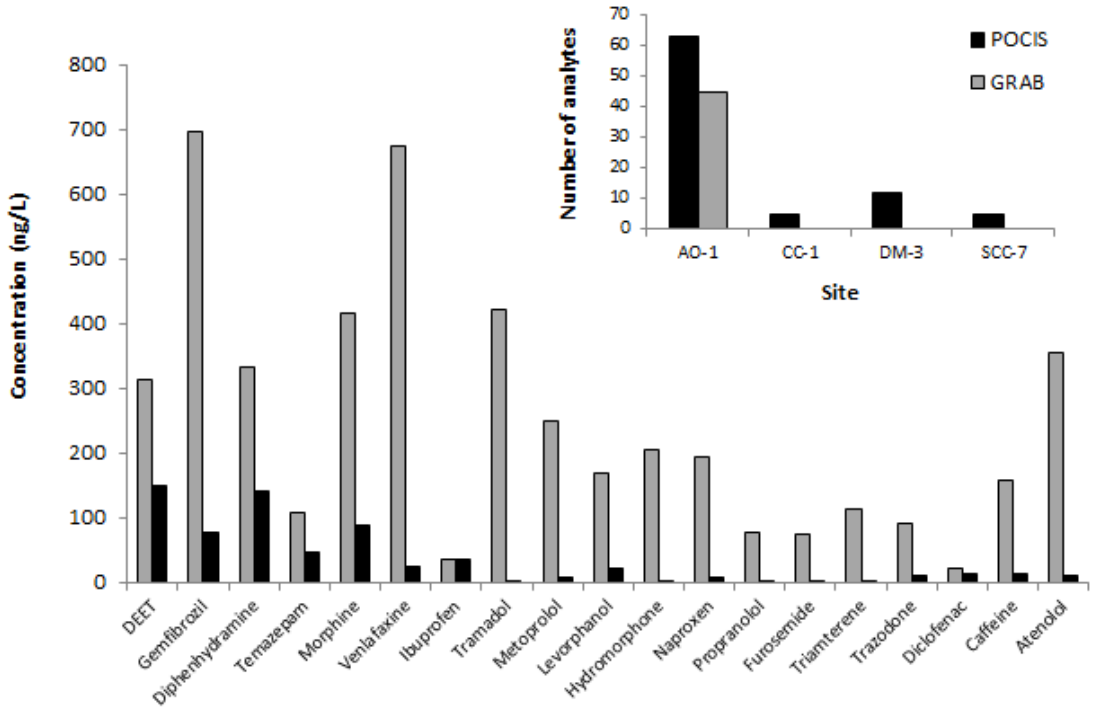

Figure 2.8 Time-weighted average water concentration for 30 days polar organic chemical integrative sampler (POCIS) and grab samples in ng/L of PCPs found at higher concentration. And number of analytes detected for each sampling technique from South Crestone (SCC-7)m Crestone (CC-1), Deadman creek (DM-3), and WWTP effluent (AO-1). Samples were collected in May 2012. *DEET: N,N-Diethyl-meta-toluamide.

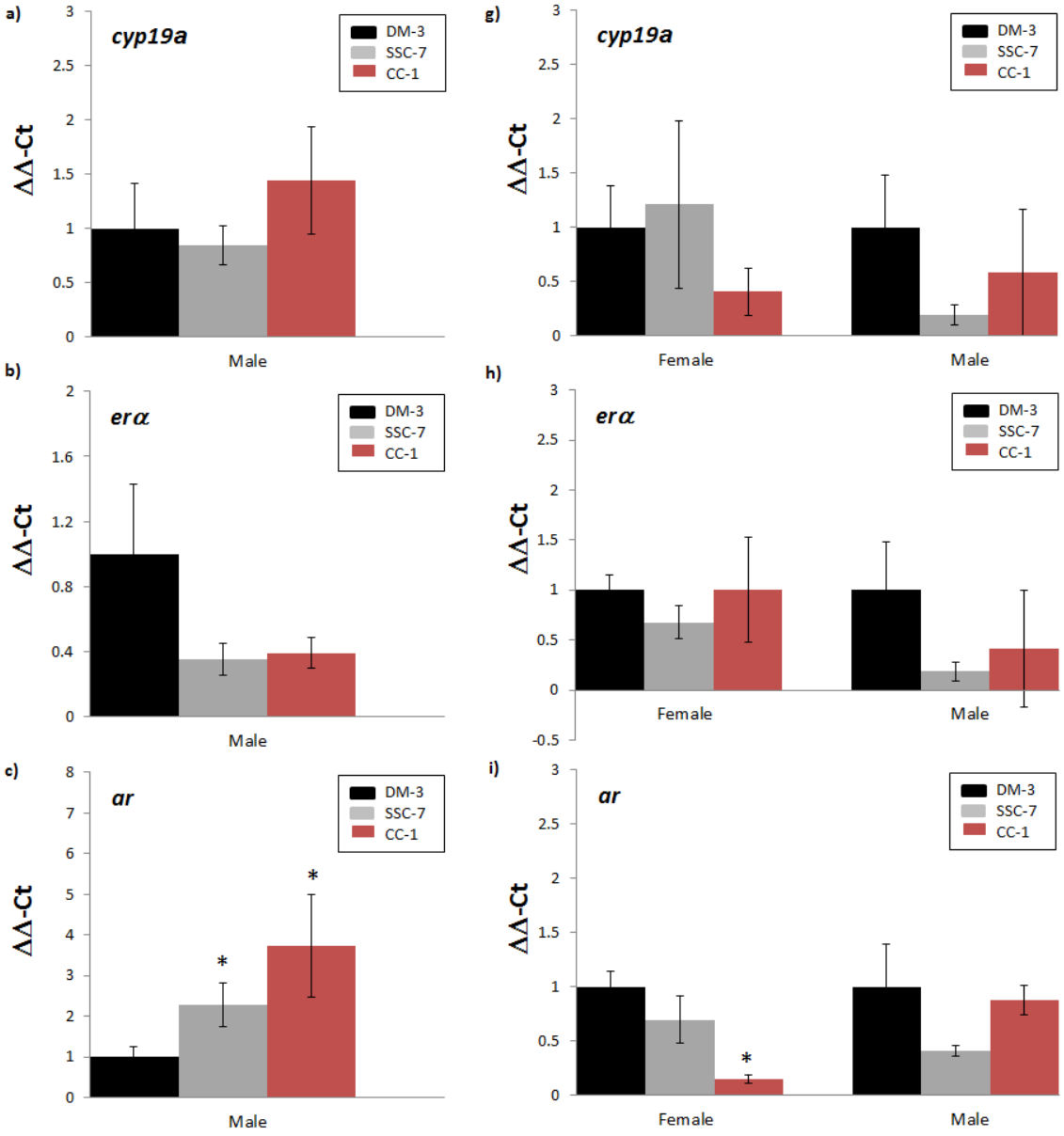

Figure 2.9 Relative gene expression of aromatase (cyp19a), estrogen receptor alpha (erα), and androgen receptor (ar) in female and male fathead minnow collected from South Crestone (SCC-7), Crestone (CC-1) and Deadman creek (DM-3) in a-c) 2011 (left panels) and g-i) 2012 (right panels). Shown are means ± SE in relation to controls using the $\Delta\Delta$Ct method. Significant differences, $P < 0.05$, are depecited by asterisks.

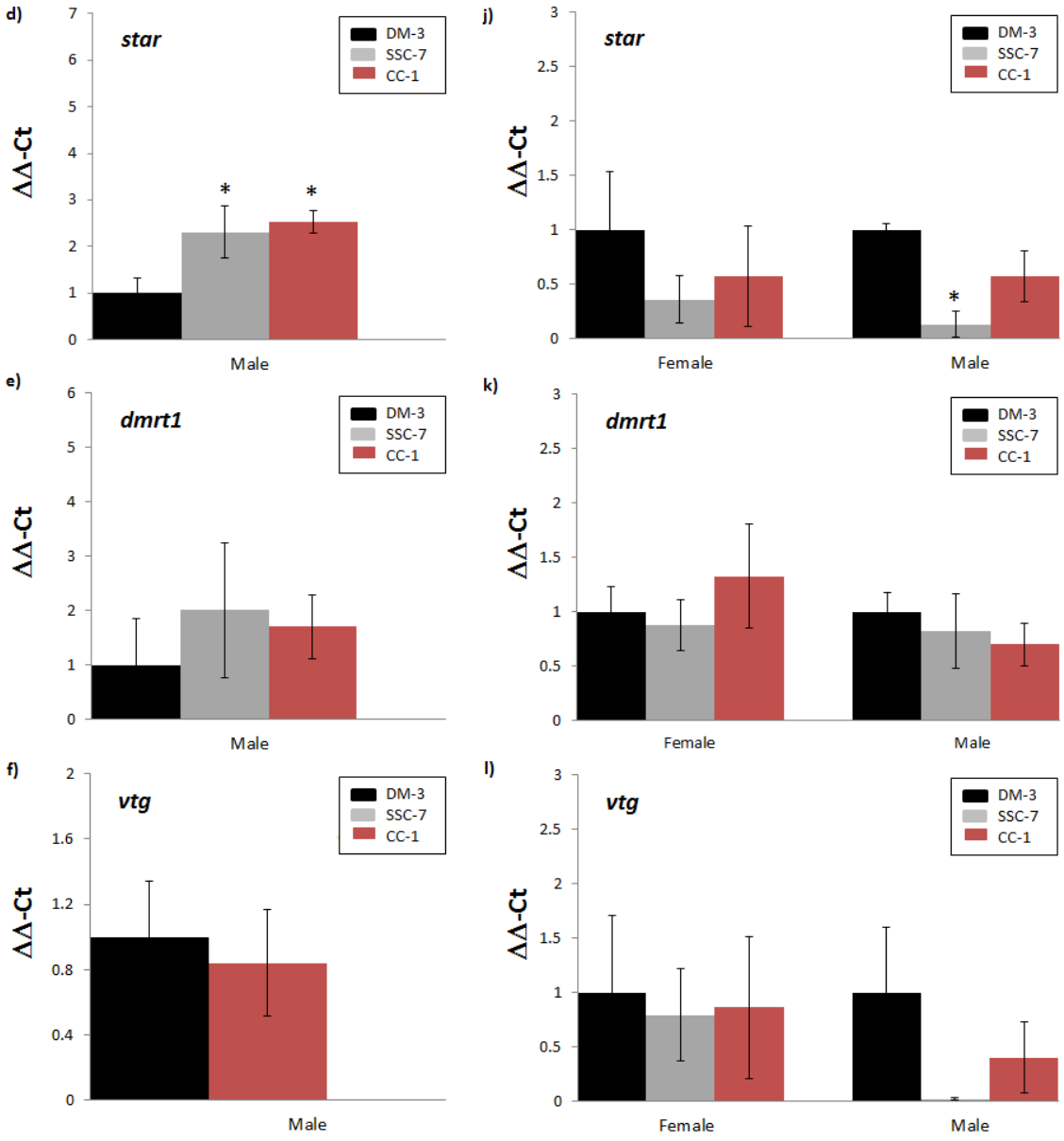

Figure 2.10 Relative gene expression of steroidogenic acute regulatory protein (star), doublesex and mab-3 related transcription factor 1 (dmrt1), and vitellogenin (vtg) in female and male fathead minnows collected from South Crestone (SCC-7), Crestone (CC-1) and Deadman creek (DM-3) in d-f) 2011 (left panels) and j-l) 2012 (right panels). Shown are means ± SE in relation to controls using the ΔΔCt method. Significant differences, $P < 0.05$, are depicted by asterisks.

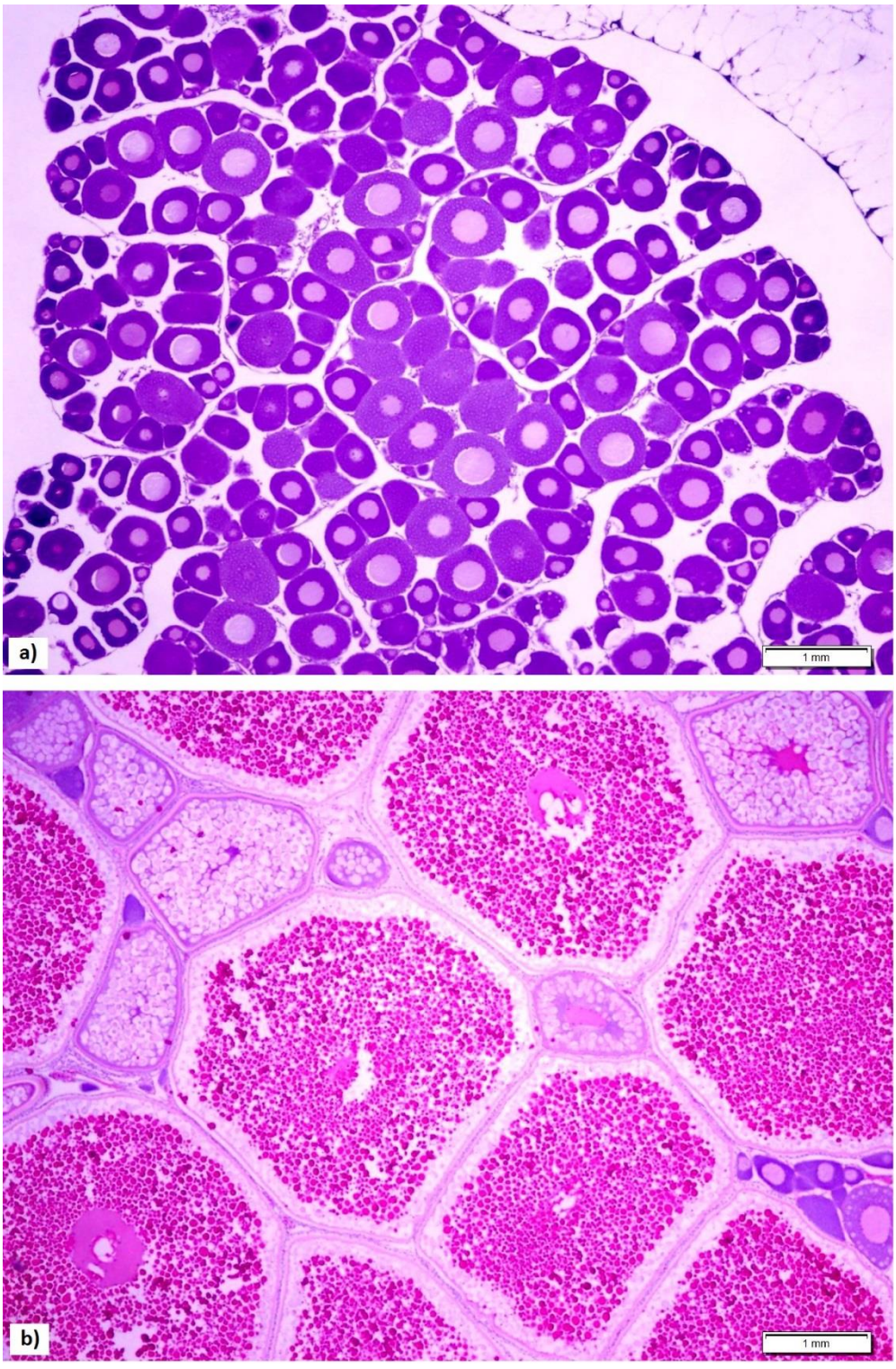

Figure 2.11 Histological sections showing gonadal maturation stages of female fathead minnows. a) Immature fish ovaries collected in 2011 and b) mature fish ovaries collected in 2012 (paraffin, H&E).

