Genomic and phenotypic response of hornyhead turbot exposed to municipal wastewater effluents

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Abstract

Laboratory tests with marine flatfish were conducted to investigate associations among gene expression, higher biological responses and wastewater effluent exposure. In the present study, male hornyhead turbot (*Pleuronichthys verticalis*) were exposed to environmentally realistic (0.5%) and higher (5%) concentrations of chemically enhanced advanced-primary (PL) and full-secondary treated (HTP) effluents from two southern California wastewater treatment plants (WWTP). Hepatic gene expression was examined using a custom low-density microarray. Alterations in gene expression (*vs*. controls) were observed in fish exposed to both effluent types. Fish exposed to 0.5% PL effluent showed changes in genes involved in the metabolism of xenobiotics, steroids, and lipids, among other processes. Fish exposed to 5% PL effluent showed expression changes in genes involved in carbohydrate metabolism, stress responses, xenobiotic metabolism, and steroid synthesis, among others. Exposure to 5% HTP effluent changed the expression of genes involved in lipid, glutathione and xenobiotic metabolism, as well as immune responses. Although no concentration-dependent patterns of response to effluent exposure were found, significant Spearman correlations were observed between the expression of 22 genes and molecular and/or higher biological responses. These results indicate that microarray gene expression data correspond to higher biological responses and should be incorporated in studies assessing fish health after exposure to complex environmental mixtures.

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Introduction

The study of contaminants of emerging concern (CECs) is becoming more common and many of these compounds are increasingly being determined to cause adverse effects in exposed fish (Kidd *et al*. 2007). Several current-use pesticides and personal care products, as well as most CECs, are not required to be monitored or regulated under the National Pollutant Discharge Elimination System (NPDES). Effects caused by exposure to CECs capable of disrupting endocrine systems in humans and aquatic organisms are of particular concern (Baker 2005, Hutchinson *et al*. 2006, Swan 2008a, Vandenberg *et al*. 2009, Brodin *et al*. 2013). Exposure to CECs can alter plasma concentrations of steroids and thyroid hormones (Oehlmann *et al*. 2008, Heindel and VomSaal 2009, Baker 2011). Treated municipal wastewater effluents are a significant source of CECs in aquatic environments (Tusher *et al*. 2001; Swan 2008a,b; Vandenberg *et al*. 2009). In southern California (USA), more than one billion gallons of these effluents are discharged into the ocean everyday (Lyon *et al*. 2006). To assess marine environmental condition, local sanitation districts monitor outfall discharge sites for biological impacts, including effects on hornyhead turbot (*Pleuronichthys verticalis*; Allen *et al*. 1998).

A comprehensive study previously conducted in the Southern California Bight (SCB) observed biological responses that suggest endocrine disruption in hornyhead turbot living in southern California (Bay *et al*. 2012). This study found unexpectedly high concentrations of plasma estradiol (E2) and the presence of vitellogenin (VTG) in male hornyhead turbot. These findings could not be attributed to effluent exposure, since these responses were present among fish which were collected near effluent discharges and also at a reference area (Forsgren *et al*. 2012, Reyes *et al*. 2012). However, concentrations of thyroxine (T4) hormone were significantly lower in males and females collected near outfall discharge areas (Bay *et al*. 2012). Previous field studies identified several CECs in the discharged effluents, receiving seawater, as well as in sediment and fish collected at the effluent discharge areas (Maruya *et al*. 2012, Vidal-Dorsch *et al*. 2012). In addition, another study found priority pollutants such as legacy pesticides (e.g., DDTs) and PCBs in sediments and fish tissue that may have also influenced biological responses in the turbot (Maruya *et al*. 2012). However, the independent and additive effects of these CEC mixtures on fish remain unknown.

The present study investigated the effects of exposure to advanced primary- and secondary-treated effluents in hornyhead turbot (*Pleuronichthys verticalis*) under controlled laboratory conditions. A laboratory approach was used to resolve some of the confounding factors, such as the presence of historical DDT and PCB contamination, which complicate interpretation of data derived from field studies in the SCB. To investigate responses in different biological systems, hepatic gene expression was measured in male hornyhead turbot. Males of this species were chosen because unexpected biological responses were observed in previous field and laboratory studies (Forsgren *et al*. 2012, Reyes *et al*. 2012). A custom microarray was used to investigate genetic responses in fish exposed to two types of treated municipal wastewater effluent. The experiments included negative (natural seawater) and positive (30 nM estradiol) controls, as well as two concentrations of each effluent type: an environmentally realistic concentration (0.5% v/v) expected to be representative of the outfall discharge sites, and a ten-fold higher concentration to examine dose response relationships.

