Biological responses of marine flatfish exposed to effluent

Doris E. Vidal-Dorsch, Steven M. Bay, Monica A. Mays, Darrin J. Greenstein, Diana Young, Daniel Schlenk[1] and Kevin M. Kelley[2]

Abstract

There is an increasing concern about the presence of pharmaceutical compounds, personal care products, and other chemicals collectively known as contaminants of emerging concern (CECs) in municipal effluents. Yet, knowledge about potential environmental impacts related to these compounds is still limited. In this study we conducted laboratory exposures that examined estrogenic, androgenic, and thyroid-related endocrine responses in marine hornyhead turbot (*Pleuronichthys verticalis*) exposed to CECs from municipal wastewater effluents with two degrees of treatment. Plasma concentrations of estradiol (E2), vitellogenin (VTG), 11-keto testosterone (11-KT), and thyroxine (T4) were measured to assess endocrine responses. In addition, 32 effluent CECs were analyzed using gas chromatography/mass spectrometry to investigate the presence of these compounds in the effluents. Several pharmaceuticals and personal care products were present in the effluent treatments used during the exposures. No significant responses were observed in fish exposed to environmentally realistic concentrations of effluent. Elevated E2 concentrations were observed in males exposed to ammonia concentrations similar to those found in effluents. Exposure to ammonia did not result in high male VTG concentrations. The results of this study highlight the importance to conduct research with sentinel organisms to understand the environmental significance of the presence of CECs in aquatic systems.

Introduction

Although the presence of pharmaceutical compounds, personal care products, and industrial and commercial chemicals, commonly referred to as CECs in municipal effluents has been well documented, knowledge about potential environmental impacts related to these contaminants remains limited. Because publicly owned treatment works (POTWs) plants were not designed to remove CECs, effluents from these facilities represent a primary source of these compounds in aquatic environments (Baronti *et al*. 2000). Recent studies indicate that exposure to environmental CECs can cause adverse estrogenic responses in fish and their populations (Folmar *et al*. 2001, Kidd *et al*. 2007). Since there are thousands of chemicals that could produce a wide range of effects, it is important to investigate and prioritize which chemicals or chemicals mixtures we should truly be concerned about.

The environmental presence of CECs has spurred further research because of the potential risk that these compounds pose to aquatic life (Daughton and Ternes 1999, Jorgensen and Halling-Sorensen 2000). Some municipal wastewater CECs are known to accumulate in fish tissue (Snyder *et al*. 2004). Furthermore, in some fish species, biological responses have been documented after environmental exposure to CECs (Tilton *et al*. 2002, Todorov *et al*. 2002, Liney *et al*. 2006, Barber *et al*. 2007, Alvarez *et al*. 2009). Most studies investigating effects from effluent CECs exposure to fish focused mainly on responses that measure VTG and E2 concentrations (Hemming *et al*. 2001, Tilton *et al*. 2002, Barber *et*

[1] *University of California, Riverside, CA*
[2] *California State University, Long Beach, CA*

al. 2007, Thorpe *et al*. 2008). Although the study of E2 and VTG responses in fish is very important and should be continued because of their involvement in reproductive processes, it is necessary to investigate additional types of endocrine responses since CECs could affect diverse biological processes (Sanderson *et al*. 2004).

Biological responses indicative of exposure to endocrine disruptors were found in marine flatfish living near POTW outfall discharges in southern California. Responses observed in hornyhead turbot (*P. verticalis*) included widespread elevated concentrations of VTG and E2 in males collected in different areas around the Southern California Bight (unpublished data). In addition, different types of CECs were found in POTW effluents, seawater and sediments collected near the effluent discharge areas (unpublished data). However, legacy contaminants such as dichloro diphenyl trichloroethane (DDT) were also found in sediments near some of the POTW discharge sites. Thus, the chemical parameters responsible for the biological alterations found in previous field studies are still unclear since legacy contaminants may cause effects similar to those caused by currently discharged endocrine disruptors.

In this study, laboratory exposures were conducted to determine the potential for POTW effluent constituents to cause endocrine responses in marine flatfish. We selected a laboratory approach to remove some confounding factors present in the field (e.g., presence of legacy contaminants) and directly examine the contribution of POTW constituents to endocrine responses. We examined two effluents types (primary and secondary treated effluents), which allowed us to investigate biological responses produced by different types of CEC mixtures and effluent treatment processes. We also examined two different effluent concentrations of which one represented maximal environmentally realistic levels of exposure, to determine the effects of effluent dilution and quantify dose responses. In addition, we investigated endocrine responses to ammonia, an important POTW constituent and potential confounding factor.

Methods

Experimental Design

To investigate endocrine responses resulting from exposure to POTW effluents, two laboratory experiments were conducted using effluent from San Diego (Point Loma; SD) and Los Angeles (Hyperion; LA). The SD effluent received advanced primary treatment and LA effluent received secondary treatment. Treatments used during exposures consisted of negative controls (seawater), positive controls (E2), and 0.5% and 5% effluent concentrations. During the SD exposure an ammonia (NH_3) treatment was also added to investigate the effects of this common effluent constituent on endocrine responses. The 0.5% concentration of effluent was selected to represent an environmentally realistic level of exposure. The 5% effluent concentration represented a level at which biological responses were expected. The NH_3 concentration used was based on the ammonia levels present in the 5% effluent concentration. Each exposure lasted 14 days and had 10 replicates per treatment.