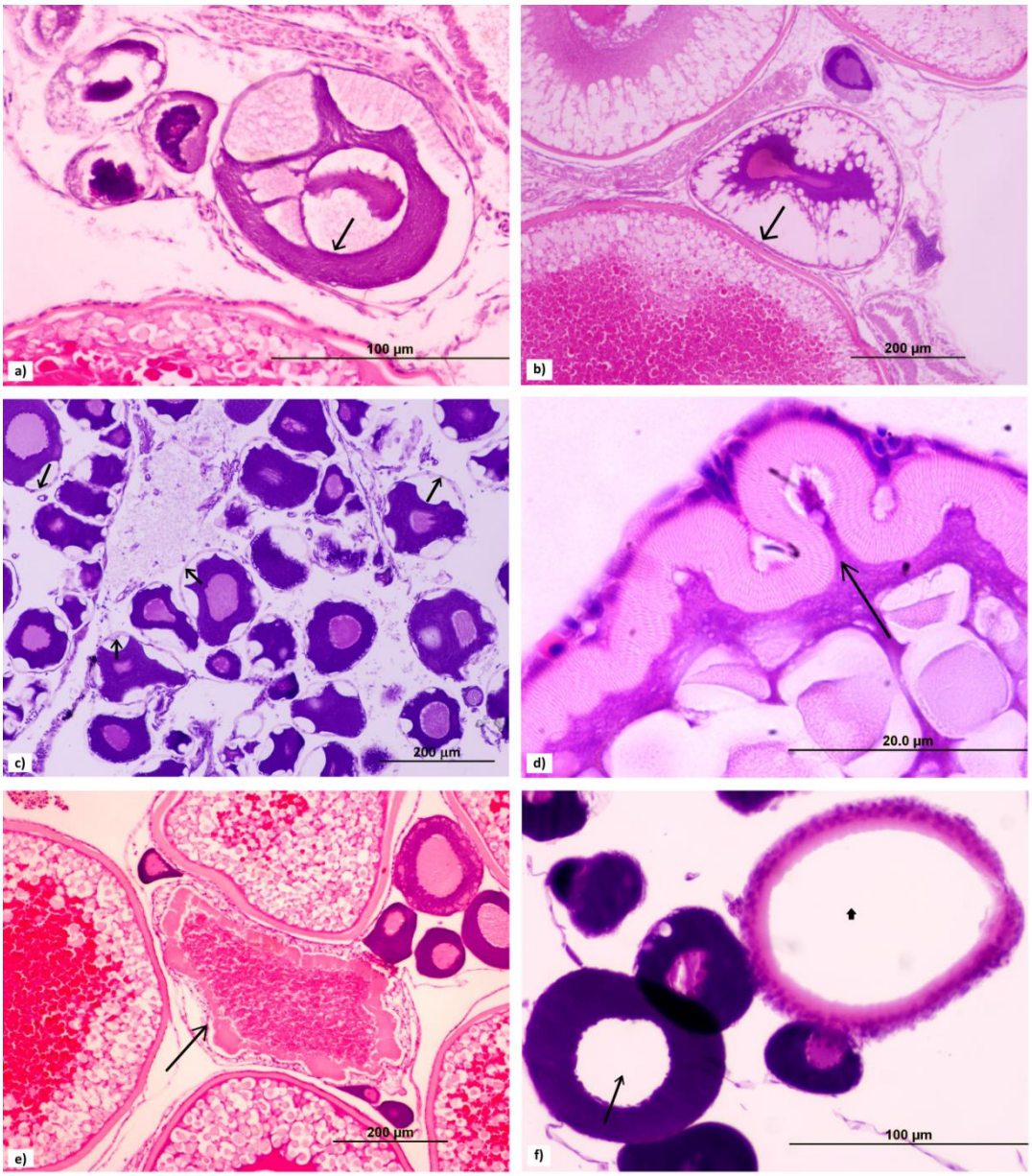

Figure 2.12 Ovarian cellular changes: (a) Ovary from a female Rio Grande chub, from South Crestone creek (SCC-7) showing Karyoplasmic retraction (arrow). Females Rio Grande chubs from (b) South Crestone (SCC-7) and (c) Crestone creek (CC-1), showing cytoplasmic retraction. (d) Cortical alveolar oocyte from a female fathead minnow from South Crestone Creek (SCC-7) displaying a sinusoidal chorionic shape (arrow). (e) Atretic oocyte (arrow) is evident in female fathead minnow from Deadman Creek (DM-3). (f) Ovary from a female Rio Grande chub from South Crestone Creek (SCC-7) exhibiting an anucleated oocyte (arrow) and empty follicle (arrowhead). Paraffin, H&E.

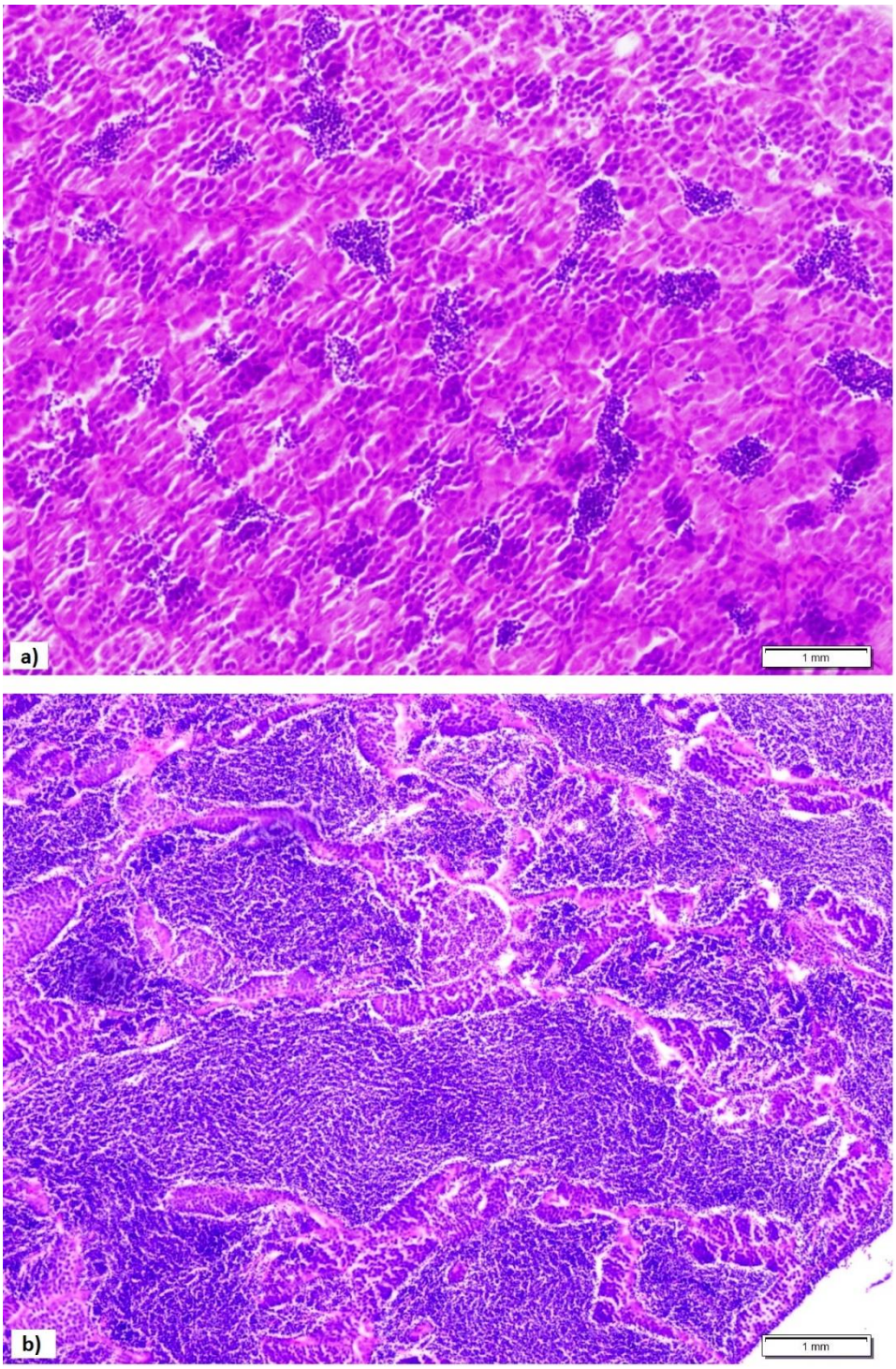

Figure 2.13 Histological sections showing gonadal maturation stages of male fathead minnows. a) Immature fish testis collected in 2011 and b) mature fish testis collected in 2012. Paraffin, H&E.

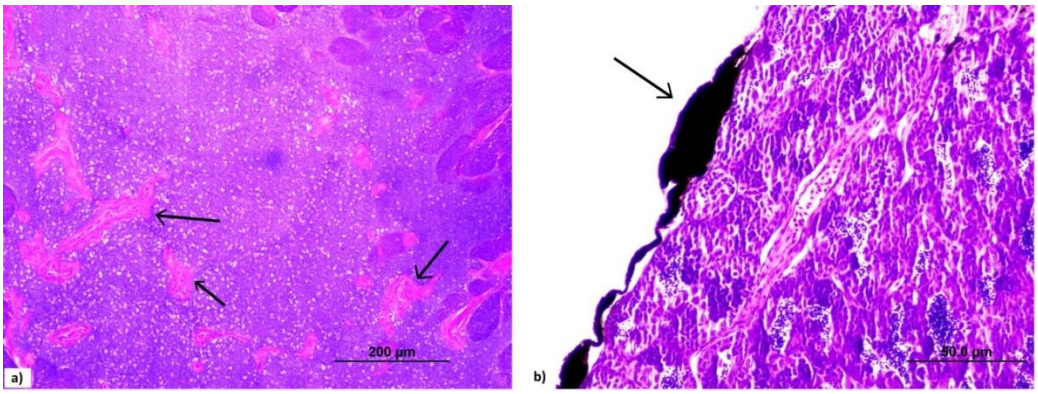

Figure 2.14 Testicular cellular changes: (a) Proteinaceous fluid (arrow) in the testis of adult male Rio Grande chub, from South Crestone Creek (SCC-7). (b) Testis from a male fathead minnow from Deadman creek (DM-3) showing melanomacrophage centers (MMCs, arrow). Paraffin H& E.

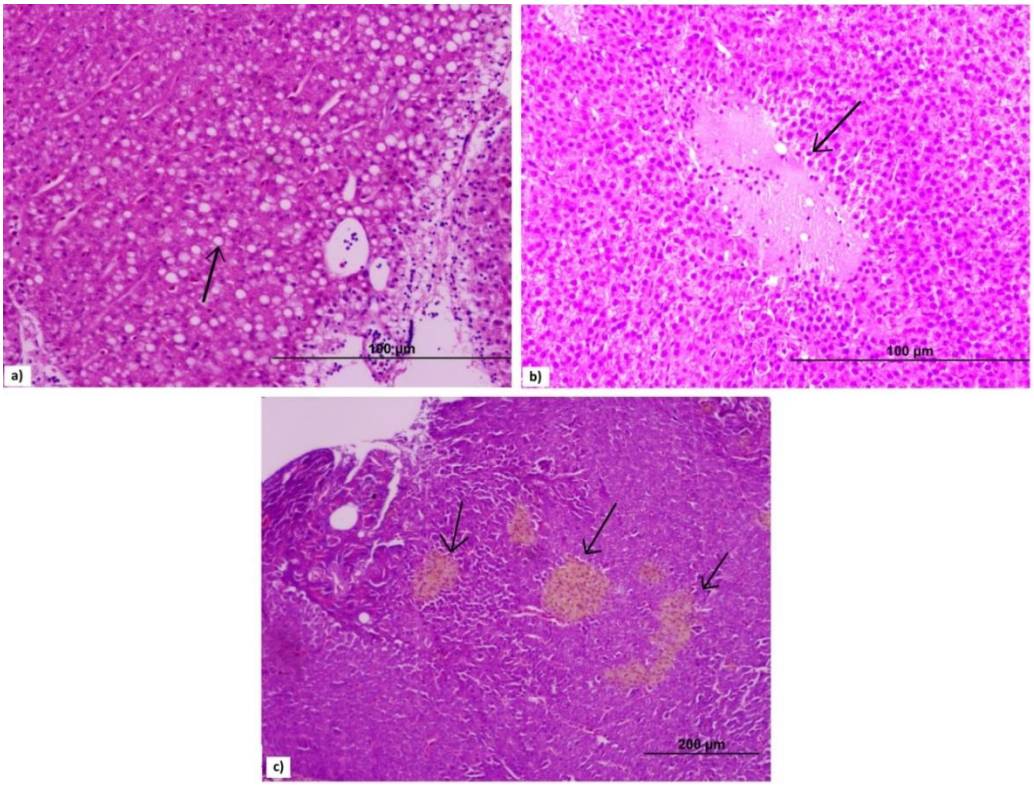

Figure 2.15 Hepatic cellular changes: (a) Liver from an adult male fathead minnow from SCC-7 showing cytoplasmic vacuolization (arrow). (b) Liver from a juvenile female Rio Grande chub from SCC-7 displaying a proteinaceous fluid accumulation (arrow). (c) Liver from an adult female fathead minnow from SCC-7 showing melanomacrophage centers (arrow). Paraffin H&E.

Table 2.1 Sampling rate (Rs) values from the literature for the 14 chemicals detected using POCIS

Analyte	Category	Rs (L/d)	Reference
Atenolol	Beta Blocker	0.094	(Li, Helm et al. 2010)
Caffeine	Stimulant	0.127	(Li, Helm et al. 2010)
Cannabidiol	Sedative	2.15*	
DEET	Insect repellent	0.19	(Bartelt-Hunt, Snow et al. 2009)
Diphenhydramine	Antihistamine	0.15	(Bartelt-Hunt, Snow et al. 2009)
Gemfibrozil	Hypolipidemic drug	0.350	(Li, Helm et al. 2010)
Ibuprofen	Anti-inflammatory	0.348	(Li, Helm et al. 2010)
Levorphanol	Pain reliever (opiate)	0.20*	
Naproxen	Pain reliever (opiate)	0.391	(Li, Helm et al. 2010)
Progesterone	Steroid hormone	0.436	(Bartelt-Hunt, Snow et al. 2009)
Tetrahydrocannabinol	Sedative	2.15*	
Triclocarban	Anti-bacterial	2.15*	
Triclosan	Anti-bacterial	2.15	(Li, Helm et al. 2010)
Trimethoprim	Antibiotic	0.436	(Li, Helm et al. 2010)

* Values selected include highest R_s values reported from non-polar compounds and the average for polar ones.