Methods

Experimental Design

This manuscript describes gene expression results in male hornyhead turbot and it is a part of a larger study that investigated hormonal responses in male and female fish of this species after exposure to wastewater effluents (Vidal-Dorsch *et al*. 2010). A custom low-density microarray was used to examine changes in expression of genes involved in hormone responses and xenobiotic metabolism (Baker *et al*. 2009). Quantitative *polymerase chain reaction* (q*PCR*) methods were used to validate the microarray results. Fish were exposed in separate experiments to advanced-primary or full-secondary treated effluent from two of the largest wastewater dischargers in the SCB.

Two separate experiments were conducted to carry out the exposures. In the first experiment, wastewater effluent from the Point Loma Wastewater Treatment Plant (PL), in San Diego, CA was used. In the second experiment, effluent from the Hyperion Treatment Plant (HTP), in Playa Del Rey, CA, near Los Angeles was used. The PL effluent received

chemically enhanced advanced-primary treatment, and the HTP effluent received full-secondary treatment. Further information regarding the effluents used can be found elsewhere (Vidal-Dorsch *et al*. 2012).

The experimental treatments used in this study included negative (seawater only) and positive (estradiol; 30nM nominal) controls, and two effluent concentrations (0.5% and 5%). During the PL exposure, an ammonia (NH_3) treatment was also included to investigate the effects of this common effluent constituent on endocrine responses. The NH_3 concentration represented ammonia levels found at 5% effluent concentrations (2 mg/L = 1.17 µM/L). Not enough fish were available to conduct an NH_3 exposure during the HTP experiment.

The fish were exposed to the experimental treatments for 14 days. Individual fish were placed in separate glass aquaria containing seawater. Ten replicate aquaria were used per treatment. At the conclusion of each experiment, external evaluations of gross morphology were conducted on each fish. The fish were then sacrificed and dissected. The sex of each fish was determined after dissection. Tissues of interest were harvested for subsequent storage and analysis.

Blood plasma was collected to measure hormone (T4, E2, and 11-keto testosterone (11-KT)) and VTG concentrations. The liver and the right side gonad were weighed to determine the liver somatic index (LSI) and the half gonad somatic index (½ GSI). To characterize the composition of the effluents and seawater a suite of 31 CECs was analyzed.

Effluents and Controls

At the treatment plant the PL influent was screened and aerated, followed by chemically-assisted sedimentation for particulate removal. In addition to screening and primary sedimentation, the full-secondary influent treatment at HTP included aeration in oxygen reactors and subsequent settling of activated sludge in clarifier basins (Vidal-Dorsch *et al*. 2010). The effluent was transported in Nalgene® carboys and stored at 4°C until use. The effluent samples were peak flow grabs (collected in the late morning or early afternoon) of final effluent from each sanitation district. Three effluent batches were used for each experiment. The batches were sequentially used to prepare the experimental treatment in order to avoid long term storage; fish were exposed for 5 days to each batch. The control and dilution water was natural seawater collected from Redondo Beach (CA), which was filtered (0.45 µm) and treated with activated carbon before use.

An estradiol positive control (8 µg/L = 30 nM) was included. This concentration was selected on the basis that it would elicit a strong induction of many of the mRNA targets under study, and thereby would evaluate the performance of the microarray tool. A master-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 and the E2 was plated 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; subsequently this solution was transferred to a 20-L carboy where it was mixed with 10 L of seawater to create a final E2 stock solution. The final E2 solution was manually delivered to test aquaria where it was mixed with seawater to achieve the desired concentration. The E2 concentration measured in the test aquaria was 5.08 ±2.28 µg/L.