At the end of the exposure blood plasma was collected from each hornyhead turbot to measure estrogenic, androgenic, and thyroid related indicators. Biological responses investigated included measurements of plasma E2, 11-KT, T4, and VTG concentrations in female and male fish. A suite of 32 CECs was analyzed to investigate the presence of these compounds in the effluent treatments and seawater. Adult hornyhead turbot were collected by trawl from Dana Point (California) and brought to the laboratory to conduct the effluent exposures (Figure 1). Fish collections took place in April and May of 2009. Fish that were injured during collection or did not appear to be healthy after the acclimation period were not used for the experiments.

Effluents and Seawater

In this study, effluents from two major POTW facilities in southern California (San Diego and Los

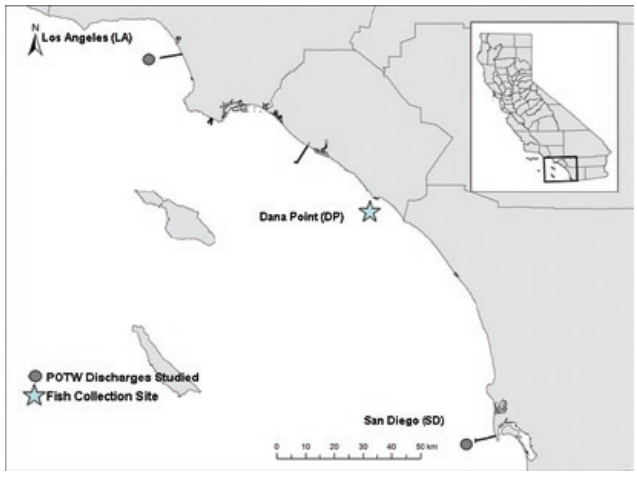

Figure 1. Hornyhead turbot collection sites.

Angeles) were used, while seawater for the analyses was collected near Redondo Beach (California). Effluent from SD was provided by the Point Loma Wastewater Treatment Plant (California). The SD effluent received advanced primary treatment in which wastewater entering the facility (influent) underwent screening through a 0.6 inch traveling screen, followed by aerated grit removal and chemically assisted sedimentation before discharge via the Point Loma Ocean Outfall. Effluent provided by the City of Los Angeles (LA) was from the Hyperion Treatment Plant (HTP) located in Playa Del Rey (California). The LA influent was treated with sedimentation during the primary treatment process with the aid of ferric chloride and polymer. This primary treatment was followed by a secondary treatment that consisted of aeration in oxygen reactors and settling of activated sludge in clarifier basins. The seawater used during the exposures was stored at SCCWRP in 1000 to 2000 gallon polyethylene tanks following collection. The seawater was then pumped through filters (0.45 µm, 1 to 5 µm, 50 to 100 µm, and carbon) and chilled at 15 ±1°C before it was added to exposure tanks.

Exposure Conditions

During the exposures, each fish was considered a replicate and placed in an all-glass 60 L tank containing 40 L of seawater or treatment solution. Fish were kept under flow through conditions for acclimation periods of two weeks. The fish had a regime of 16 hour light: 8 hour dark photoperiod. After acclimation, the fish were randomly selected and assigned to different exposure treatments. The fish were fed *ad libitum* pieces of worm and squid every other day. Water quality measurements were taken and recorded three times a week. Fish were maintained at a temperature of 15 ±1°C and at a salinity of 33 ±2 parts per thousand. During acclimation, dissolved oxygen averaged 7.97 ±0.19 mg/L (average ± standard deviation), NH_3 0.27 ±0.10 mg/L, and pH 7.97 ±0.03.

Throughout exposure to POTW effluents and control treatments, the fish remained in their original acclimation tanks, but received treatment solutions or seawater by static renewal. Daily 50% static renewals consisted of removing 20 L of water from each tank and then adding a 20 L of treatment solution. Negative seawater controls received seawater during water renewals with similar methods and equipment used for other treatments. Photoperiod, temperature, salinity and feeding regimes were kept similar to conditions previously described for acclimation. Water quality measurements were taken three times a week during exposures. Dissolved oxygen averaged 7.36 ±0.13 mg/L, and pH 7.86 ±0.02 in both exposures. Total NH_3 concentration for the negative controls and 0.5% effluent and E2 treatments was 0.66 ±0.18 mg/L. Total NH_3 concentration for the ammonia and 5% effluent treatments was 1.99 ±0.13 mg/L. A total of 32 males and 18 females were used for exposure to SD effluent, and 19 males and 15 females for exposure to LA effluent. The fish

Table 1. Number of fish (N) per treatment type, mean length and weight, and respective standard deviation (sd) by gender and location.