Table 2.2 Summary of the number of fathead minnow (FHM) and Rio Grande chub (RGC) collectedin (a) August 2011 and (b) May 2012 from South Crestone (SCC-7), Crestone (CC-1) and Deadman creek (DM-3).

a)

2011	SCC-7	CC-1	DM-3	Total
FHM	12	12	12	36
Female	4	5	2	11
Male	8	7	10	25
RGC	12	11		23
Female	7	8		15
Male	5	3		8

b)

2012	SCC-7	CC-1	DM-3	Total
FHM	12	12	12	36
Female	8	6	6	20
Male	4	6	6	16
RGC	12	12		24
Female	7	6		13
Male	5	6		11

Table 2.3 Gene specific primers used for qPCR.

Gene	Forward primer	Reverse primer	Efficiency	R^2	Slope	Amplification
rpl8	CTCCGTCTTCAAAGC CCATGT	TCCTTCACGATCCC CTTGATG	95.68	0.998	-3.430	1.96
cyp19a	CAGGAGTTACAGGAT GCCATCA	CCGACCAGCTAAAA CAGTTTCC	102.61	0.993	-3.261	2.03
dmrt1	AGGTCGTGGGTGATG TGAAT	GGCCACTGCAGAG CTTAGAG	91.8	0.999	-3.536	1.92
star	TGTCCGCTGTGCCAA AC	GCTCTTACAAATCC TTTCTTCTC	108.44	0.966	-3.135	2.08
erα	CACCCACCAGCCCTC AG	CACCTCACACAGAC CAACAC	108.1	0.943	-3.142	2.08
ar	GTGGAGGGGCTGAA GAGTCAG	TTTGGCACCTGGAC GGAGAT	93.69	0.96	-3.483	1.94
vtg	GCTCTCGTCTCCAAT CTTGC	GCTAAGCCTGTCCA GTTTGC	110.73	0.989	-3.089	2.11

Table 2.4 Concentration of nitrogen (N) and phosphorous (P) from waste water treatment plant effluent (AO-1), Crestone (CC-1), Deadman (DM-3), and South Crestone (SCC-7) creeks. Only one sample was collected in May 2012 per site.

Sample ID	N (mg/L)	P (mg/L)
AO-1	3.26	0.308
CC-1	1.87	0.150
DM-3	1.47	0.136
SCC-7	0.12	0.063

Table 2.5 Biological indices of male and female fathead minnow collected in 2011. Asterisks and letters indicate significant differences compared to the control (DM-3) and among South Crestone (SCC-7) and Crestone creek (CC-1) respectively (ANOVA, $P < 0.05$). No statistics were made for female fathead minnows.

	SCC-7		CC-1		DM-3	
Fathead Minnow	Male (n=8)	Female (n=4)	Male (n=7)	Female (n=5)	Male (n=10)	Female (n=2)
Body weight (g)	2.99 ± 1.08	2.12 ± 0.86	3.23 ± 0.69	2.65 ± 0.40	2.41 ± 0.34	2.76 ± 0.78
Total length (mm)	65.38 ± 8.09	59.25 ± 5.80	68.29 ± 4.19	64.80 ± 3.56	63.60 ± 4.12	67.00 ± 5.66
Condition factor	1.05 ± 0.19	0.98 ± 0.10	1.00* ± 0.05	0.97 ± 0.04	0.93 ± 0.07	0.90 ± 0.03
Gonad weight (g)	0.02 ± 0.01	0.05 ± 0.01	0.02 ± 0.01	0.07 ± 0.01	0.01 ± 0.01	0.08 ± 0.01
GSI	0.63 ± 0.21	2.52 ± 0.75	0.50 ± 0.25	2.86 ± 0.62	0.52 ± 0.29	2.85 ± 0.33
Liver weight (g)	0.03 ± 0.021	0.03 ± 0.014	0.03 ± 0.015	0.02 ± 0.008	0.03 ± 0.008	0.02 ± 0.005
HSI	1.02 ± 0.33	1.14 ± 0.15	0.83 ± 0.45	0.77 ± 0.20	1.10 ± 0.30	0.93 ± 0.43

GSI = Gonadosomatic index
HSI = Hepatosomatic index

Table 2.6 Biological indices of male and female Rio Grande chub collected in 2011. Different letters indicate significant differences among SCC-7 and CC-1. No statistics were made for female and male Rio Grande chubs.

	SCC-7		CC-1	
Rio Grande chub	Male (n=5)	Female (n=7)	Male (n=7)	Female (n=5)
Body weight (g)	12.61 ± 2.33	21.78 ± 7.91	4.98 ± 1.22	7.75 ± 3.92
Total length (mm)	114.20 ± 7.69	135.43 ± 14.42	79.00 ± 7.55	89.50 ± 13.08
Condition factor	0.84 ± 0.05	0.85 ± 0.06	1.00 ± 0.06	1.01 ± 0.08
Gonad weight (g)	0.10 ± 0.06	0.70 ± 0.25	0.03 ± 0.02	0.18 ± 0.20

GSI	0.77 ± 0.32	3.22 ± 0.59	0.60 ± 0.28	1.86 ± 1.08
Liver weight (g)	0.11 ± 0.04	0.23 ± 0.08	0.06 ± 0.01	0.11 ± 0.06
HSI	0.85 ± 0.23	1.09 ± 0.17	1.11 ± 0.08	1.40 ± 0.18

GSI = Gonadosomatic index
HSI = Hepatosomatic index

Table 2.7 Biological indices of male and female fathead minnow collected in 2012. Asterisk and letters indicate significant differences compared to the control and among SCC-7 and CC-1 respectively (ANOVA, P < 0.05).

	SCC-7		CC-1		DM-3	
Fathead Minnow	Male (n=4)	Female (n=8)	Male (n=6)	Female (n=6)	Male (n=6)	Female (n=6)
Body weight (g)	3.33 ± 1.01	3.24 ± 0.51	5.14 ± 1.05	3.60 ± 0.69	3.71 ± 0.80	1.95 ± 0.47
Total length (mm)	67.75 ± 5.38	66.13 ± 2.70	75.00 ± 4.05	66.67 ± 3.78	65.67 ± 3.61	54.17 ± 3.76
Condition factor	1.04* ± 0.08	0.99 ± 0.41	1.20 ± 0.11	1.21 ± 0.12	1.29 ± 0.13	1.21 ± 0.12
Gonad weight (g)	0.10 ± 0.05	0.66 ± 0.25	0.14 ± 0.03	0.65 ± 0.32	0.12 ± 0.04	0.30 ± 0.13
GSI	2.96 ± 0.71	19.68 ± 6.19	2.77 ± 0.45	17.36 ± 7.73	3.27 ± 0.78	16.09 ± 7.34
Liver weight (g)	0.10 ± 0.03	0.12 ± 0.03	0.17 ± 0.02	0.13 ± 0.04	0.12 ± 0.04	0.10 ± 0.03
his	2.94 ± 0.43	3.91* ± 0.95	3.60 ± 1.71	3.59* ± 0.74	3.11 ± 1.03	5.30 ± 0.45

GSI = Gonadosomatic index
HSI = Hepatosomatic index

Table 2.8 Biological indices of male and female Rio Grande chub collected in 2012. Different letters indicate significant differences among SCC-7 and CC-1. No statistics were made for female and male Rio Grande chubs.

Rio Grande chub	SCC-7		CC-1	
	Male (n=5)	Female (n=7)	Male (n=6)	Female (n=6)
Body weight (g)	8.18 ± 3.09	17.78 ± 9.07	8.66 ± 3.79	18.99 ± 9.98
Total length (mm)	92.80 ± 13.63	117.00 ± 20.82	94.50 ± 11.98	116.67 ± 18.50
Condition factor	0.97 ± 0.12	1.03 ± 0.16	0.98 ± 0.05	1.10 ± 0.10
Gonad weight (g)	0.38 ± 0.21	1.26 ± 0.67	0.37 ± 0.17	2.59 ± 1.24
GSI	3.06 ± 2.25	7.27 ± 2.30	4.29 ± 2.14	14.71 ± 5.58
Liver weight (g)	0.14 ± 0.08	0.43 ± 0.31	0.20 ± 0.10	0.58 ± 0.41
HSI	1.90 ± 1.06	2.30 ± 1.06	2.29 ± 0.42	2.88 ± 0.80

GSI = Gonadosomatic index
HSI = Hepatosomatic index

Table 2.9 Qualitative cellular changes in ovaries of female fathead minnow and Rio Grande chub in 2011 from South Crestone (SCC-7), Crestone (CC-1), and Deadman creek (DM-3).

Fathead minnows		**Range of severity**	
Histopathologic finding	SCC-7	CC-1	DM-3
Cytoplasmic retraction	Absent to Moderate	Minimal to Severe	Absent to Mild
Atretic oocytes	Absent to Moderate	Mild to Moderate	Mild to Severe
Karyoplasmic retraction	Mild to Severe	Minimal to Mild	Moderate to severe
Sinusoidal chorionic shape	Absent to Mild	Absent to Severe	Absent
Empty follicles	Absent to Minimal	Absent to Minimal	Absent
Anucleated oocytes	Absent to Minimal	Absent to Minimal	Absent

Rio Grande chubs	**Range of severity**	
Histopathologic finding	SCC-7	CC-1
Cytoplasmic retraction	Mild to Severe	Mild to Severe
Atretic oocytes	Minimal to Severe	Absent to Moderate
Karyoplasmic retraction	Moderate to Severe	Mild to Severe
Sinusoidal chorionic shape	Absent to Severe	Absent to Severe
Empty follicles	Minimal to Severe	Absent to Moderate
Anucleated oocytes	Absent to Mild	Absent to Mild

Table 2.10 Qualitative cellular changes in ovaries of female fathead minnows and Rio Grande chubs in 2012 from South Crestone (SCC-7), Crestone (CC-1), and Deadman creek (DM-3).

Fathead minnows		**Range of severity**	
Histopathologic finding	SCC-7	CC-1	DM-3
Cytoplasmic retraction	Minimal to Severe	Absent to Severe	Minimal to Severe
Atretic oocytes	Absent to Severe	Minimal to Severe	Minimal to Severe
Karyoplasmic retraction	Absent to Severe	Absent to Minimal	Absent to Moderate
Sinusoidal chorionic shape	Mild to Severe	Absent to Moderate	Mild to Severe
Empty follicles	Absent to Mild	Absent to Severe	Absent to Moderate
Anucleated oocytes	Absent to Minimal	Absent to Minimal	Absent to Minimal

Rio Grande chubs	**Range of severity**	
Histopathologic finding	SCC-7	CC-1
Cytoplasmic retraction	Absent to Severe	Minimal to Severe
Atretic oocytes	Minimal to Severe	Minimal to Severe
Karyoplasmic retraction	Minimal to Severe	Absent to Severe
Sinusoidal chorionic shape	Absent to Severe	Minimal to Moderate
Empty follicles	Absent to Mild	Absent to Mild
Anucleated oocytes	Minimal to Severe	Absent to Mild

CHAPTER 3. EFFECTS OF TRICLOCARBAN, DEET AND A MIXTURE OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS ON FATHEAD MINNOWS (*PIMEPHALES PROMELAS*)

3.1 Abstract

Pharmaceuticals and personal care products (PPCPs) have been detected widely in aquatic ecosystems, but little is known about their mechanisms of toxicity. We exposed adult fathead minnows (*Pimephales promelas*) for 48 hours to triclocarban (1.4 µg/L), DEET (0.6 µg/L) or to a PPCP mixture consisting of: atenolol (1.5 µg/L), caffeine (0.25 µg/L), diphenhydramine (0.1 µg/L), gemfibrozil (1.5 µg/L), ibuprofen (0.4 µg/L), naproxen (1.6 µg/L), triclosan (2.3 µg/L), progesterone (0.2 µg/L), triclocarban (1.4 µg/L), and DEET (0.6 µg/L). Quantitative real-time polymerase chain reaction (qPCR) revealed an up-regulation in *vtg* (vitellogenin) in livers of females and males exposed to triclocarban. Also, an up-regulation of hepatic *lpl* (lipoprotein lipase) and a down-regulation of *ar* (androgen receptor) and *star* (steroidogenic acute regulatory protein) were observed in testes. The group treated with DEET only showed a significant decrease in *ar* in females. In contrast, the PPCP mixture down-regulated *vtg* in females and males, and expression of *er*α (estrogen receptor alpha), *star*, and *thra1* (thyroid hormone receptor alpha 1) in testes. Our results show the molecular 'estrogenic' effects of triclocarban are eliminated (males) or

reversed (females) when dosed in conjunction with several other PPCPs, once again showing that results from single exposures could be vastly different from those observed with mixtures.

3.2 Introduction

There is growing concern about the presence of pharmaceutical and personal care products (PPCPs) in natural bodies of water. World-wide, many studies have reported the presence of these emerging contaminants in wastewater treatment plant (WWTP) effluents and receiving waters, in some instances having been also found in drinking and ground waters (Ferrari, Mons et al. 2004; Glassmeyer, Furlong et al. 2005; Lee and Rasmussen 2006; Loraine and Pettigrove 2006; Osorio, Marce et al. 2012; Li, Zheng et al. 2013; Perez, De Sylor et al. 2013). Some of the most commonly detected PPCPs are the bactericides triclocarban and triclosan; the insect repellent DEET; the non-steroidal anti-inflammatory drugs naproxen and ibuprofen; and caffeine. Water concentrations range in the parts per trillion to low parts per billion from <0.1 – 2.0> μg/L (Kim, Jang et al. 2009; Blake, Martinovic et al. 2010; Li, Helm et al. 2010; Brausch and Rand 2011; Brozinski, Lahti et al. 2011; Gomez-Martinez 2011; DeQuattro, Peissig et al. 2012).

Triclocarban (3,4,4'-trichlorocarbanilide) is a synthetic antimicrobial used mainly in household products being found in 76% of liquid and 29% of bar soaps (Perencevich, Wong et al. 2001). Despite its extensive production and use, its fate and mode of action is unknown. However, due to its structural similarity,

function and usage to triclosan, triclocarban is predicted to have a similar environmental fate and mode of action (Halden and Paull 2005; Ying, Yu et al. 2007). In a previous study have shown these bactericides have endocrine disrupting properties enhancing estradiol- and testosterone-dependent activation of the estrogen receptor (*er*) (Ahn, Zhao et al. 2008). In Japanese medaka (*Oryzias latipes*), triclosan caused masculinization (measured as changes in anal fin length) (Foran, Bennett et al. 2000). Additional studies with castrated rats reported a synergistic action of triclocarban with testosterone with an increase in the size of male accessory organs (Chen, Ahn et al. 2008). In contrast, feminizing effects (evidenced as an increase in the female-specific protein vitellogenin, VTG) after exposures to high concentrations of triclosan have been reported in male medaka (20 and 100 μg/L) (Ishibashi, Matsumura et al. 2004) and mosquitofish (*Gambusia affinis*;101 μg/L) (Raut and Angus 2010). Disruption of thyroid hormone-associated gene expression has been reported from American bullfrogs (*Rana catesbiana*) exposed to environmentally relevant concentrations of triclosan (0.15 ug/L) (Hinther, Bromba et al. 2011).