Fish Exposures

Hornyhead turbot were collected from Dana Point, a location distant to the largest municipal wastewater discharges in southern California, which was previously used as a reference site (Brown and Steinert 2003, Deng *et al*. 2007). This area is located near a small municipal wastewater discharge (the effluent receives secondary treatment). Yet, previous studies found that the Dana Point area is less contaminated chemically when compared to other areas near major wastewater outfalls. Because this area is not pristine, after collection the fish were brought to the laboratory and acclimated for a period of four weeks to allow depuration. Fish of a standard length >13 cm were collected by trawl (in the spring of 2009).

During acclimation, each fish was placed in a glass tank containing 40 L of seawater. A regime of 50% daily renewal at 15°C (±1) and a photoperiod of 16 hours light and 8 hours dark was used. The fish were fed pieces of frozen squid and worm every other day *ad libitum*. Water quality (total ammonia (NH_3), dissolved oxygen, conductivity, pH, and temperature) was recorded three times a week. Dissolved oxygen averaged 7.97±0.19 mg/L (average ±standard deviation), NH_3 averaged 0.27 ±0.10 mg/L, and pH averaged 7.97 ±0.03.

After acclimation, the fish were randomly assigned to different exposure treatments. Ten replicates were used in each treatment. Each replicate consisted of one fish in an individual aquarium. During the exposure period, the fish remained in their original acclimation aquaria and received daily renewals of exposure solutions. For the renewals, 20 L of seawater or treatment solution were removed from each tank, followed by the addition of 20 L of seawater or treatment solution.

Two effluent concentrations were tested in each experiment. The 0.5% effluent was used to represent environmental conditions as each outfall produced an initial dilution of at least 100:1. The 5% effluent concentration was used with the expectation that this concentration would produce greater biological responses and concomitant changes in gene expression. Further information regarding the preparation of 0.5 and 5% effluent treatments has been previously described (Vidal-Dorsch *et al*. 2010).

During the two week exposures, the temperature, photoperiod, and feeding regimes were kept similar to those used in the acclimation period. Dissolved oxygen averaged 7.36 ±0.13 mg/L and pH 7.86 ±0.02 in both experiments. Total NH_3 concentration for the negative controls, the 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 2 ±0.13 mg/L.

Fish survival ranged between 90 and 100% in both experiments. However, fish that did not seem alert when approached or that did not eat during the acclimation and exposure periods were not selected for analysis (<11%), in order to reduce potential bias introduced by outliers. The size of the fish used ranged from 13 to 16 cm in both experiments. Chemical measurements from the exposure tanks are shown in Figure SI-1 (SI; ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2013AnnualReport/ar13_153_168SI.pdf).

Biological Measurements

Indicators measured in male hornyhead turbot included hepatic gene expression, and higher physiological responses such as plasma hormone and VTG concentrations (Vidal-Dorsch *et al*. 2010). After effluent exposure the fish were anesthetized with MS-222 (0.2 g/L; Sigma-Aldrich, San Louis, MO), weighed, and measured for standard length. Fish were considered anesthetized when they stopped moving and did not respond to gentle touch. Fish blood was collected with a heparinized syringe from the caudal vein and centrifuged for plasma collection. Subsequently, the fish were humanely sacrificed with a cervical dislocation in accordance with established SCCWRP protocols (Vidal-Dorsch *et al*. 2011). The livers were removed post-mortem, weighed, and thin slices were cut and preserved in RNAlater (Qiagen, Valencia, CA) for microarray analysis. The right side gonad was then removed and weighed.

Blood plasma hormones were analyzed using a radioimmunoassay for E2 and enzyme immunoassay methods for 11-KT and T4 (Reyes *et al*. 2012). Plasma VTG was analyzed using a competitive enzyme-linked immunosorbant assay (Rempel *et al*. 2006). Detection limits were 4.4 pg/ml (6.2×10^{-6} M) for E2, 3.9 ng/ml (2×10^{-4} M) for T4, 1.3 pg/ml (2.5×10^{-7} M) for 11-KT, and 1.25 ng/ml for VTG. Hormone samples were analyzed in duplicate, and VTG samples were analyzed in triplicate at 1:50, 1:1500, and 1:45000 dilutions.

Gene expression was quantified using a custom one-color Agilent microarray (8 x 15, 000 platform). Further description and validation of the original microarray development and probes can be found in (Baker *et al*. 2009) and in the SI. The microarray used in the current study represents a refined version of this tool which contains genes with defined roles in endocrine processes. This refined version contains probes for 39 genes (Table SI-1). The genes in the microarray were selected to investigate responses in reproduction, growth, development, stress responses, contaminant metabolism and detoxification, response to infection, and hormone activity (Ribecco *et al*. 2011, Baker *et al*. 2012, Ribecco *et al*. 2012).