Location/ Treatment Type	Males					Females				
	N	Length (cm)		Weight (g)		N	Length (cm)		Weight (g)	
		Mean	sd	Mean	sd		Mean	sd	Mean	sd
San Diego										
Seawater Control	6	14.5	1.9	78.3	20.4	4	18.1	2.3	166.3	65.7
0.50%	7	14.5	1.2	71.4	15.7	2	17.4	0.9	112.5	3.5
5%	6	15.4	1.3	90.8	25.8	4	15.7	1.5	95.0	31.6
E2	8	14.7	1.2	74.4	19.4	2	18.0	2.5	140.0	56.6
NH_3	5	13.7	2.1	66.0	25.3	3	17.1	2.4	121.7	58.4
Los Angeles										
Seawater Control	6	15.0	1.1	72.2	22.6	3	17.7	1.6	136.7	45.4
0.50%	1	14.7	0.0	85.0	0.0	5	18.1	2.9	140.6	78.7
5%	6	14.0	1.7	80.8	33.5	3	17.7	1.8	77.7	41.8
E2	6	14.0	1.9	56.0	21.4	4	16.9	1.4	108.8	21.4

standard length and weights were statistically similar within each gender for both exposures (Table 1).

Biological Measurements

After the POTW effluent exposures, blood plasma samples were collected to measure VTG and hormone concentrations of E2, T4, and 11-KT from each fish. After every exposure period, surviving fish were weighed, measured for standard lengths, bled, sacrificed, dissected, and sexed. Fish were anesthetized with a solution of MS-222 (Sigma-Aldrich, San Louis, MO). Once anesthetized and measured, the fish were sampled for blood. Bleeding was carried out while the heart was still beating. The blood was removed with a syringe from the caudal veins and arteries that run along the ventral side of the spine. Then the blood was centrifuged for plasma collection.

Hornyhead turbot blood plasma hormones were analyzed using a radioimmunoassay (RIA) for E2. For 11-KT and T4 an enzyme immunoassay (EIA) was used and VTG was analyzed using a competitive enzyme-linked immunosorbant assay (ELISA) developed for this species (Rempel *et al*. 2006, Brara *et al*. 2010). Detection limits for the hormone assays were 4.4 pg/ml (E2), 3.9 ng/ml (T4) and 1.3 pg/ml (11-KT). All hormone samples were analyzed in duplicate. Plasma VTG detection limit was 1.25 ng/ml. All VTG samples were analyzed in triplicate and at three different dilutions (1:50, 1:1500, 1:45 000).

Preparation of Positive Controls and Effluent Treatments

Estradiol positive controls were used in addition to two POTW effluent concentrations in the exposures. The E2 treatments were used to investigate fish estrogenic responses during the effluent exposure. Estradiol positive controls of 8 µg/L were used. An initial stock solution was prepared by dissolving E2 powder in HPLC grade acetone (Fisher Scientific Pittsburgh, PA), in a 10 L glass jar. Subsequently, the acetone was evaporated at room temperature for one hour; this step was followed to plate the E2 onto the jar surface. Then 10 L of seawater were added to the glass jar to create a second stock solution which was mixed for 2 hours. The second stock solution was subsequently transferred to a 20 L carboy where it was mixed with another 10 L of seawater to create a final E2 stock solution. The final E2 solution was manually delivered to test tanks where it was mixed with seawater to achieve the target concentration. The E2 concentration measured in treatment tanks was 5.08 ±2.28 µg/L.

Two effluent concentrations were used in each exposure: 0.5 and 5 %. To prepare the 0.5% effluent stock solution, 1 L of whole effluent was mixed with 19 L of seawater. Preparation of the 5% effluent stock solution consisted of mixing 10 L of raw final effluent and 10 L of seawater. Then the 20 L of each effluent treatment solution were mixed for 5 minutes in a carboy. Afterward, 2 L of the corresponding effluent stock solution were manually delivered to each test tank; seawater was also simultaneously added to achieve the target concentration and volume in each tank.

In the exposure conducted with SD POTW effluent an additional treatment with spiked ammonia was included. This NH_3 treatment was used to determine if some of the biological responses in the fish could be influenced by ammonia since it is a very common constituent in POTW effluents. This treatment was not used during the LA exposure because not enough fish were available. The NH_3 treatment had a concentration of 1.97 ±0.46 mg/L.

Effluent CECs Measurements

Several effluent and seawater CECs were measured in samples collected through the POTW effluent exposures to investigate the presence of these constituents in the effluent treatments. The CECs were analyzed using liquid chromatography/tandem mass spectrometry (LC-MS/MS) and gas chromatography/tandem mass spectrometry (GC-MS/MS) methods. The samples were analyzed by the Southern Nevada Water Authority (Las Vegas, NV). Seawater samples were composites collected from each of the negative control tanks at the beginning and end of each exposure. The CEC concentrations in the 5% treatments were also analyzed at the beginning and end of each exposure in composite samples collected from each tank of the treatment. For the 0.5% treatments CECs were measured in composite samples collected from each tank of the treatment, but measured only once at the end of each exposure since most target CECs were expected to be below detection limits. Effluent and seawater samples were collected in two 1 L, silanized, glass, amber bottles. Prior to sample collection, each bottle received 1 g of sodium azide and 50 mg of ascorbic acid to prevent microbial degradation and to reduce

the presence of residual oxidants (unpublished data). Full descriptions of the methods used for chemical analyses have been previously published (Trenholm *et al*. 2006, Vanderford and Snyder 2006). A list of the 32 CECs measured for this study and the detection limits for each constituent can be found in Table 2.