DEET (N,N-Diethyl-meta-toluamide) is commonly used as the active ingredient in insect repellents (Fradin and Day 2002). This organic pollutant is found frequently in surface waters at concentrations ranging between 0.04 to 3.0 μg/L (Costanzo, Watkinson et al. 2007). DEET is slightly toxic to fish (LC_{50}-96 hr = ~75 mg/L, U.S. EPA, 1998) or aquatic invertebrates (EC_{50} = ~67 mg/L, (USEPA 1998); however, not much is known regarding its potential chronic effects. Chronic effects in invertebrates showed DEET interferes with olfactory

receptor neurons sensitive to attractans (Davis 1985). The only DEET toxicology study conducted in vertebrates used rodents. Rats were exposed to high levels (191 μg/L) for 30 min resulting in an decrease in acetylcholinesterase activity (Corbel, Stankiewicz et al. 2009).

An important research gap in this field is the limited information available on effects of PPCP mixtures. This is a reflection of the complexity of the issue, since in some sites it is not uncommon to detect several hundred compounds. Overall, studies have reported an antagonistic interaction among PPCPs measured as suppression or reduction of toxicity. For example, in adult zebrafish (*Danio rerio*), acetaminophen and gemfibrozil caused liver damage when administered alone and plasma 11-ketotestosterone levels were reduced by carbamazepine, but none of these effects were reported in the mixture exposure (Galus, Jeyaranjaan et al. 2013). In another study, fathead minnow (*Pimephales promelas)* larvae were exposed to fluoxetine, sertraline, venlafaxine, and bupropion individually and in mixture. A significant increase in body length was observed only in larval fish exposed to fluoxetine and bupropion, while a reduction was evident in larvae exposed to the mixture (Painter, Buerkley et al. 2009). In a similar study, Schultz et al. (Schultz, Painter et al. 2011) exposed adult fathead minnows to fluoxetine, sertraline, venlafaxine, or bupropion alone and in mixture and all effects observed in single exposures (e.g., mortality, increased vitellogenin, and ‘masculinization’ evidenced as increased number of nuptial tubercles, and size and color of dorsal pad) disappeared in the mixture exposure. Decrease in spermatozoa production was more pronounced in

zebrafish exposed to carbamazepine, fenofibrate, propranolol hydrochloride, sulfamethoxazole, and trimethoprim in mixture compared to single exposures (Madureira, Rocha et al. 2011).

In order to help fill this research gap, the goal of the present study was to evaluate molecular and whole organ/organism responses of adult fathead minnows to an acute exposure to triclocarban, DEET or a mixture of PPCPs consisting of atenolol, caffeine, diphenhydramine, gemfibrozil, ibuprofen, naproxen, triclosan, progesterone, triclocarban, and DEET. We hypothesized that triclocarban would elicit estrogenic effects, while DEET would cause effects to the nervous system. We also hypothesized that the PPCP mixture would induce additive effects for those PPCPs sharing common mechanisms of action such as triclocarban and triclosan. These endpoints and PPCPs were selected based on data from an ongoing field study at the Baca National Wildlife Refuge, Colorado that is examining the presence and potential effects of these compounds in native fish (Zenobio, Sanchez et al. 2013). In these studies, triclocarban and DEET have been found at the highest concentrations during the fish spawning season at the Refuge (May-August) (Zenobio et al., Purdue University, West Lafayette, Indiana, unpublished data) with an accumulative concentration of 30 days (measured with Polar Organic Chemical Integrative Samplers POCIS) ranging from <36 – 143> µg/L. Because data on single PPCP concentrations from the refuge surface water was not well established at the times these experiments were conducted, we instead chose a range of environmental

concentrations from the literature to provide more realistic exposure conditions (see Table 1).

3.3 Materials and methods

3.3.1 Exposed organisms

Sexually mature (6 months old) male and female fathead minnows were purchased from a reputable source (Aquatic Eco-Systems, Apopka, FL, USA) and acclimated in 110-L tanks (~ 50 fish/tank) for two weeks after arrival at Purdue University and prior to the experiments. Fish were maintained in reconstituted reverse osmosis water and were fed brine shrimp until satiation once per day. Tanks were monitored daily for mortality and basic water quality parameters which were maintained at: pH 6.6 ± 0; temperature 25.7 ± 0.57°C; dissolved oxygen 8.10 ± 0.21 mg/L; and 0 mg/L for ammonia, nitrite, and nitrate.

3.3.2 Experimental design

Fish were exposed for 48 hours under standard conditions including a 16L:8D light cycle. Three separate experiments were conducted with triclocarban, DEET, and a PPCP mixture. Fish were tested in glass tanks holding 40-L each stocked with 3 females and 3 males. A total of 108 fish were used (36 per experiment, divided in three replicates of 6 fish each for controls and treated). For the single compound experiments (DEET and triclocarban) three replicate tanks were included for the solvent control (either acetone for triclocarban or ethanol for DEET, see more on this below) and three replicate

tanks for each of the chemicals. The PPCP mixture experiment consisted of three replicate tanks for controls (acetone and ethanol, which contained the same concentration used in the mixture exposure) and three replicates of a PPCP mixture containing triclocarban, triclosan, DEET, gemfibrozil, ibuprofen, progesterone, diphenhydramine, naproxen, atenolol, and caffeine. Triclocarban (99% purity), DEET (97%), gemfibrozil (>99%), ibuprofen (≥98%), naproxen (≥98%), atenolol (≥98%), progesterone (≥99), caffeine (>99%), triclosan (>99%), and diphenhydramine (≥99%) were obtained from either Sigma-Aldrich (St Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA). Superstock solutions (5 mg/mL) were prepared in ethanol, acetone, or water according to their solubility (Table 1). Daily stock aliquots were prepared by diluting 1 mL superstock with 700 mL Milli-Q water for a final concentration of 3.6 µg/ml. All stock aliquots were wrapped in aluminum foil and stored in amber glass vials at 4°C until usage. Solution concentrations were chosen to be the highest concentration reported by other studies in surface water (Table 1) (Buser, Poiger et al. 1999; Stumpf, Ternes et al. 1999; Zuccato, Castiglioni et al. 2005; Kim, Jang et al. 2009; Berninger, Du et al. 2011; Brausch and Rand 2011; Brozinski, Lahti et al. 2011; Gomez-Martinez 2011; DeQuattro, Peissig et al. 2012; Skolness, Durhan et al. 2012; Al-Odaini, Zakaria et al. 2013). Nominal concentrations (in µg/L) of PPCPs are presented in Table 2. Chemicals were added to the conditioned water after daily water exchanges (100% daily static renewal). Three water samples were collected from each tank in pre-rinsed 40 mL amber glass vials with Teflon septa. The first and second water samples (day 0 and day 1) were taken 5 min after

water exchange to verify a constant PPCP concentration during the exposure time. The last water sample (day 2, no water exchange) was taken 5 min before fish were removed to estimate the degradation rate using a first-order decay. All water samples were stored at 4°C for later chemical analyses by the USEPA Region 8 Laboratory (Golden, CO, USA).

3.3.3 Chemical measurements

Water samples were shipped overnight to the U.S. EPA Region 8 Laboratory (Golden, CO, USA) after each experiment was performed. Samples arrived at the USEPA within 72 h and upon arrival were stored at -20°C until they were extracted at 96 h. According to Vanderford et al. (Vanderford, Mawhinney et al. 2011) no substantial degradation should be observed when water samples preserved at 4°C are extracted within a short period of time (72h). PPCP concentrations were determined following the U.S. EPA method protocol 1694 and using an Acquity ultra-performance liquid chromatography (UPLC, Waters, Milford, MA, USA) and liquid chromatography-tandem mass spectrometry (LC/MS/MS). Briefly, samples were separated into two aliquots of 1000 mL each. One aliquot was acidified with HCl to pH 2.0 and the other one was basified with NH_4OH to pH 10. Following this pH adjustment, both aliquots were weighed and filtered separately through a glass-fiber filter (Whatman GMF 150/1um, Whatman, NJ, USA). Labeled compounds for the analytes of interest were spiked into their respective acid or base aliquots and 500 mg of $Na_4EDTA.2H_2O$ was added only to the acid aliquot for stabilization. Solid phase extraction (SPE) was used to

extract the target analytes. Hydrophilic-lipophilic balance (HLB) 20 cm^3 cartridges were preconditioned with 20 mL of methanol and 6 mL of reagent water (only for the acid aliquot water was adjusted to a pH 3). Then, the samples were loaded with a flow rate of approximately 5-10 mL/min and the acid aliquot was washed with 10 mL extra of reagent water to remove the EDTA. The cartridges were rinsed with 12 mL of methanol for the acid aliquot and 6 mL of methanol followed by 9 mL of 2% formic acid solution for the base aliquot. An extra 6 mL of acetone:methanol (1:1) was used for triclocarban and triclosan. Next, cartridges were dried under a gentle stream of nitrogen and analytes eluted using 3 mL of methanol. Labeled injection acid and base internal standards were added and the final volume was adjusted to 4 mL with 0.1% formic acid solution. Finally, 1 mL of each sample was injected for HPL/MS/MS analysis and the remaining 3 mL was stored in a refrigerator as backup.

3.3.4 Determination of pharmaceutical decay rates

Average of the first and second water samples (day 0 and day 1) were used as initial concentration (C_0), while the last water sample (day 2) was used as concentration at 24h (C_t) to calculate degradation rates for each PPCP. Degradation rates were fitted to a first-order exponential decay model. Decay rates (*k*) were determined for each PPCP using the following equation:

$$C_t = C_0 * e^{(-kt)}$$

where C_t (μg/L) is the concentration at a specific time t; C_0 (μg/L) is the initial concentration at time zero; k (day^{-1}) is the decay rate coefficient; and t (day) is time.

3.3.5 Fish endpoints

At the end of the exposures, fish were euthanized in 300 mg/L of tricaine methanesulfonate solution (MS-222) and whole body weights and total lengths measured. Gonads, livers, and brains were excised and weighed. These measurements were used to calculate gonadosomatic index (GSI = gonad weight/whole-body weight x 100); hepatosomatic index (HSI = liver weight/whole-body weight x 100); and condition factor (CF = (body weight/total $lenght^3$) x 100,000). A portion of each tissue was placed in a cryovial and flash frozen in liquid nitrogen and stored at -80°C for qPCR analysis.

3.3.6 RNA extraction and cDNA synthesis

A set of target genes was selected according to the mode of action of the PPCPs tested (Tables 1 and 3). Genes were quantified in gonads, livers, or brains depending on their major target organ of expression so that 7 genes were quantified in gonads, 2 in livers, and 1 in brains (see more on this below). Tissues were homogenized in 1 mL of TriSure (Bioline, Tauton, MA, USA) following resuspension of RNA in diethyl pyrocarbonate (DEPC)-treated water. RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at $absorbance_{260nm}$ and stored at -80°C. Only

samples with a ratio of A260/A280 >1.8 were used for qPCR analyses. Samples were treated with DNase I (Fermentas Inc., Glen Burnie, MD, USA) to remove genomic DNA contamination. cDNA was prepared using a high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA, USA) and primed with random primers. All processes were performed according to the manufacturer's instructions.

3.3.7 Real-time PCR

Real-time PCR assays were developed for ten target genes: aromatase (*cyp19a*), estrogen receptor alpha (*er*α), androgen receptor (*ar*), thyroid hormone receptor alpha 1 (*thra1*), prostaglandins endoperoxide synthase 2 (*pges2*), steroidogenic acute regulatory protein (*star*), doublesex and mab-3 related transcription factor 1 (*dmrt1*) in gonads; *vtg* and lipoprotein lipase (*lpl*) in livers; and acetylcholinesterase (*ache*) in brains (Table 3). These genes were chosen because they have been shown to play a significant role in the regulation of growth, development, and reproduction and/or are associated with reported mechanisms of toxicity in mammalian systems for the pharmaceuticals tested. Ribosomal protein L8 (*rpl8*) was used as a stable internal control gene for normalization (Filby and Tyler 2007). All primers were selected from primary literature or developed from our previous study (Zenobio, Sanchez et al. 2013). Primers were purchased through Integrated DNA Technologies (Coralville, IA, USA) and gene expression quantified using a Bio-Rad CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA). All qPCR reactions were performed in

duplicate with no-template negative controls and Ct values between replicates were averaged. Each reaction (20 µL total volume) contained 7 µL 2x qPCR mix (Bio-Rad Laboratories, Hercules, CA, USA), 0.5 µL each of gene specific primers (forward and reverse), 10 µL of nuclease-free water, and 2 µL of cDNA template (15 ng/µL). Conditions used to amplify samples were: 95 °C for 3 min; 40 cycles of 95 °C for 10 sec; 58 °C for 30 sec; 72 °C for 30 sec; and final extension at 65 °C to 95 °C in increments of 0.5 °C for 5 sec. A melting curve was generated for each run to confirm the specificity of the assay. Expression of the target gene was normalized relative to the expression of the reference gene (*rpl8*). Relative gene expression was calculated with the ΔΔCt method (Schmittgen and Livak 2008).

3.3.8 Statistical analyses

All data were analyzed using SPSS 20.0. First, data normality was verified using a Shapiro-Wilk test and non-normal data was log-transformed prior to analyses. All data were assessed for differences compared to the control treatment using one-way analysis of variance (ANOVA). Significant differences were considered at $P < 0.05$. All data are presented as mean ± standard error (SE).

3.4 Results

3.4.1 Concentrations of PPCPs

Nominal and corresponding observed concentrations for each PPCP in the single and mixture treatments are summarized in Table 2. The only compounds that showed some discrepancy between nominal and observed values were triclocarban for both the single and mixture experiments and triclosan for the mixture experiment, with concentrations about half of the target. In addition, progesterone was not detected in the mixture experiment (Table 2).

3.4.2 Degradation of PPCPs

Degradation modeling for all the PPCPs is presented in Figures 1 and 2, except for progesterone which was not detected. Based on first-order exponential decay analysis, the calculated half-lives ($t_{1/2}$) and decay rate constants (k) at a pH = 6.6, T = 25°C, and a 16L:8D light cycle for all PPCPs are shown in Table 4. Under our conditions, triclocarban and triclosan degraded fastest (~10 hours), whereas ibuprofen, DEET, and caffeine had the lowest degradation rates with half-lives of 545, 300, and 266 hours, respectively, thus indicating high persistence in water.

3.4.3 Fish endpoints

No mortalities were observed during the course of these experiments. No significant differences were detected in CFs or GSIs for any of the treatments (Figures 3-5). The only organ response observed was a small decrease in HSI in

females exposed to DEET (*P* = 0.005) compared to controls. As expected, gender differences were observed for GSIs with higher values in females (7.7 ± 1.1%) than males (1.1 ± 0.1%). These values are indicative of fish that were reproductively mature (Ankley, Jensen et al. 2001; Jensen, Korte et al. 2001).

3.4.4 Gene expression

Significant differences were detected in gene expression in all three treatments with most changes occurring in the triclocarban group (2 up- and 2 down-regulated); followed by the PPCP mixture (3 down-regulated); and DEET (1 down-regulated). *Vtg* was increased in livers of males (*P* = 0.001) and females (*P* = 0.006) exposed to triclocarban (Figure 6). In addition, triclocarban down-regulated *ar* and *star* in testes (*P* = 0.009 and *P* = 0.010) and up-regulated *lpl* in livers of males (*P* = 0.036). Only *ar* was down-regulated in the ovaries of females (Figure 7) exposed to DEET (*P* = 0.049). In the mixture exposure, a down-regulation of *vtg* in livers of females (*P* = 0.0004); and of *er*α, *star,* and *thra1* in testes (*P* = 0.003, *P* = 0.032, and *P* = 0.035, respectively) were detected (Figure 8).