Different strategies were used to develop and validate the microarray since hornyhead turbot gene sequences were not readily available. In brief, the majority of the sequences to develop the microarray were collected from GenBank using BLAST (Altschul *et al*. 1990). The sequences represent 60mer oligonucleotides, which were constructed for each probe. The sequences used to develop the probes were collected from multiple species including, Tetraodoniformes and Perciformes, which were phylogeneticly close to Pleuronectiformes (hornyhead turbot). Additionally, hornyhead turbot-specific cDNA sequences of interest were obtained by degenerate PCR cloning and Sanger sequencing. The microarray design is contained in the European

Bioinformatics Institute (EBI) database, Aquatic MultiSpecies Array V2.0; array express accession: A-MEXP-2291.

For microarray assays, RNA was isolated from five biological replicates of male hornyhead turbot liver samples, except for the Estradiol PL treatment where N = 4. RNA was extracted following previously published procedures (Baker *et al*. 2009). RNA was assessed for integrity and purity with the 6000 Nano LabChip assay from Agilent, (Palo Alto, CA). The quality of all samples used in this study had RIN numbers >7 which were measured using bioanalyzer procedures following Agilent protocols. If contaminating DNA was present the sample was again treated with RNase-free DNase. Total RNA (100 ng) was converted into fluorescently labeled Cy3 cRNA using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent). Fluorescent targets were purified to remove unincorporated nucleotides using RNeasy (Qiagen).

Absorbance (OD) at 260 nm was used to quantify the cRNA concentrations, and absorbance at 550 nm was used to measure the efficiency of Cy3 dye incorporation. An incorporation efficiency of 9 pmol/µg or greater was deemed necessary before proceeding with hybridization. For each sample, 1 µg of fragmented cRNA was hybridized to the multispecies microarray in accordance with single color Agilent hybridization protocols. The Agilent hybridization conditions were such that the hybridization was carried out under high salt conditions to facilitate probe and target interactions. The stringency washing of the arrays was done initially with high salt solutions followed by low salt solutions (Talapatra *et al*. 2003). Data were collected using an Agilent Microarray Scanner and Feature Extraction Software (V10.5).

Relative turbot liver mRNA transcript levels were also measured by real-time quantitative PCR with a LightCycler 480 (Roche, Indianapolis, IN). The genes analyzed with qPCR were vitellogenin-1 and -2 (*VTG1* and *VTG2*) and cytochrome P450, family-1, subfamily A (*CYP1A*). Primers used for these transcripts are described in the supplemental information. Total RNA was extracted from liver samples as described above and reverse-transcribed using the Roche Transcriptor Kit (Indianapolis, IN) and 50 ng cDNA were quantified using LightCycler 480 SYBR Green Master kit (QIAGEN). The *β-actin* transcript served as an internal control for normalization. Each sample was run in duplicate using the selected primer pairs. Thermocycling protocols consisted of 5 minutes at 95°C, 45 cycles of 10 seconds at 60°C, 10 seconds at 72°C, and then 10 seconds at 95°C. Amplification and melting curve analyses for these assays indicated that the amplifications were primer specific. Normalized gene expression values were obtained using LightCycler Relative Quantification software. Relative gene copy numbers were derived using the formula $2^{\Delta CT}$, where ΔCT is the difference in amplification cycles required to detect amplification product from equal starting concentrations of turbot liver RNA.

CEC Measurements

Three whole-effluent samples and negative controls were analyzed for CECs. The first effluent sample was collected at the beginning of each exposure, the second at the end of the first week, and the last one towards the end of the second week to characterize each effluent batch used. In addition, composite samples from the tanks were collected at the beginning and end of each experiment to characterize chemical exposure. Each effluent or composite sample was analyzed for a suite of CECs (analytes and detection limits shown in Table SI-2). The Southern Nevada Water Authority (Las Vegas, NV) analyzed the samples using liquid chromatography/tandem mass spectrometry (LC-MS/MS) and gas chromatography/ tandem mass spectrometry (GC-MS/MS).