Statistical Analysis

To analyze the target endocrine responses of fish exposed to the POTW effluent or positive control treatments, data were statistically compared to their respective negative seawater controls (SWC). Statistical tests were conducted with JMP version 8

Table 2. Reporting limits (RL) for contaminants of emerging concern (CEC) present in the 5% treatment effluent, by location and contaminant type, along with respective mean (the average of two samples collected at the beginning and end of the exposures), minimum (Min), and maximum (Max) concentrations in µg/L. For the 0.5% effluent treatment samples, CEC concentrations in µg/L were only measured at the end of exposure. PPCP = Pharmaceutical and Personal Care Products. ND = not detected.

CEC by Type	RL	San Diego				Los Angeles			
		5% Treatment			0.5% Treatment	5% Treatment			0.5% Treatment
		Mean	Min	Max	End	Mean	Min	Max	End
PPCP									
Triclosan	0.01	0.06	0.03	0.09	0.01	0.06	0.01	0.11	ND
Sulfamethoxazole	0.0025	0.140	0.120	0.160	0.015	0.039	0.027	0.050	0.005
Trimethoprim	0.0025	0.069	0.065	0.073	0.007	0.035	0.034	0.035	ND
Fluoxetine	0.0005	0.001	0.001	0.002	ND	0.002	0.002	ND	ND
Meprobamate	0.0025	0.043	0.042	0.044	0.004	0.018	0.017	0.019	ND
Carbamazepine	0.005	0.017	0.016	0.018	ND	0.012	0.01	0.013	ND
Dilantin	0.0011	0.011	0.010	0.011	ND	0.005	ND	0.010	ND
Primidone	0.0005	0.010	0.009	0.011	ND	0.003	ND	0.007	0.001
Diclofenac	0.0025	0.011	0.010	0.012	0.003	0.005	ND	0.010	ND
Ibuprofen	0.05	1.5	1.0	2.0	0.1	ND	ND	ND	ND
Naproxen	0.005	1.030	0.850	1.200	0.097	0.005	ND	0.009	ND
Atenolol	0.0011	0.260	0.240	0.270	0.024	0.073	0.071	0.075	ND
Atorvastatin	0.0025	0.011	0.0055	0.015	ND	0.001	ND	0.002	ND
Musk Ketone	0.025	ND	ND	ND	ND	ND	ND	ND	ND
Gemfibrozil	0.0025	0.295	0.28	0.31	0.029	0.14	0.14	0.14	0.013
Caffeine	0.0053	4.750	4.200	5.300	0.550	0.007	ND	0.011	ND
Diazepam	0.0003	ND	ND	ND	ND	ND	ND	ND	ND
Iopromide	0.011	ND	ND	ND	ND	0.015	ND	0.025	ND
Current Use Pesticides									
Lindane	0.001	0.009	0.0069	0.012	ND	0.003	ND	0.004	ND
Atrazine	0.0003	ND	ND	ND	ND	ND	ND	ND	ND
NN-Diethyl-meta-toluamide (DEET)	0.001	0.0255	0.021	0.03	0.008	0.067	0.045	0.088	0.007
Hormones									
Estradiol	0.0005	0.001	ND	0.0023	0.001	0.08	ND	0.16	0.003
Estrone	0.0002	0.007	0.005	0.008	0.003	0.016	0.002	0.031	0.001
Progesterone	0.0005	0.014	ND	0.027	ND	ND	ND	ND	0.001
Testosterone	0.0005	0.004	ND	0.007	ND	ND	ND	ND	ND
Etynylestradiol	0.001	ND	ND	ND	ND	ND	ND	ND	ND
Industrial/Commercial									
Tris(1-chloro-2-propyl) phosphate (TCPP)	0.11	ND	ND	ND	ND	ND	ND	ND	ND
Tris(2-chloroethyl) phosphate (TCEP)	0.01	0.008	ND	0.011	ND	0.012	ND	0.018	ND
Benzophenone	0.053	0.086	0.061	0.11	ND	ND	ND	ND	ND
Bisphenol A	0.05	ND	ND	ND	ND	0.009	ND	0.015	ND
Nonylphenol	0.08	0.541	0.345	0.737	0.27	0.505	0.36	0.65	0.102

(SAS, USA). Comparisons were made within the SD or the LA exposure. The data were not normally distributed and were log transformed prior to analysis. Data variances were analyzed using one-way analysis of variance (ANOVA; $p = <0.05$), followed by a comparison to seawater controls using Dunnett test. Data in the bar graphs of this manuscript are presented as the geometric mean ± standard error.

Results

CECs Measurements

The occurrence and concentration of CECs differed in the investigated effluent types. In general, more of the investigated CECs were found at detectable levels in SD effluent and at higher concentrations (Table 2). The CECs detected at the highest concentrations in SD effluent treatments were caffeine, ibuprofen, naproxen, nonylphenol and gemfibrozil. Concentrations of detected CECs in SD effluent treatments ranged from 0.003 µg/L for diclofenac to 4.75 µg/L for caffeine. In the LA effluent treatments, the CECs detected at the highest concentrations were nonylphenol, triclosan, gemfibrozil, DEET, and sulfamethoxazole. In LA effluent treatments, the CEC concentrations ranged from 0.003 µg/L for estradiol to 0.57 µg/L for nonylphenol. Some of the CECs detected in the effluent treatments are known to cause estrogenic responses in fish (e.g. nonylphenol, and estradiol).