3.5 Discussion

Over the last decade, the global use of pharmaceuticals has almost doubled from 2 to 3.9 billion annual prescriptions (Tong, Peake et al. 2011). This has resulted in the constant release of PPCPs from SWWTPs into surface waters. Numerous studies have shown PPCPs are found in complex mixtures of up to

several hundred compounds. However, data on the effects of PPCP mixtures on fish are scarce. Our results show the molecular 'estrogenic' effects of triclocarban are eliminated (males) or reversed (females) when dosed in conjunction with several other PPCPs, once again stressing the point that results from single exposures could be vastly different from those observed with mixtures.

In the present study, measured concentrations of PPCPs in treatment tanks were close to target with the exception of triclocarban (51% from nominal), triclosan (60%), and progesterone (0%). Our data indicates that out of all the PPCPs tested here these three had the shortest half-lives. This issue might significantly affect PPCP effects and thereby affect the accuracy of data interpretation. Similar half-lives were reported by Zhao (Zhao, Ying et al. 2010) who suggested photo-degradation might be occurring with triclocarban and triclosan. Despite these fast degradation rates, these two bactericides are commonly found downstream from SWWTPs in the ppb level indicating they are constantly being discharged into the environment at high concentrations. Breakdown products of triclosan by direct photolysis include chlorophenols and dioxin, mainly 2,8-dichlorodibenzo-p-dioxin (2,8-DCDD) and 2,4-dichlorophenol (2,4-DCP) (Latch, Packer et al. 2005; Aranami and Readman 2007). 2,8-DCDD, a toxic intermediate product, is a type of dioxin that persist longer than triclosan (Aranami and Readman 2007). 2,4-DCP, a USEPA priority pollutant, is considered an endocrine disruptor chemical responsible of adverse effects in female sex organs by interruption of ER-mediated processes (Zhang, Zha et al. 2008). Degradation pathway of triclocarban is still unclear. However, in 2007

Sires et. al (Sires, Oturan et al. 2007) proposed a reaction pathway for the degradation of triclocarban suggesting that hydroquinone and 1-chloro-4-nitrobenzene may be their chemical breakdown products. Although reproductive effects in fish have not been reported for any of these metabolites, studies in rats showed reduction of male fertility (USEPA 1987) and evidence of carcinogenic effects (Gold, Slone et al. 1989) after the exposure to hydroquinone or 1-chloro-4-nitrobenzene, respectively. Progesterone was not detected in any of the tanks. Microbial degradation is the most important process in steroid degradation (Jurgens, Holthaus et al. 2002; Zuo, Zhang et al. 2006), and it is likely that progesterone was quickly degraded in water rich in microbes coming from fish and feed. Moreover, light can also play a role in the degradation of this hormone since rapid photolysis of steroid hormones under environmental conditions has been observed before (Yang 2010), with a half-life of ~5 hours for progesterone. Vanderford et al. (Vanderford, Mawhinney et al. 2011) reported a significant degradation of progesterone (65% ± 1.9) within a short period of time (72h) when water samples were kept at 4ºC and no chemical preservation and extraction done. Therefore, we believe that microbial and photo-degradation occurred during the laboratory exposure and sample transportation (72h) which resulted in progesterone not being detected in any of the water samples. The two principal pathways for progesterone are testosterone acetate and 17α-hydroxyprogesterone (carlstrom). 17α-hydroxyprogesterone is a steroid hormone that interferes in shrimp reproduction increasing the biosynthesis of yolk protein in the ovaries of shrimps (Quackenbush 1987). While testosterone induces sex

reversal in fish (pandian 1995). To the contrary, ibuprofen, DEET and caffeine showed the lowest degradation rates, with half-lives ranging from 11 – 23 days. This might be due to their low photo-transformation rate since they do not absorb sunlight (Buerge, Poiger et al. 2003; Tixier, Singer et al. 2003; Kim and Tanaka 2009). Consequently, they are frequently detected in surface water at much higher levels compared to other PPCPs.

PPCPs evaluated in the present study caused no mortalities. The only organ-level effect observed was a small decline in HSI in females exposed to DEET. Although declines in HSI by DEET has not been previously reported, studies with another insect repellent (fenvalerate) also showed a reduction of HSI in the African catfish (*Clarias gariepinus*) due to a rapid release of hepatic glycogen due to stress (Bhattacharya and Kaviraj 2009). Thus this fast decline in liver size after exposure to DEET could be related to loss of hepatic glycogen. No changes in HSI were detected in the mixture treatment which may be associated to the presence of PPCPs such as gemfibrozil, which has been reported to interfere with glycogen metabolism (Khan, Saxena et al. 2008).

Up-regulation of *vtg* has been confirmed to be a sensitive indicator of estrogenic exposure in both laboratory and field studies and it is also commonly used as an indicator of possible reproductive alterations. Relative expression of *vtg* was significantly increased in females (~2-fold change) and males (~2.5-fold change) treated with triclocarban. A similar response in female (1.58 μg/L) and male (0.56 μg/L) fathead minnows exposed to triclocarban for 21 days was reported by Schultz (Schultz, Bartell et al. 2012), but changes in plasma VTG

levels were not significant in that study. In males exposed to triclocarban, we also observed a down-regulation in the expression of *ar* (0.5-fold change) and *star* (0.2-fold change). These results are in overall agreement with previous studies with triclosan that have reported it as having both estrogenic and androgenic activity (Foran, Bennett et al. 2000; Ishibashi, Matsumura et al. 2004; Raut and Angus 2010).

Star transports cholesterol from the outer to the inner mitochondrial membrane and thus plays a critical role in steroidogenesis. Decreased *star* expression has been reported with triclosan *in vivo* and *in vitro* as a consequence of a decreased synthesis and availability of cholesterol (Kumar, Balomajumder et al. 2008; Kumar, Chakraborty et al. 2009). Down-regulation of both genes (*ar* and *star*) in males was also observed in fathead minnows from our field study in 2012 (Zenobio, Sanchez et al. 2013).

An up-regulation of *lpl* (1.9-fold change) was observed in males exposed to triclocarban. The function of this gene is to hydrolyze triglycerides, decreasing their circulating levels. Thus an up-regulation of this gene results in a down-regulation of genes involved in lipid biosynthesis (Staels, Dallongeville et al. 1998) and in a reduction in cholesterol levels (Velasco-Santamaria, Korsgaard et al. 2011). Our results suggest that triclocarban might impact steroidogenesis through declines in cholesterol levels and inhibition of *star* expression, the later having been reported with triclosan (Ishii, Hasegawa et al. 2002). Whether these two mechanisms are connected is not clear at this time. In contrast to what was reported in amphibians, no effects were observed in the expression of *thra1*.

In females, only one gene was statistically down-regulated (*ar* = 0.4-fold change) from the DEET treatment. Permethrin, a synthetic chemical used as insect repellent, has been reported to have hormonal activity and therefore it may disrupt endocrine function (Kim, Lee et al. 2005). However, no studies have reported androgenic or estrogenic activities by DEET in fish. More research is required to better understand any possible endocrine effects associated with DEET.

Contrary to the single triclocarban treatment, a significant decrease in *vtg* (0.1-fold change) was observed in females exposed to the PPCP mixture. In addition, in males, the up-regulation of *vtg* expression was not observed and *er*α, *star*, and *thra1* were down-regulated (0.2, 0.4 and 0.7-fold change, respectively). The only study exposing fish to both bactericides as single compounds or mixture reported variable effects in plasma VTG concentrations in male and female fathead minnows comparing the single and mixture treatments, although neither was statistically significant (Schultz, Bartell et al. 2012). These results point to an antagonistic interaction between triclocarban, triclosan, and the other PPCPs as it relates to *vtg*. It also suggests that other endocrine effects (thyroid metabolism) might be occurring when fish are exposed to PPCPs mixtures likely to be found downstream from SWWTPs.

In summary, our results suggest that environmental levels of triclocarban are weakly estrogenic as evidenced by increased *vtg* expression in males and females and that the mechanisms of toxicity of this bactericide are similar to those reported for the better studied triclosan. In addition, triclocarban effects on

lpl and *star* suggest it might negatively impact steroidogenesis. Gene expression changes, however, were very different when a mixture of PPCPs resembling a 'natural' mixture was tested. Therefore, further studies are required to elucidate the mechanisms of toxicity and effects of PPCP mixtures.

Acknowledgment -- We would like thank the U.S. EPA Region 8 Laboratory (Golden, CO, USA) for assistance in chemical analyses of PPCPs. We would also like to thank Luis A. Jauregui for his help with fish dissections.

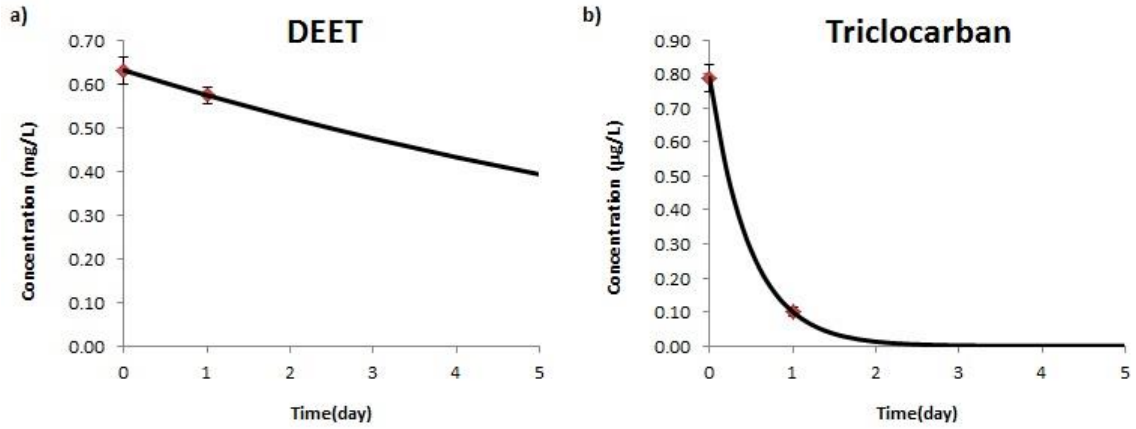

Figure 3.1Degradation of a) N,N-Diethyl-meta-toluamide (DEET) and b) triclocarban in water from the single compound treatment.

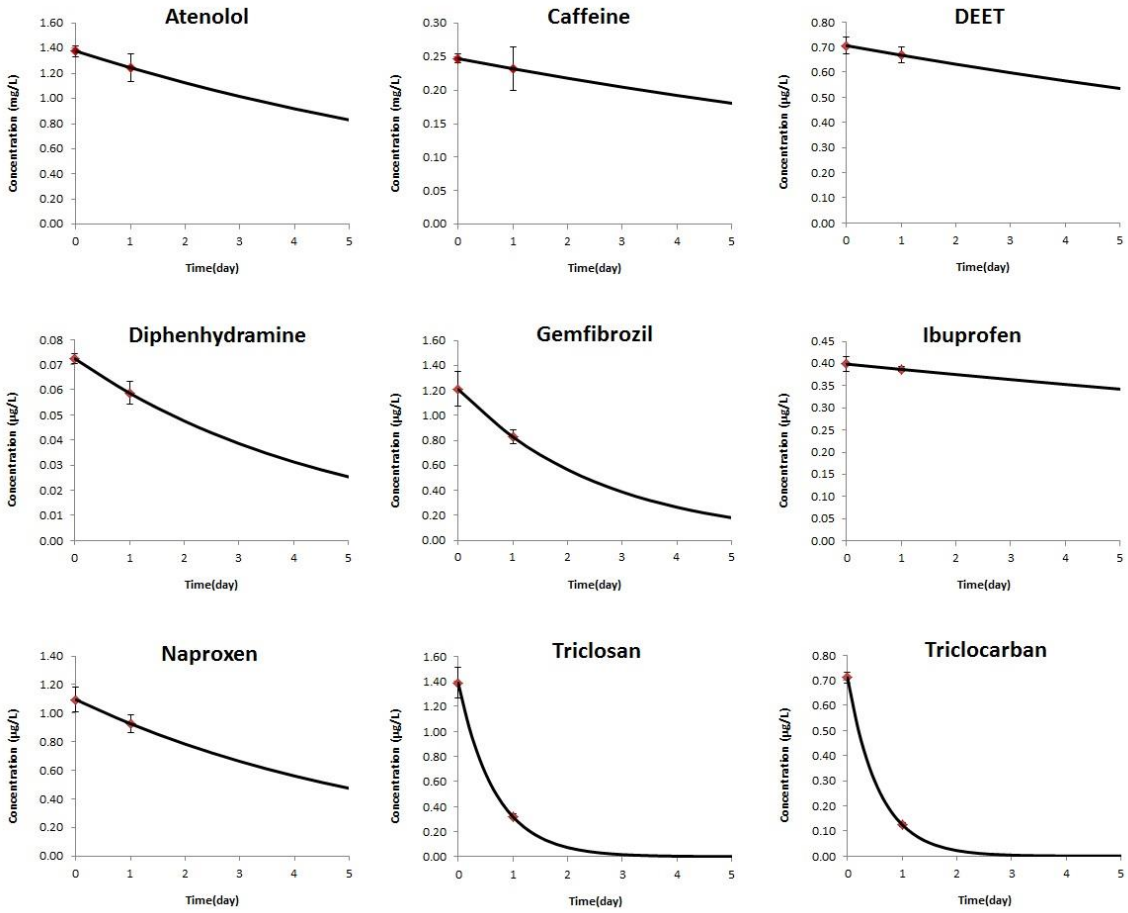

Figure 3.2 Degradation of the nine PPCPs (atenolol, caffeine, DEET, diphenhydramine, gemfibrozil, ibuprofen, naproxen, triclocarban, and triclosan) in water from the mixture treatment.