Quality-control measures for chemical analysis consisted of procedural blanks, duplicate analyses, and spike analyses. Laboratory spikes and blanks were analyzed with every set of 10 samples. In addition, standard surrogates were used to determine analyte loss during processing. Isotopically-labeled standards were available for the majority of the compound classes investigated, and the standard recoveries ranged from 80 to 120%. The chemical concentrations were corrected for blanks and control contamination. If contamination was found, the analyte value was adjusted by subtracting the concentration found in the blanks and/or controls. Full descriptions of the methods used for sample collection and analyses have been previously published (Trenholm *et al*. 2006,Vanderford and Snyder 2006, Vidal-Dorsch *et al*. 2012).

Data Analysis

To determine differential gene expression in control and exposed fish, the Significance Analysis of Microarrays (SAM) procedure as described by Tusher

et al. (2001) was used. To analyze the responses in fish exposed to effluent or control treatments, the data were statistically compared to respective negative water controls. Genes with scores greater than a threshold were considered to be significant. A false discovery rate (FDR) threshold of <0.2 was used to classify genes that were significantly up-regulated or down-regulated; except for the E2 PL data where the FDR used was <0.3 (due to the smaller number of fish in this treatment). No analysis was conducted for 0.5% HTP treatment. All data resulting from the microarray analysis were submitted to the European Bioinformatics Institute (EBI) database (accession number: E-MEXP-3851).

Spearman correlation analyses were conducted to investigate the relationship between gene expression and the concentrations of plasma hormones, plasma vitellogenin, ½ GSI, LSI, standard length and weight (analysis conducted with JMP Genomics V5; SAS, Carry, NC). Q-PCR data presented in this paper are in the form of mean values. The results of the qPCR analysis were examined using a one-way ANOVA followed by a Tukey test (P <0.05).

RESULTS

Gene Expression

While gene expression variability was observed among fish exposed to the same effluent type, 10 out of the 39 genes contained in the microarray were differentially expressed (i.e., significantly different from negative controls) in livers of male fish exposed in at least one of the treatments. These genes were: *GSTα, G3PDH, FXR, HAMP, HSP90, LXR, VTG1, VTG2, ZP2*, and *ZP3*. Most of these genes were down-regulated, except for *GSTα* and *HSP90* which were up-regulated, but only after exposure to HTP effluent (Figure 1). Gene-expression patterns varied according to effluent type and concentration (Table 1). Only *HAMP* was down-regulated in fish exposed to both effluent types.

Livers of male fish exposed to PL effluent (which received advanced-primary treatment) had down-regulation of *FXRα, HAMP, LXR, VTG1, VTG2, ZP2*, and *ZP3* genes. The 0.5% PL effluent down-regulated the expression of *FXRα, VTG1, VTG2, ZP2*, and *ZP3* genes; and the 5% concentration down-regulated the expression of *HAMP, LXR, VTG1, VTG2, ZP2*, and *ZP3*. The *VTG* and *ZP* genes