A few CECs were detected at very low levels in the seawater used for controls and dilutions. The CECs detected in seawater included, nonylphenol, bisphenol A, progesterone, DEET, estrone, sulfamethoxasole, and estradiol. However, the concentrations of these CECs were very close to detection limits ranging from 0.0006 to 0.0056 µg/L, except for nonylphenol (0.25 ±0.16 µg/L; average ± standard deviation) and estrone (0.001 ±0.01 µg/L). Effluent dilutions used in 0.5 and 5% treatments were accurate as indicated by the comparison to CEC concentrations in whole effluent (Table 3).

Biological Measurements

No significant patterns of response were observed in fish exposed to effluent when compared to controls even at concentrations ten times what is encountered in the environment. However, significant patterns of response were observed, in fish exposed to the positive control estradiol and ammonia treatments. Significant estrogenic responses in fish exposed to effluents were not observed but there are some patterns that may be indicative of potential responses. Specifically, females exposed to 0.5% SD and LA effluent treatments had lower mean VTG concentrations than their respective controls, although the differences were not significant ($p > 0.05$; Figure 2) and did not follow a classical concentration-response relationship. Considerable variability in individual female responses reduced statistical power of the analysis and was likely due to variation of sexual maturity stages (gonadosomatic index and VTG regression yielded $r^2 > 0.5$; $p < 0.05$) found in these fish. Females exposed to E2 showed elevated plasma estradiol and VTG concentrations, but these differences were only significantly higher than control responses in the LA effluent treatments. Ammonia had no influence on the measured estrogenic responses in female fish.

Estrogenic responses were also not observed in male fish exposed to the POTW effluent concentrations. No significant differences were observed in E2 or VTG concentrations ($p > 0.05$) when compared to controls (Figure 2). The VTG concentrations observed in male fish were low and, in many individuals, close to the method detection limits. The E2 concentrations were below detection or statistically similar to the concentrations observed in control males. Estrogenic responses were observed in males exposed to ammonia and the positive control, E2. Male fish exposed to NH_3 had significantly higher concentrations of plasma E2 when compared to controls ($p < 0.05$). Exposure to NH_3 did not result in a significant increase in male VTG production. As expected, exposure of male fish to the positive control, E2, resulted in a significant increase in both plasma E2 and VTG production in both exposure periods. Significant androgenic responses were not observed in fish exposed to either wastewater effluent, but significant anti-androgenic responses were observed following exposure to the positive control, E2. Specifically, female and male plasma 11-KT concentrations were not affected by exposure to different effluent treatments. Fish exposed to effluent treatments showed similar 11-KT concentrations when compared to their respective controls ($p > 0.05$; Figure 3). However, 11-KT concentrations were significantly lower upon exposure to E2 in both male and female fish. Slightly lower levels of 11-KT were observed in fish of both genders exposed to ammonia, but the decrease was not statistically significant ($p > 0.05$).

Table 3. Mean contaminants of emerging concern (CEC) concentrations (μg/L) and respective standard deviation (sd), by type, in whole effluent samples. Mean and sd calculated using three samples. PPCP = Pharmaceutical and Personal Care Products. ND = not detected.

CEC by Type	Common Use	RL	San Diego		Los Angeles	
			Mean	sd	Mean	sd
PPCP						
Triclosan	Antibacterial	0.01	1.08	0.1	0.4	0.1
Sulfamethoxazole	Antibiotic	0.0025	1.83	0.2	1.26	0.3
Trimethoprim	Antibiotic	0.0025	1.09	0.3	0.67	0.0
Fluoxetine	Antidepressant	0.0005	0.02	0.0	0.04	0.0
Meprobamate	Antidepressant	0.0025	0.7	0.1	0.37	0.0
Carbamazepine	Antiepileptic	0.005	0.48	0.2	0.22	0.0
Dilantin	Antiepileptic	0.0011	0.19	0.1	0.18	0.0
Primidone	Antiepileptic	0.0005	0.16	0.0	0.11	0.0
Diclofenac	Anti-inflammatory	0.0025	0.2	0.0	0.18	0.0
Ibuprofen	Anti-inflammatory	0.05	18.13	11.8	ND	ND
Naproxen	Anti-inflammatory	0.005	15.3	5.1	0.17	0.0
Atenolol	Beta Blocker	0.0011	4.17	1.3	1.4	0.1
Atorvastatin	Cholesterol regulator	0.0025	0.29	0.2	0.21	0.2
Musk Ketone	Fragrance	0.025	ND	ND	ND	ND
Gemfibrozil	Lipid Regulator	0.0025	5.33	1.3	3.07	0.4
Caffeine	Stimulant	0.0053	99	1.7	0.3	0.1
Diazepam	Tranquilizer	0.0003	0.01	0.0	ND	ND
Iopromide	X-ray contrast medium	0.011	ND	ND	0.5	0.5
Current Use Pesticides						
Lindane	Insecticide	0.001	0.21	0.1	0.14	0.0
Atrazine	Herbicide	0.0003	ND	ND	ND	ND
DEET	Insect repellant	0.001	0.51	0.1	1.06	0.4
Hormones						
Estradiol	Hormone	0.0005	0.01	0.0	0.01	0.0
Estrone	Hormone	0.0002	0.1	0.0	0.01	0.0
Progesterone	Hormone	0.0005	0.18	0.3	ND	ND
Testosterone	Hormone	0.0005	0.12	0.1	ND	ND
Etynylestradiol	Synthetic hormone	0.001	ND	ND	ND	ND
Industrial/Commercial						
Tris(1-chloro-2-propyl) phosphate (TCPP)	Flame retardant	0.11	ND	ND	1.05	1.0
Tris(2-chloroethyl) phosphate (TCEP)	Flame retardant	0.01	0.16	0.1	0.35	0.0
Benzophenone	UV blocker	0.053	1.08	0.9	ND	ND
Bisphenol A	Plasticizer	0.05	0.39	0.2	0.26	0.0
Nonylphenol	Surfactant	0.08	2.34	0.4	1.63	0.3