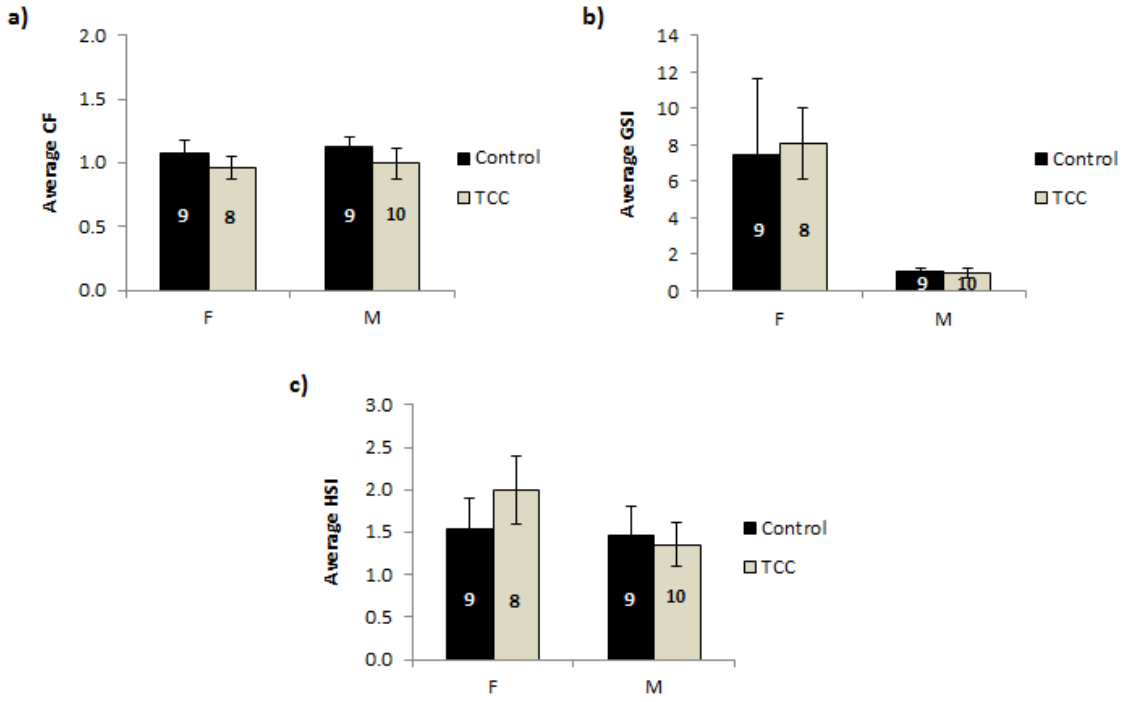

Figure 3.3 Effects of triclocarban (TCC) on female and male fathead minnows (a) condition factor (CF), (b) gonadosomatic index (GSI), and (c) hepatosomatic index (HSI). Data are mean ± SE. Sample sizes are shown inside each bar

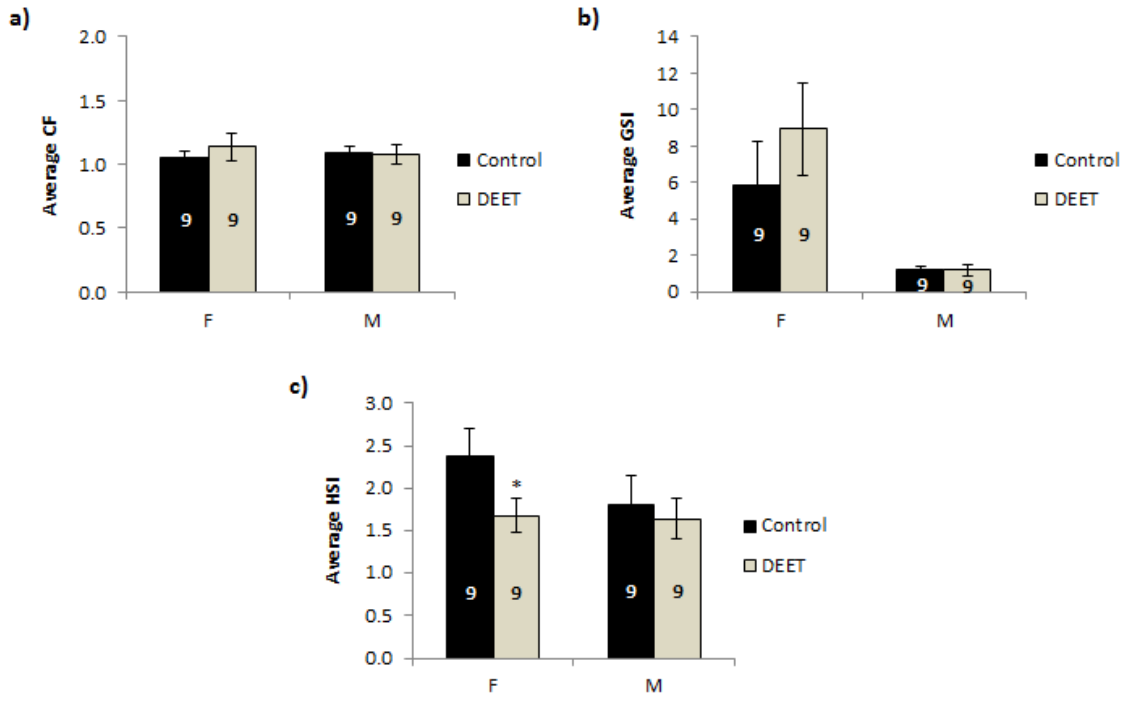

Figure 3.4 Effects of N,N-Diethyl-meta-toluamide (DEET) on female and male fathead minnows (a) condition factor (CF), (b) gonadosomatic index (GSI), and (c) hepatosomatic index (HSI). Data are mean ± SE. Significant differences, $P < 0.05$, are depicted by asterisks.Sample sizes are shown inside each bar.

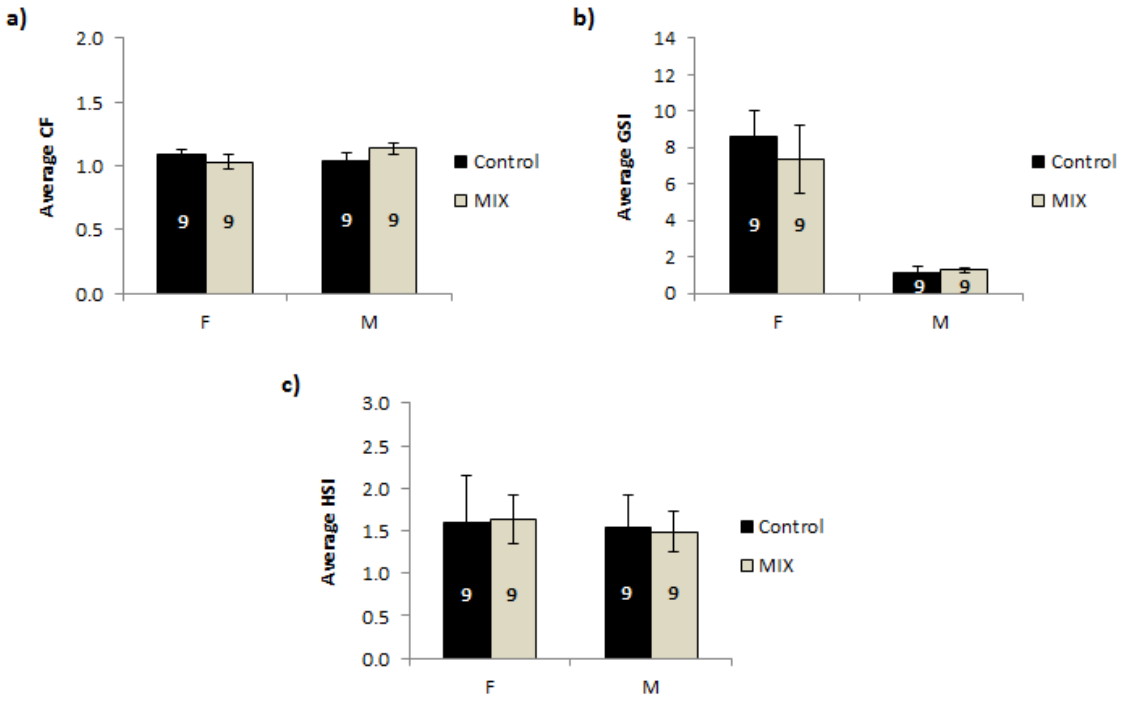

Figure 3.5 Effects of PPCPs mixture (triclocarban, DEET, gemfibrozil, ibuprofen, progesterone, naproxen, atenolol, caffeine, triclosan, and diphenhydramine) on female and male fathead minnows (a) condition factor (CF), (b) gonadosomatic index (GSI), and (c) hepatosomatic index (HSI). Data are mean ± SE. Sample sizes are shown inside each bar.

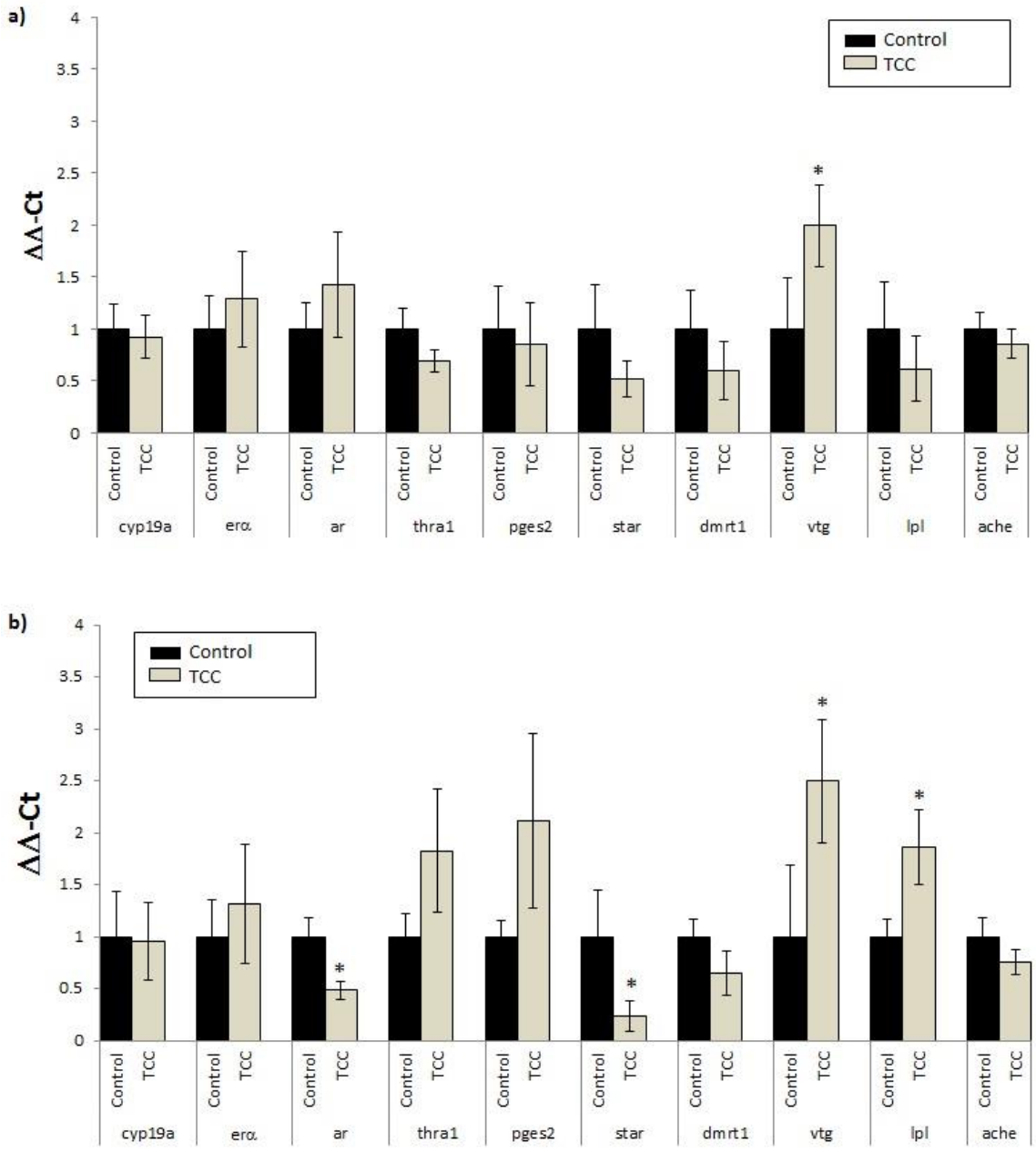

Figure 3.6 Relative gene expression from gonads (aromatase (*cyp19a*), estrogen receptor alpha (*er*α), androgen receptor (*ar*), thyroid hormone receptor alpha 1 (*thra1*), prostaglandin endoperoxide synthase 2 (*pges2*), steroidogenic acute regulatory protein (*star*), and and doublesex and mab-3 related transcription factor 1 (*dmrt1*), livers (vitellogenin (*vtg*) and lipoprotein lipase (*lpl*)), and brains (acetylcholinesterase (*ache*)) in (a) female and (b) male fathead minnows exposed to triclocarban. Shown are means ± SE in relation to controls using the $\Delta\Delta$Ct method. Significant differences, $P < 0.05$, are depicted by asterisks.

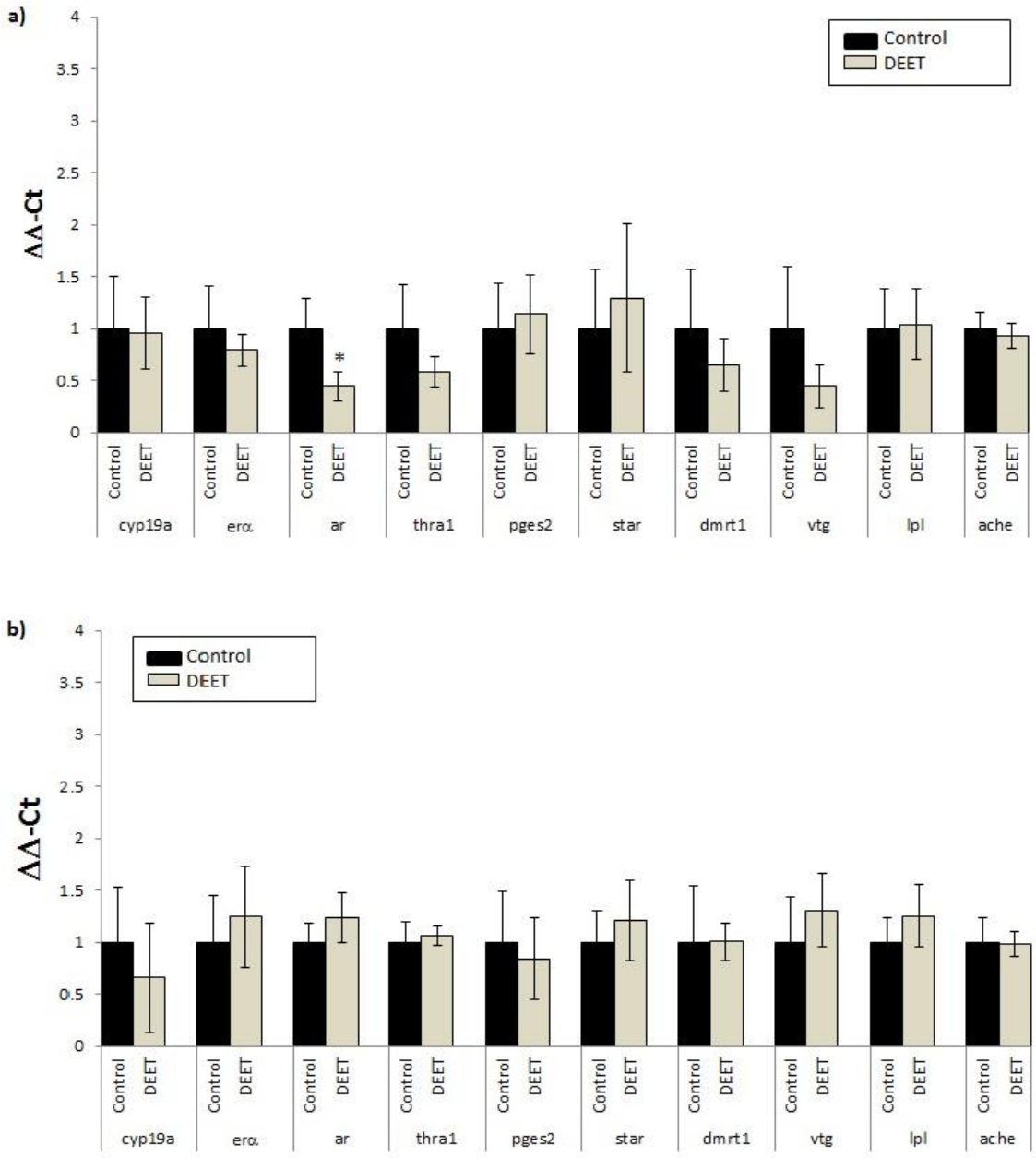

Figure 3.7 Relative gene expression from gonads (aromatase (cyp19a), estrogen receptor alpha (*er*α), androgen receptor (*ar*), thyroid hormone receptor alpha 1 (*thra1*), prostaglandins endoperoxide synthase 2 (*pges2*), steroidogenic acute regulatory protein (*star*), and doublesex and mab-3 related transcription factor 1 (*dmrt1*)), livers (vitellogenin (*vtg*) and lipoprotein lipase (*lpl*)), and brains (acetylcholinesterase (*ache*)) in (a) female and (b) male fathead minnows expose to N,N-Diethyl-meta-toluamide (DEET). Shown are means ± SE in relation to controls using the $\Delta\Delta$Ct method. Significant differences, $P < 0.05$, are depicted by asterisks.