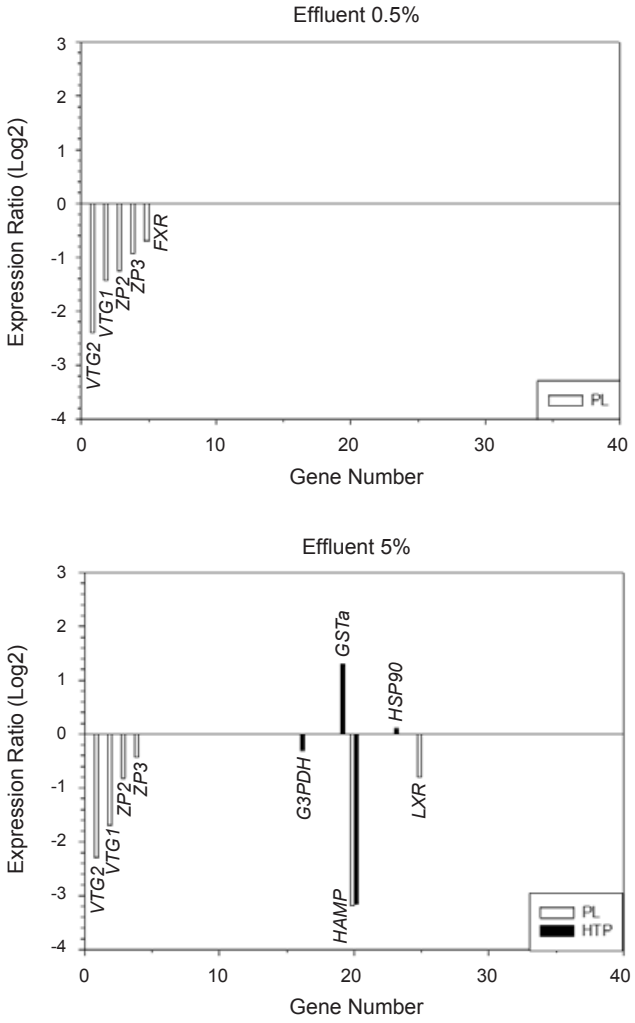

Figure 1. Liver gene expression (expressed as station averages (Log2) of fold change over expression of control samples) for hornyhead turbot exposed to advanced-primary treated effluent (PL) or to full-secondary treated effluent (HTP). All genes were arranged from most up-regulated to most down-regulated for the 0.5% PL effluent.

were down-regulated by both PL effluent concentrations with a relatively similar degree of expression (Figure 1).

Fish exposure to 5% HTP effluent showed up-regulation of *GSTα* and *HSP90*, and down-regulation in the expression of *HAMP* and *G3PDH*. Gene expression was not evaluated for the 0.5% HTP effluent since only one male was exposed. Hornyhead turbot are not sexually dimorphic and it was not possible to predetermine the number of males in each treatment.

Exposure to estradiol and to NH_3 also caused differential gene expression when compared to their respective negative controls (Table 1). The largest

Table 1. Gene expression ratios for genes that were differentially expressed (vs. controls) in at least one treatment, after exposure to advanced-primary treated effluent (PL), full-secondary treated effluent (HTP), spiked ammonia (NH3) during the PL experiment, or estradiol (E2). The exposures to E2 were conducted during the PL and the HTP experiments. Negative control fish N = 5. NS = Not significant change.

Gene ID	PL Effluent		HTP Effluent	PL E2	HTP E2	NH3
	0.5% (N= 5)	5% (N= 5)	5% (N= 5)	(N= 4)	(N= 5)	(N= 5)
AHR	NS	NS	NS	1.1	1.2	NS
AR	NS	NS	NS	1.1	NS	NS
CYP1A	NS	NS	NS	1.1	NS	NS
CYP3A	NS	NS	NS	0.8	NS	NS
ER1	NS	NS	NS	2.5	1.5	NS
ER2	NS	NS	NS	1.6	1.3	NS
FXRα	0.6	NS	NS	1.1	NS	NS
FXRβ	NS	NS	NS	1.2	NS	NS
GSTα	NS	NS	2.5	1.3	1.5	NS
G3PDH	NS	NS	0.8	NS	0.9	NS
HAMP	NS	0.1	0.1	1.5	1.1	NS
HSD11B1	NS	NS	NS	0.6	1.2	NS
HSP90	NS	NS	1.1	NS	NS	NS
LXR	NS	0.6	NS	1.1	NS	0.6
MT1	NS	NS	NS	0.8	0.8	NS
PPARα	NS	NS	NS	2	2	NS
SHBG	NS	NS	NS	0.8	0.5	NS
TR	NS	NS	NS	0.8	1.1	NS
VDR	NS	NS	NS	1.1	1.3	NS
VTG1	0.4	0.3	NS	32	39	0.4
VTG2	0.2	0.2	NS	26	37	0.3
ZP2	0.4	0.8	NS	23	28	NS
ZP3	0.5	0.6	NS	52	60	0.5

number of genes differentially expressed was found in fish exposed to estradiol treatments. During the PL experiment 21 genes were differentially expressed after exposure to the E2 treatment and a total of 16 genes were differentially expressed during the HTP experiment. Up-regulation of the *VTG* and *ZP* genes was observed after exposure to estradiol in both experiments. The estradiol treatment was primarily used in order to validate that the microarray was detecting expected changes in gene expression.