Concentrations of T4 in fish exposed to effluent, E2, and NH_3 treatments were not significantly different than controls. For females the average T4 concentrations in the control fish differed by more than two-fold between the two exposures studies, making it difficult to determine if any trends existed among the female responses to effluents or controls (E2 and NH_3). Different T4 concentrations patterns were observed in male fish exposed to either SD or LA effluents (Figure 4). Males exposed to SD effluent treatments had lower T4 concentrations than controls and displayed a concentration-response relationship,

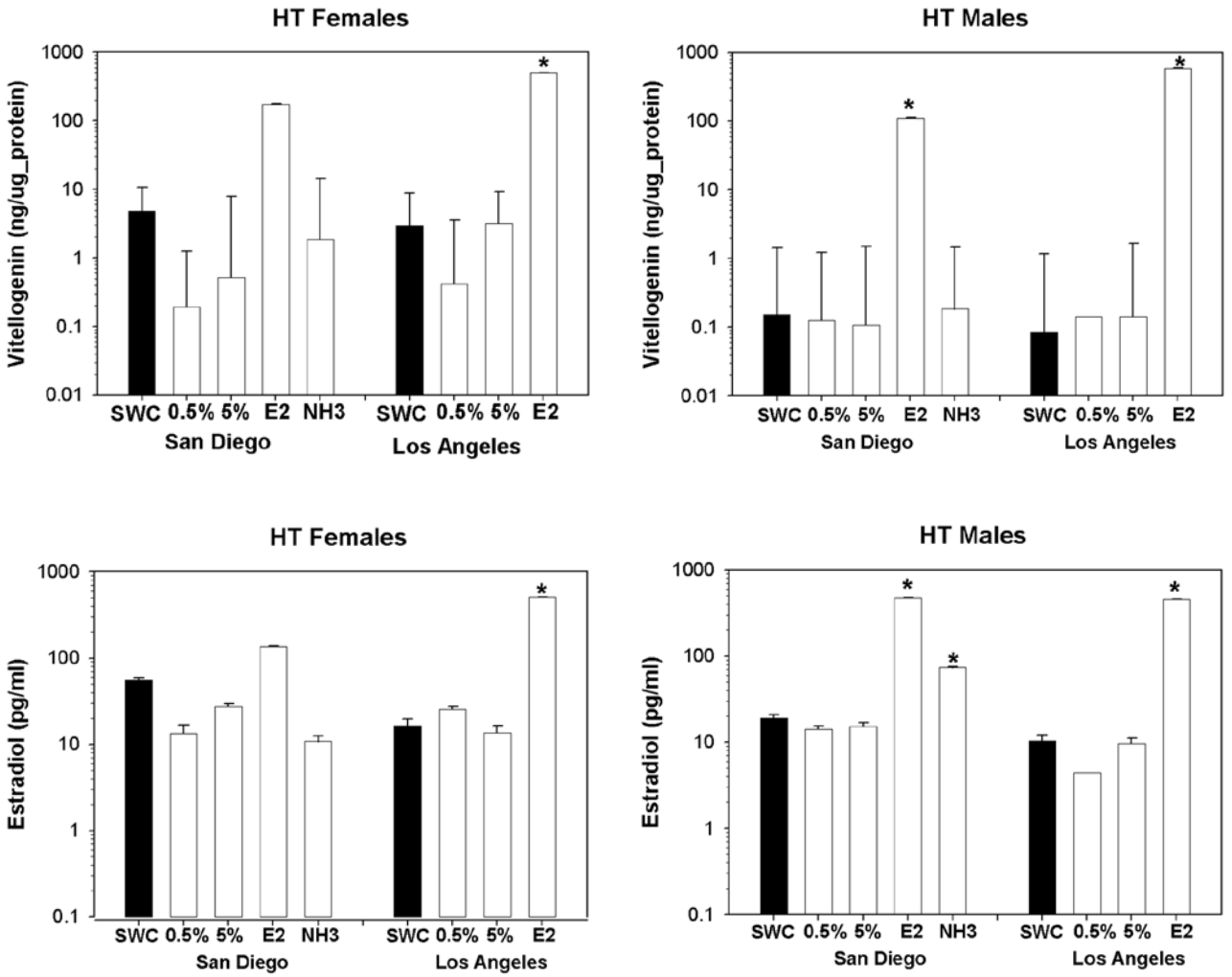

Figure 2. Female and male estradiol and vitellogenin concentrations in hornyhead turbot. Bars represent geometric mean concentrations ± standard errors. * indicates a significant increase when averages are compared to the control for each effluent exposure for seawater (SWC), estradiol (E2) and ammonia (NH3) controls, and for 0.5 and 5% effluent treatments.

but these differences were not statistically significant ($p > 0.05$). Male fish exposed to ammonia had higher T4 concentrations but the difference was not significantly different ($p > 0.05$).