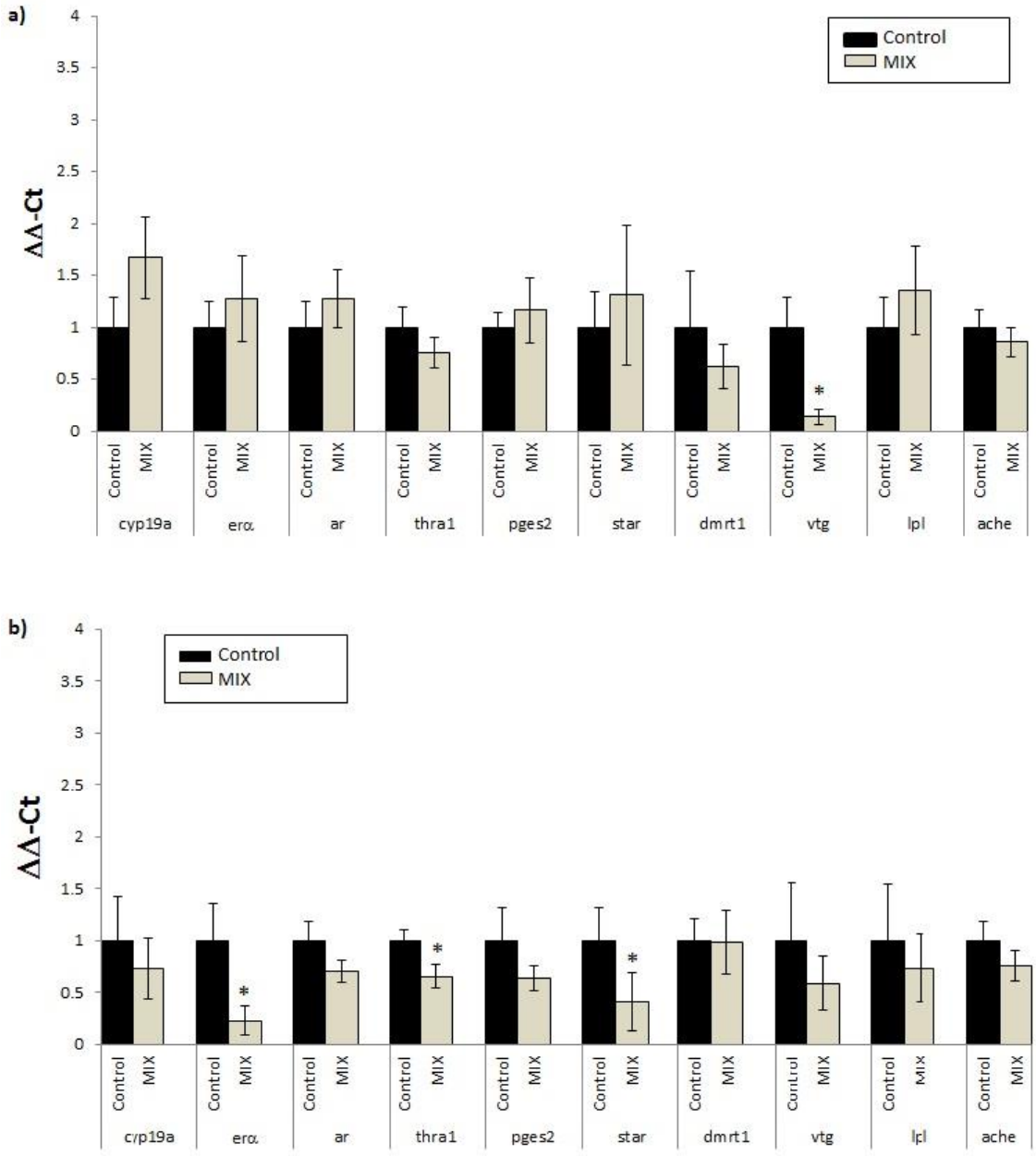

Figure 3.8 Relative gene expression from gonads (aromatase (*cyp19a*), estrogen receptor alpha (*er*α), androgen receptor (*ar*), thyroid hormone receptor alpha 1 (*thra1*), prostaglandins endoperoxide synthase 2 (*pges2*), steroidogenic acute regulatory protein (*star*), and doublesex and mab-3 related transcription factor 1 (*dmrt1*)), livers (vitellogenin (*vtg*) and lipoprotein lipase (*lpl*)), and brains (acetylcholinesterase (*ache*)) in (a) female and (b) male fathead minnows expose to a PPCP mixture. Shown are means ± SE in relation to controls using the $\Delta\Delta$Ct method. Significant differences, $P < 0.05$, are depicted by asterisks.

Table 3.1 List of pharmaceuticals and personal care products used for the laboratory exposure and their solvent media.

Analyte	CAS #	Solvent	Mechanism of action	Range in surface water (μg/L)
Bactericides				
Triclocarban*	101-20-2	Acetone	Disruption of bacterial cell membranes	0.02 – 1.43[a]
Triclosan	3380-34-5	Acetone	Inhibition of bacterial fatty acid synthesis	0.0001 – 2.30 [a]
Nonsteroidal anti-inflammatory drugs (NSAIDs)				
Ibuprofen	15687-27-1	Ethanol	Inhibition of the enzyme cyclooxygenase (COX)	0.002 – 0.40[b]
Naproxen	22204-53-1	Ethanol	Inhibition of the enzyme cyclooxygenase (COX)	0.01 – 1.60[c]
Beta blockers				
Atenolol	29122-68-7	Ethanol	Blocks beta-adrenergic receptors in the heart and juxtoglomerular apparatus	0.02 – 1.50[d]
Stimulant				
Caffeine	58-08-2	Water	Blocks adenosine receptors	0.01 – 0.25[e]
Insect repellent				
DEET	134-62-3	Ethanol	Inhibition of the central nervous system enzyme acetylcholinesterase	0.01 – 0.66[a]
Anti-histamines				
Diphenhydramine	58-73-1	Water	Blocks histamine H1-receptors	0.01 – 0.10[f]
Lipid-regulating				
Gemfibrozil	25812-30-0	Ethanol	Inhibition of lipolysis and reduction of hepatic fatty acid uptake	0.01 – 1.50[g]
Steroid hormone				
Progesterone	57-83-0	Ethanol	Binds to the progesterone and estrogen receptors	0.01 – 0.20[h]

[a](Brausch and Rand 2011). *Presumptive mechanism of action within the hypothalamic-pituitary-gonadal axis.
[b](Buser, Poiger et al. 1999; Kim, Jang et al. 2009)
[c] (Stumpf, Ternes et al. 1999; Brozinski, Lahti et al. 2011)
[d] (Zuccato, Castiglioni et al. 2005; Al-Odaini, Zakaria et al. 2013)

[e](Gomez-Martinez 2011)
f (Berninger, Du et al. 2011)
[g] (Skolness, Durhan et al. 2012)
[h](DeQuattro, Peissig et al. 2012)

Table 3.2 Nominal and measured concentrations (at time 0) of pharmaceuticals and personal care products (μg/L) for each of the treatments (n = 3).

	Triclocarban		**DEET**		**Mixture**	
	Nominal Conc. (μg/L)	Measured Conc. (μg/L)	Nominal Conc. (μg/L)	Measured Conc. (μg/L)	Nominal Conc. (μg/L)	Measured Conc. (μg/L)
Bactericides						
Triclocarban	1.40	0.79 ± 0.096			1.40	0.71 ± 0.054
Triclosan					2.30	1.39 ± 0.300
Nonsteroidal anti-inflammatory drugs (NSAIDs)						
Ibuprofen					0.40	0.34 ± 0.041
Naproxen					1.60	1.10. ± 0.209
Beta blockers						
Atenolol					1.50	1.34 ± 0.107
Stimulant						
Caffeine					0.25	0.25 ± 0.017
Insect repellent						
DEET			0.60	0.60 ± 0.075	0.60	0.54 ± 0.083
Anti-histamines						
Diphenhydramine					0.10	0.07 ± 0.005
Lipid-regulating						
Gemfibrozil					1.50	1.21 ± 0.339
Steroid hormone						
Progesterone					0.20	0

Table 3.3 Genes selected for qPCR analyses and their primers.

Gene Symbol	Name	Forward primer	Reverse primer
rpl8	Ribosomal protein L8 (reference)	CTCCGTCTTCAAAGCCCATGT	TCCTTCACGATCCCCTTGATG
cyp19a	Aromatase	CAGGAGTTACAGGATGCCATCA	CCGACCAGCTAAAACAGTTTCC
erα	Estrogen receptor alpha	CACCCACCAGCCCTCAG	CACCTCACACAGACCAACAC
Ar	Androgen receptor	GTGGAGGGGCTGAAGAGTCAG	TTTGGCACCTGGACGGAGAT
thra1	Thyroid hormone receptor alpha 1	ATGACCCAGAGAGCGAGAC	CATCAGACACCACTCCTAACC
pges2	Prostaglandins endoperoxide synthase 2	GGTCCCATTTGGTCGACAGT	CCTCTGTGGATCAGGGATGAA
Star	Steroidogenic acute regulatory protein	TGTCCGCTGTGCCAAAC	GCTCTTACAAATCCTTTCTTCTC
dmrt1	Doublesex and mab-3 related transcription factor 1	AGGTCGTGGGTGATGTGAAT	GGCCACTGCAGAGCTTAGAG
Vtg	Vitellogenin	GCTCTCGTCTCCAATCTTGC	GCTAAGCCTGTCCAGTTTGC
Lpl	Lipoprotein lipase	TACAGCGTCAACAAAGTCCG	GGGATATTCTCCTTCTCGCC
Ache	Acetylcholinesterase	GCTAATGAGCAAAAGCATGTGGGCTTG	TATCTGTGATGTTAAGCAGACGAGGCAGG

Table 3.4 Calculated decay coefficients and half-lives for all pharmaceuticals and personal care products tested in the present study (n = 3).

	Triclocarban		DEET		Mixture	
Target Analyte	Decay Coefficient k (day^{-1})	Half-life $t_{1/2}$ (days)	Decay Coefficient k (day^{-1})	Half-life $t_{1/2}$ (days)	Decay Coefficient k (day^{-1})	Half-life $t_{1/2}$ (days)
Atenolol					0.1011	6.85 ± 0.08
Caffeine					0.0627	11.06 ± 0.90
DEET			0.0947	7.32 ± 0.68	0.0553	12.53 ± 0.39
Diphenhydramine					0.2095	3.31 ± 0.91
Gemfibrozil					0.3787	1.83 ± 0.16
Ibuprofen					0.0305	22.70 3.66
Naproxen					0.1672	4.15 ± 0.45
Triclocarban	2.0621	0.34 ± 0.007			1.7357	0.40 ± 0.008
Triclosan					1.4695	0.47 ± 0.02

CHAPTER 4. CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

Chapter 2

A field study was conducted in the Baca National Wildlife Refuge (BNWR), CO to measure the impact of the Aspen WWTP effluent in the Refuge's creeks and fish populations. Our results indicate that PPCPs are present in the WWTP effluent and in the Refuge's creeks. Prescription drugs were the chemical groups most frequently detected by grab (79%) and POCIS (83%). Thirty-seven PPCPs were detected by grab (average from 2010-2012) and sixty-three using POCIS (only in 2012) from the WWTP effluent. POCIS detected PPCPs in all of the fish sites (including control site), with caffeine, triclosan and DEET being found at the highest concentration. Significantly more chemicals were isolated from POCIS compared to grab samples. POCIS detected PPCPs at lower concentrations because of their ability to collect chemical data for several days detecting episodic pollution events. Therefore, POCIS deployed for long periods (~ 1 month) represent a better alternative sampling method to conventional grab sampling for monitoring PPCPs.

During the years of study, gene and cellular changes in gonads and livers were observed in fish from all sites, including controls (SCC-7, CC-1, and DM-3). However, because of the lack of a reference site, further studies are needed at the Refuge. Source(s) of PPCPs to the reference site is unknown at this time.

Chapter 3

Based on the findings from our field study, a laboratory PPCP exposure study was performed with fathead minnows (Pimephales promelas). All of the PPCPs used in this experiment were detected in the Refuge creeks and used at environmental relevant concentrations. This study focused on examines the mechanisms of toxicity and effects of a selected number of PPCPs. We observed significant changes in genes involved in reproduction (vitellogenin (vtg), androgen receptor (ar), steroidogenic acute regulatory protein (star), and estrogen receptor alpha (erα)), lipid metabolism (lipoprotein lipase (lpl)), and thyroid metabolism (thyroid receptor alpha (thra)).

The estrogenic nature of triclocarban was clearly identified through the significant increase of hepatic vtg in male and females and a decrease of ar and star expression in males. Expression of lpl was up-regulated by triclocarban in males. DEET exposure also elicited endocrine effects decreasing ar expression in females. In contrast, the PPCP mixture down-regulated vtg in females and males, and expression of erα and thra1 in testes. Our results show the molecular 'estrogenic' effects of triclocarban are eliminated (males) or reversed (females) when dosed in conjunction with several other PPCPs, once again showing that

results from single exposures could be vastly different from those observed with mixtures. In conclusion, this study provides an insight on the mechanisms of toxicity of commonly found PPCPs. More studies are needed that focus on population-level effects of these contaminants on fish.

4.2 Implications

Results of this research clearly show that PPCPs are present in surface water in low concentrations. PPCP levels in sewage effluents are high; suggesting that direct disposal of sewage down surface water may be a contributing source of PPCP contamination. POCIS are a good alternative to monitor PPCPs in aqueous environment due to its sensitivity detecting organic contaminants (better than grab sampling) and its simplicity to handle, preserve, and transport. POCIS could be used to mimic chemical accumulation occurring when fish are exposed to contaminated waters.