During the PL experiment, exposure to estradiol caused an increase in the production of *AHR*, *AR*, *CYP1A*, *ER1*, *ER2*, *FXRα, FXRβ*, *GSTα*, *HAMP*, *LXR*, *PPARα*, *VDR, VTG* and *ZP* transcripts, while *CYP3A*, *HSD11B1*, *MT1*, *SHBG* and *TR* were decreased. During the HTP experiment fish exposed to estradiol showed an increase in the production of *AHR*, *ER1*, *ER2*, *GSTα*, *HAMP*, *HSD11B1*, *PPARα*, *TR* and *VDR* transcripts (in addition to *VTG* and *ZP*), and a decrease in the regulation of *G3PDH*, *MT1*, and *SHBG* transcripts. Males exposed to NH_3 experienced a decrease in *LXR, VTG1, VTG2* and *ZP3* transcripts, these results could indicate that ammonia, an important municipal effluent constituent, may have properties that affect estrogenic responses and influenced in part the results found after exposure to PL effluent.

The qPCR assays were consistent with the results of the microarray analysis. For example, both the qPCR and the microarray analyses showed that the expression of *VTG* genes were down-regulated in fish exposed to advanced-primary effluent (Figure 2). Another example was the lack of statistical differences between negative controls and advanced-primary effluent-exposed fish for the *CYP1A* gene. Both the qPCR and the microarray analyses found that *CYP1A* gene was not differentially expressed. The qPCR results showed that exposure to estradiol and NH_3 caused differential gene expression, which also corroborated the microarray data.

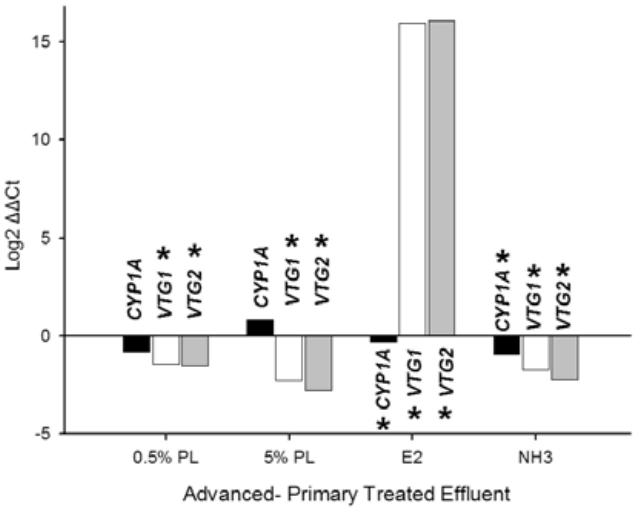

Figure 2. Genes analyzed using qPCR in fish exposed during the PL experiment; these genes were not differentially expressed in fish exposed to 5% HTP effluent. Symbols (*) represent averages that are statistically different when compared to negative control fish.

Physiological Measurements

Fish condition as measured by LSI in effluent-exposed males was not significantly different from the condition observed in negative controls. Average LSI values were also not statistically different ($p > 0.05$) among negative control males and males exposed to estradiol (Table 2). Average LSI values in fish used for both experiments ranged between 0.007 and 0.011. Likewise, ½ GSI values were not affected by exposure to effluent or positive controls in both experiments. Average ½ GSI values in fish used in both experiments ranged between 0.0004 and 0.0051.

The concentrations of plasma VTG and hormones were not significantly different between negative controls and effluent-exposed fish for both effluent types. (Table 2). Considerable protein concentration variability was observed in the effluent-exposed fish. In the positive controls, plasma E2 concentrations were significantly higher ($p < 0.05$) and 11-KT significantly lower than negative controls. Plasma VTG concentrations in positive control fish were significantly higher ($p < 0.05$) when compared to negative controls in both experiments.

Correlation Analysis

The expression of estrogen-responsive genes and other endocrine targets was associated with plasma E2 and VTG levels (Table 3). In fish exposed during the PL experiment, significant and positive Spearman correlations were observed between plasma estradiol and plasma VTG with *ZP3, ZP2, VTG2, VDR, PPARα*, *ERα,* and *ERβ*; furthermore, negative correlations were found with *TR*, *MT1* and *CYP3A*. In fish exposed during the HTP experiment, positive correlations were found between plasma estradiol and plasma VTG with *HSD11B1, HAMP, ZP3, ZP2, VTG2, VTG1, ERα, ERβ,* and *VDR,* while *SHBG* was negatively correlated. Plasma E2 and plasma VTG concentrations were positively correlated ($p = 0.014$; $R = 0.7$).