Discussion

The presence and concentrations of effluent CECs varied in the studied effluent types. In general, the SD effluent had more chemicals detected, and at higher concentrations, than the LA effluent. The effluents differed likely because the LA effluent received biological treatment (secondary) that removed CECs more effectively (Snyder *et al*. 2003). In both effluents, most of the target CECs found at detectable levels had low concentrations (in the parts per trillion range). The concentrations of estrogenic compounds detected in the effluents used were lower than levels known to cause estrogenic effects in other fish species (Christensen *et al*. 2009). For example, estradiol and ethynylestradiol are known to cause estrogenic responses in fish larvae at concentrations of 0.10 and 0.03 µg/L (Hagino *et al*. 2001). The concentrations found in SD and LA whole effluents were 0.01 µg/L for estradiol and ethynylestradiol was not detected (in both effluents). Another example is nonylphenol, a chemical known to cause endocrine responses in fish at concentrations higher than 50 µg/L (Brooke and Thursby 2005). The nonylphenol concentrations found in SD and LA whole effluent samples were 2.34 and 1.63 µg/L. The presence of these chemicals at such low concentrations in the effluent prior to further dilution (20X and 200X) for the fish exposure studies may be

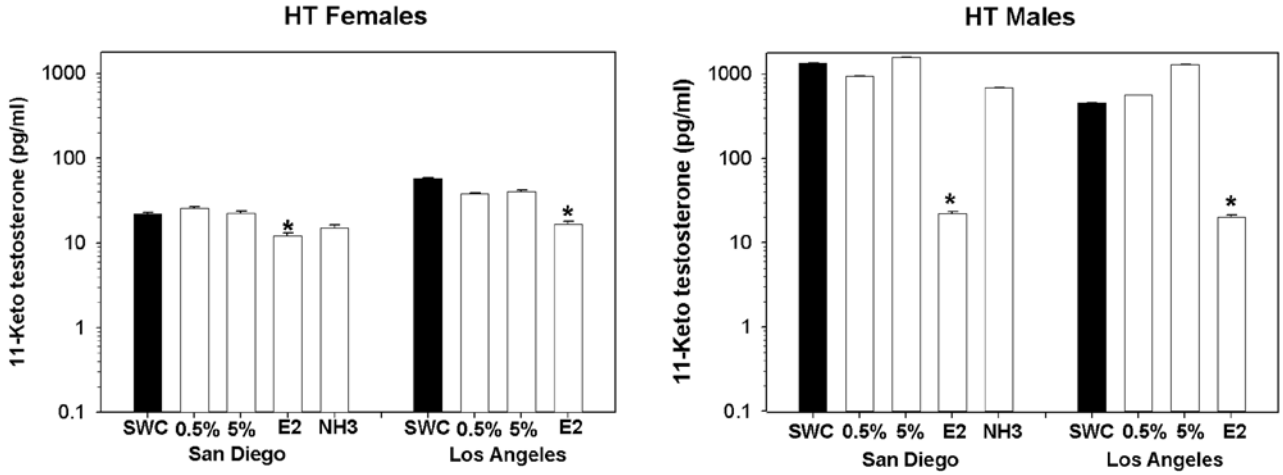

Figure 3. Hornyhead turbot female and male 11-keto testosterone concentrations measured. Bars represent geometric mean concentrations ± standard errors. * indicates a significant decrease when averages are compared to the control for each effluent exposure.

the primary reason why no significant biological responses were observed in our study. However, the selected effluent exposure concentrations are representative of maximal environmental exposures in the coastal environment (0.5% effluent treatments) or at ten times the maximal concentration (5% effluent treatments) representing a standard margin of safety.

In fish exposed to the effluent treatments we observed no significant changes of the target biological responses. The responses that were found after effluent exposure varied by effluent type, effluent concentration and fish gender. The E2 and VTG levels were lower in females exposed to SD effluent than in the LA effluent, but did not follow a concentration-response relationship. Female E2 and VTG concentrations were lower than controls in the 0.5% effluent treatments, but not in the 5% effluent treatment. A possible explanation is that the higher 5% effluent concentrations decreased the fish ability to respond, thus causing minimal responses when compared to negative controls. This biphasic response pattern has been observed with other indicators in fish exposed to low dose contaminants (Huggett *et al*. 2002, Calabrese 2008). The responses found also had a relation to the fish gender. For example, no estrogenic responses were observed in male fish when compared to their respective controls. Although these patterns were observed, none were statistically significant and could be representative of natural response variability.