Aquatic organisms present in water bodies receiving wastewater treatment are exposed to PPCPs throughout their entire life-cycle. Alterations in the reproductive system due to PPCP exposure may cause changes in sexual behavior, unfair sex organ development, and resulting in decline or extinction of local populations.

4.3 Future Research

Other conventional methods (i.e. composite sampling) for monitoring surface water should be compared with POCIS to determine the more efficient

and easy method for the field work. Determination of additional sampling rate data used to estimate the water concentration of emerging chemicals by POCIS is necessary. Also, future work is needed to evaluate the influence of water quality parameters on the sampling rates for POCIS. WWTPs were not designed to remove PPCPs, alternative treatment process that help to reduce PPCPs levels from sewage should be explored.

PPCPs are present in the environment in complex mixtures, which may result in additive, synergistic, or antagonistic effects from exposure to multiple PPCPs sharing common mechanism of action. Chemicals continually entering the aquatic environment can lead to a continuous exposure for aquatic organisms. Thus, a multigenerational exposure to determine population-level effects should be considered. Therefore, studies exploring the toxicodynamics (mode of action) and toxicokinetics (accumulation) of PPCPs in fish and wildlife are necessary to understand the risk to biota of individual compounds and mixtures.

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APPENDIX

APPENDIX

Table A 1Concentration of pharmaceuticals and personal care products (ng/L) from waste water treatment plant effluent (AO-1), Crestone (CC-1, CC-2, and CC-3), Crestone ditch (CD-1, CD-2, and CD-3), Cottonwood (CWC-2 and CWC-4), Deadman (DM-1, DM-2, and DM-3), North Crestone (NCC-1), South Crestone (SCC-1, SCC-6, and SCC-7), Spanish (SC-2 and SC-3), and Willow (WC-2) creeks by grab sampling.

AO-1	CAS No.	Use	Aug-10	Aug-11	May-12	Aug-12
(+/-)11-nor-9-carboxy-delta-THC	64280-14-4	Metabolite of THC			99.0	
17B-Estradiol-glucuronide	14892-12-8	Metabolite of 17b-estradiol	109.0			
2-(4-Chlorophenoxy)-2-methylpropionic acid	882-09-7	Herbicide	38.3			
Albuterol	18559-94-9	Bronchodilator	14.6			
Amitriptyline	549-18-8	Antidepressant	71.1		28.4	
Amitriptyline (+/-)-E-10-hydroxylated	64510-05-4	Antidepressant			10.8	
Atenolol	29122-68-7	Beta blocker	526.0	348.0	355.0	260.0
Benzoylecgonine	519-09-5	Metabolite of cocaine		29.2		
Bupropion	34841-39-9	Antidepressant			62.2	91.8
Butalbital	77-26-9	Barbiturate			12.5	
Caffeine	58-08-2	Stimulant			160.0	96.3
Carbamazepine	298-46-4	Anti-convulsive	284.0	69.0		13.0
Carisoprodol	78-44-4	Muscle relaxant		49.1		
Chlorpheniramine	132-22-9	Anti-histamine			14.2	
Cimetidine	51481-61-9	H_2 blocker		10.3	381.0	27.6
Cocaine	53-21-4	Recreational drug	57.4			
Codeine	76-57-3	Antitussive	27.0	23.2	11.7	15.3
DEET	134-62-3	Insect repellent	112.0	256.0	315.0	10.2
Dextromethorphan	125-69-9	Antitussive	100.0	15.8		
Diclofenac	15307-86-5	NSAID		202.0	23.8	38.7
Diltiazem	33286-22-5	Calcium-channel blocker	35.5		11.7	
Diphenhydramine	58-73-1	Anti-histamine		182.0	333.0	261.0
EDDP	66729-78-0	Metabolite of methadone	140.0			
Famotidine	76824-35-6	H2 blocker		32.0	38.3	74.8
Fluoxetine	54910-89-3	Antidepressant	51.8		25.1	
Furosemide	54-31-9	Diuretic		190.0	76.4	98.1
Gabapentin	60142-96-3	Anti-convulsive		13,700.0		
Gemfibrozil	25812-30-0	Lipid-regulating	153.0		696.0	80.3
Hydrocodone	34195-34-1	Antitussive	27.9	37.3	24.9	23.6
Hydromorphone	71-68-1	Opiate analgesic	89.0	153.0	207.0	110.0
Ibuprofen	15687-27-1	NSAID			37.0	
Levorphanol	77-07-6	Opiate analgesic		121.0	169.0	176.0
Lorazepam	846-49-1	Benzodiazepine	52.7		18.3	25.4
MDA	4764-17-4	Recreational drug		13.7		
MDMA	42542-10-9	Recreational drug	10.8	69.4	33.1	23
Mefenamic acid 3-carboxy	190379-82-9	Metabolite of mefenamic acid		14.9		

Table A1: Continued.

AO-1	CAS No.	Use	Aug-10	Aug-11	May-12	Aug-12
Meprobamate	57-53-4	Tranquilizer		403.0		
Metformin	657-24-9	Antihyperglycemic		2,600.0		
Methamphetamine	51-57-0	Stimulant		13.6		
Metoprolol	56392-17-7	Beta blocker	198.0	225.0	251.0	261.0
Morphine	57-27-2	Opiate analgesic			418.0	333.0
Naproxen	22204-53-1	NSAID	83.0		195.0	275.0
Oxazepam	604-75-1	Benzodiazepine	97.9			
Oxycodone	124-90-3	Opiate analgesic	116.0	177.0	50.6	35.8
Oxymorphone	76-41-5	Opiate analgesic		177.0	37.6	65.8
Paroxetine	110429-35-1	Antidepressant			10.6	
Phentermine	122-09-8	Anorectic		17.0		
Phenytoin	57-41-0	Anti-convulsive	99.0	10.5	10.3	
Propranolol	4199-10-4	Beta blocker	327.0	56.2	78.2	37.1
Pseudoephedrine	90-82-4	Nasal decongestant		56.7		
Ranitidine	66357-35-5	H2 blocker		260.0		
Sertraline	79559-97-0	Antidepressant	84.7			
Sildenafil	171599-83-0	Erectile dysfunction			10.4	
Sotalol	959-24-0	Anti-arrhythmic	68.3			
Sulfamethoxazole	723-46-6	Antibiotic	705.0	1,620.0	20.3	101.0
Sumatriptan	103628-48-4	Migraine attack		28.7	32.4	48.5
Temazepam	846-50-4	Benzodiazepine	135.0	196.0	108.0	809.0
Theophylline	58-55-9	Bronchodilator	93.6			
Thiabendazole	148-79-8	Fungicide		15.1	30.4	21.4
Tramadol	27203-92-5	Opiate analgesic		315.0	422.0	488.0
Trazodone	19794-93-5	Antidepressant		18.6	92.3	
Triamterene	396-01-0	Diuretic	104.0	112.0	114.0	107.0
Triclocarban	101-20-2	Antibacterial	146.0		45.5	
Triclosan	3380-34-5	Antibacterial			220.0	15.5
Trimethoprim	738-70-5	Antibiotic	108.0	146.0	34.6	45.2
Venlafaxine	93413-44-6	Antidepressant		884.0	674.0	366.0
Verapamil	152-11-4	Calcium-channel blocker			23.6	
Warfarin	81-81-2	Anti-coagulant	64.9			10.1
CC-1						
Caffeine	58-08-2	Stimulant				12.3
Theophylline	58-55-9	Bronchodilator	19.7			
Triclocarban	101-20-2	Antibacterial	20.2			
CC-2						
Caffeine	58-08-2	Stimulant				21.9
DEET	58-55-9	Insect repellent			11.9	
CC-3						
Warfarin	58-08-2	Anti-coagulant	10.4			
CD-1						
17B-Estradiol-glucuronide	58-08-2	Metabolite of 17b-estradiol	26.4			
Caffeine	58-55-9	Stimulant				13.1
Theophylline	101-20-2	Bronchodilator	11.8			
CD-3						
Caffeine	58-08-2	Stimulant				22.4
CWC-2						
Caffeine	58-08-2	Stimulant				16.2
DEET	58-55-9	Insect repellent			23.5	
CWC-4						
Caffeine	58-08-2	Stimulant				17.8
DM-1						
Caffeine	58-08-2	Stimulant				19.8
DM-2						
Caffeine	58-08-2	Stimulant				11.1
DM-3						
Caffeine	58-08-2	Stimulant				18.6
NCC-1						
Caffeine	58-08-2	Stimulant				17.0

Table A1: Continued.

SCC-1	CAS No.	Use	Aug-10	Aug-11	May-12	Aug-12
DEET	58-08-2	Insect repellent			15.0	
SCC-6						
Caffeine	58-08-2	Stimulant				20.9
DEET	58-55-9	Insect repellent			16.4	
SCC-7						
Caffeine	58-08-2	Stimulant				16.7
SC-2						
Caffeine	58-08-2	Stimulant				24.5
SC-3						
Caffeine	58-08-2	Stimulant				22.2
WC-2						
DEET	58-08-2	Insect repellent			14.8	

Table A 2 Cumulative concentration of pharmaceuticals and personal care products (ng/L) from the waste water treatment plant effluent (AO-1), Crestone (CC-1), Deadman (DM-3), and South Crestone (SCC-7) creeks, by POCIS.

AO-1	CAS No.	Use	Concentration (ng/L)
Albuterol	18559-94-9	Bronchodilator	573.67
Alprazolam	28981-97-7	Benzodiazepine+C141	2,170.00
Amitriptyline	549-18-8	Antidepressant	10,100.00
Amitriptyline (+/-)-E-10-hydroxylated	64510-05-4	Antidepressant	5,186.67
Atenolol	29122-68-7	Beta blocker	12,000.00
Benzoylecgonine	519-09-5	Metabolite of cocaine	219.67
Butalbital	77-26-9	Barbiturate	6,300.00
Caffeine	58-08-2	Stimulant	20,926.67
Cannabinol	521-35-7	Metabolite of THC	461.67
Carbamazepine	298-46-4	Anti-convulsive	7,303.33
Chlorpheniramine	132-22-9	Anti-histamine	4,060.00
Clonidine	4205-91-8	Hypertension	257.33
Codeine	76-57-3	Antitussive	4,843.33
DEET	134-62-3	Insect repellent	343,443.33
Diazepam	439-14-5	Benzodiazepine	1,610.00
Diclofenac	15307-86-5	NSAID	23,700.00
Diltiazem	33286-22-5	Calcium-channel blocker	678.00
Diphenhydramine	58-73-1	Anti-histamine	256,443.67
Famotidine	76824-35-6	H2 blocker	142.67
Fentanyl	437-38-7	Opiate analgesic	291.00
Fluoxetine	54910-89-3	Antidepressant	5,263.33
Furosemide	54-31-9	Diuretic	29,366.67
Gemfibrozil	25812-30-0	Lipid-regulating	323,000.00
Glyburide	10238-21-8	Anti-diabetic	531.33
Hydrocodone	34195-34-1	Antitussive	4,273.33
Hydromorphone	71-68-1	Opiate analgesic	48,633.33
Ibuprofen	15687-27-1	NSAID	147,000.00
Levorphanol	77-07-6	Opiate analgesic	52,766.67
Loratadine	79794-75-5	Anti-histamine	1,316.67
Lorazepam	846-49-1	Benzodiazepine	2,090.00
MDMA	42542-10-9	Recreational drug	1,866.67
Metoprolol	56392-17-7	Beta blocker	56,166.67
Morphine	57-27-2	Opiate analgesic	212,000.00
Naproxen	22204-53-1	NSAID	36,233.33
n-Butylparaben	94-26-8	Preservative	310.00
Nifedipine	21829-25-4	Calcium-channel blocker	137.67
Nifedipine oxidized	67035-22-7	Metabolite of CYP3A4 Nifedipine	149.00
Norverapamil	67812-42-4	Calcium-channel blocker	2,130.00
Oxycodone	124-90-3	Opiate analgesic	7,373.33
Oxymorphone	76-41-5	Opiate analgesic	12,000.00
Paroxetine	110429-35-1	Antidepressant	314.00
Phenobarbital	50-06-6	Barbiturate	4,716.67
Phenytoin	57-41-0	Anti-convulsive	739.33
Progesterone	57-83-0	Steroid hormone	400.67
Propranolol	4199-10-4	Beta blocker	30,466.67
Sildenafil	171599-83-0	Phosphodiesterase (PDE) inhibitor	2,976.67
Simvastatin	79902-63-9	HMG-CoA reductase inhibitor	426.00
Sotalol	959-24-0	Anti-arrhythmic	184.33
Sulfamethoxazole	723-46-6	Antibiotic	10,873.33
Sumatriptan	103628-48-4	Selective serotonin receptor agonist	6,326.67
Temazepam	846-50-4	Benzodiazepine	240,333.33
Testosterone	58-22-0	Steroid hormone	399.00
THC	1972-08-3	Recreational drug	3,672.00
Thiabendazole	148-79-8	Fungicide	3,026.67
Tramadol	27203-92-5	Opiate analgesic	74,433.33
Trazodone	19794-93-5	Antidepressant	25,333.33
Triamterene	396-01-0	Diuretics	28,366.67
Triclocarban	101-20-2	Antibacterial	3,725.33

Table A2: Continued.

AO-1	CAS No.	Use	Concentration (ng/L)
Triclosan	3380-34-5	Antibacterial	9,096.67
Trimethoprim	738-70-5	Antibiotic	8,094.00
Venlafaxine	93413-44-6	Antidepressant	166,666.67
Verapamil	152-11-4	Calcium-channel blocker	4,076.67
Warfarin	81-81-2	Anti-coagulant	1,763.33
CC-1			
Caffeine	58-08-2	Stimulant	453.33
Cannabidiol	13956-29-1	Recreational drug	265.00
DEET	134-62-3	Insect repellent	110,443.33
Progesterone	57-83-0	Steroid hormone	170.67
Triclosan	3380-34-5	Antibacterial	2,046.67
DM-3			
Atenolol	29122-68-7	Beta blocker	109.00
Caffeine	58-08-2	Stimulant	1,966.67
DEET	134-62-3	Insect repellent	142,776.67
Diphenhydramine	58-73-1	Anti-histamine	19.33
Gemfibrozil	25812-30-0	Lipid-regulating	441.67
Ibuprofen	15687-27-1	NSAID	1,823.33
Levorphanol	77-07-6	Opiate analgesic	108.67
Naproxen	22204-53-1	NSAID	1,113.33
Progesterone	57-83-0	Steroid hormone	134.33
THC	1972-08-3	Recreational drug	529.00
Triclocarban	101-20-2	Antibacterial	675.33
Triclosan	3380-34-5	Antibacterial	1,913.33
SCC-7			
Caffeine	58-08-2	Stimulant	410.00
DEET	134-62-3	Insect repellent	36,410.00
Triclocarban	101-20-2	Antibacterial	24.67
Triclosan	3380-34-5	Antibacterial	300.00
Trimethoprim	738-70-5	Antibiotic	15.67