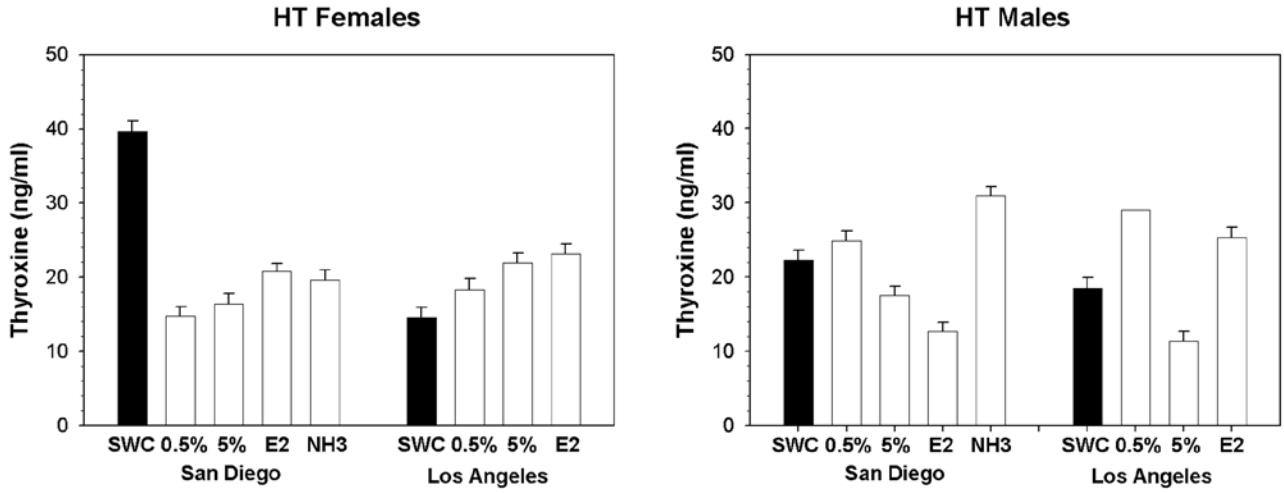

Figure 4. Thyroxine concentrations measured in female and male hornyhead turbot. Bars represent geometric mean concentrations ± standard errors.

Notably, male fish exhibited increases in plasma E2 and VTG concentrations after exposure to NH_3 although only the plasma E2 increase was statistically significant. Exposure to NH_3 is known to cause stress, thus leading to effects such as increased disease susceptibility and death (Eddy 2005). However, very little information regarding fish estrogenic responses after NH_3 exposure is available in the literature. This is a key finding because NH_3 is a very common constituent in municipal wastewater effluents. It is important to continue investigating the potential effects of this compound in other biological processes to reduce potential confounding in future studies.

Male hornyhead turbot exposed to SD and LA effluents did not exhibit the same endocrine responses that have been found in other fish species exposed to effluents under laboratory conditions. For example, studies with fathead minnow (*Pimephales promelas*) or carp (*Cyprinus carpio*) found estrogenic effects, such as significant increases in male plasma E2 and VTG concentrations when compared to controls (Hemming *et al*. 2001, Tilton *et al*. 2002, Diniz *et al*. 2005, Thorpe *et al*. 2008). However, these exposures utilized much higher effluent concentrations which were not representative of environmental exposure conditions. It is also possible that the wild-caught hornyhead turbot used in this study were pre-exposed to effluent constituents, which made them less sensitive. However, these organisms were conditioned in the laboratory for two weeks prior to testing and exposure to high doses of estrogen did elicit significant hormone responses in most cases. Control seawater was also field collected and may have contained some CECs despite being carbon-filtered prior to use. Thus, the short exposures to effluent treatments may not have triggered acute endocrine responses. A second explanation is that we did not see a lot of significant changes because of the combination of high variability among individual responses and the number of individuals used per treatment which resulted in low statistical power. A third explanation is that we did not use effluent concentrations as high as the concentrations used by other studies (e.g., 50 or 100%). Marine discharges in Southern California are engineered for maximum dilution upon discharge. Dilution ratios >100 are not uncommon. Hence, our lower concentrations of effluent exposure were consistent with our objectives to investigate biological responses after exposure to realistic ambient concentrations to help interpret data obtained in previous field studies.

Some endocrine responses observed in our laboratory study were lower than those observed in previous hornyhead turbot field studies. Responses previously observed in feral males found the presence of elevated concentrations of plasma E2, which were similar or higher than E2 concentrations in feral females (Rempel *et al*. 2006). In the laboratory acclimated fish exposed to control seawater, plasma E2 levels were lower (often by an order of magnitude) than E2 levels in feral fish. Interestingly, levels of plasma E2 in laboratory males exposed to the estradiol positive control were as high as E2 levels in feral males. However, E2 exposure did not elevate female plasma E2 levels to those found in feral females. Another example is the patterns observed for T4 concentrations. In the field, a clear pattern of decreased T4 was observed in male and female fish collected near POTW effluent discharge zones when compared to fish from a reference area. This pattern was not clearly observed in the fish exposed to effluent under laboratory conditions.

Three hypotheses may explain result differences between field and laboratory hornyhead turbot studies. First, stress responses associated with field collection, handling, and holding within a laboratory setting may alter the natural endocrine condition and mask responses to compounds present in effluents or the receiving water. Acute stress can increase cortisol concentrations, decrease sex steroid hormones and alter thyroid hormones in fish (Carraghera *et al*. 1989, Kubokawa *et al*. 1999, Zhou *et al*. 1999). A second hypothesis is that feral hornyhead turbot are being exposed to contaminants by multiple routes (e.g., sediment and food). Thus, water phase laboratory exposures may only partially simulate field exposure conditions. A third hypothesis is that, legacy contaminants (e.g., PCBs, DDT) may also contribute to the incidence and magnitude of some endocrine responses observed in field fish (Schiff and Allen 2000, Leaños-Castañeda *et al*. 2002). None of these hypotheses are mutually exclusive. Future investigations of these hypotheses, both individually and accumulatively, may help to explain the role of POTW effects observed in field collected hornyhead turbot.

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