Some correlations were found for additional biological parameters and gene expression after both experiments, in other cases the correlations were only found in one of the experiments. After the PL and HTP experiments, negative correlations were found for plasma 11-KT and *VDR*, *VTG* and *ZP*, while the *SHBG* had a positive correlation. Plasma T4 in PL fish was negatively correlated with *PPARα, ERα, ERβ, VDR and CYP1A,* and positively correlated with *CYP3A, SHBG, TR, MT1* and *HSD11B1*; there were no correlations in HTP fish. The ½ GSI values were negatively correlated with *CYP1A* expression.

The weight of the fish was negatively correlated with *VTG* gene expression, but only in fish exposed to PL. A few genes were correlated to LSI in either experiment. The fish LSI had a significant positive correlation with *AHR* after the PL experiment and *MT1* after the HTP experiment. Fish used during the HTP experiment had a positive correlation for *G3PDH* and length. The fish weight and the standard length were positively and significantly correlated during both experiments (during the PL study the spearman $r = 0.5$ and $r = 0.9$ during the HTP experiment).

Chemical Measurements

Current-use pesticides, industrial and commercial compounds, hormones, personal care products, and pharmaceuticals were detected in whole effluents. Some compounds were detected in both effluents, but several differed in occurrence and concentration when the two were compared. Twenty-seven CECs were detected in PL effluent and 24 in HTP effluent (Figure 3). Twenty-three analytes were detected in both effluents, with concentrations generally higher in PL effluent. Four analytes were detected only in PL effluent, and one was only detected in HTP. The compounds detected at the highest average concentrations ($N = 3$) in PL effluent were caffeine (99 ±2 µg/L; ±standard deviation), ibuprofen (18 ±12 µg/L), and naproxen (15 ±5

Table 2. Biological characteristics of male hornyhead turbot exposed to advanced-primary treated wastewater effluent (PL) and full-secondary treated effluent (HTP). Ammonia exposures were only conducted during the PL experiment. The summaries were calculated with five fish for each treatment, except for PL Estradiol where N = 4. ½ gonadal somatic index (½ GSI) is the wet weight of one gonad divided by the total wet body weight of the fish. Liver somatic index (LSI) is the wet weight of the liver divided by the total wet body weight of the fish.

Treatments	PL Effluent		HTP Effluent		PL Effluent		HTP Effluent	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
	Fish Weight (g)				E2 (pg/ml)			
Control	84	21	81	29	31	23	35	60
0.5% Effluent	78	15	-	-	15	9	-	-
5% Effluent	91	29	79	36	37	31	24	28
Estradiol	62	14	64	20	412*	160	687*	374
Ammonia	63	22	-	-	105	78	-	-
	Std Length (cm)				VTG (ng/µg_protein)			
Control	15	2	15	1	0.2	0.1	0.1	0.1
0.5% Effluent	15	1	16	2	0.1	0.1	-	-
5% Effluent	15	1	15	2	0.1	0.1	3	8
Estradiol	14	1	14	2	164*	69	377*	319
Ammonia	13	2	-	-	0.2	0.2	-	-
	½ GSI				11-KT (pg/ml)			
Control	0.0011	0.0007	0.005	0.0087	3127	3833	2313	3296
0.5% Effluent	0.0007	0.0003	-	-	1366	1059	-	-
5% Effluent	0.0012	0.0006	0.0051	0.0081	2707	2923	1767	2115
Estradiol	0.0004	0.0002	0.0007	0.0004	24*	11	28*	16
Ammonia	0.002	0.0034	-	-	2524	2902	-	-
	LSI				T4 (ng/ml)			
Control	0.008	0.002	0.011	0.004	28	10	25	19
0.5% Effluent	0.008	0.003	-	-	20	10	-	-
5% Effluent	0.01	0.001	0.009	0.007	22	9	18	8
Estradiol	0.009	0.002	0.007	0.002	13	3	31	17
Ammonia	0.008	0.001	-	-	31	15	-	-

* Average is significantly different when compared to the negative control.

µg/L). In HTP effluent, gemfibrozil (3 ±0.4 µg/L), total nonylphenols (1.6 ±0.3 µg/L), and atenolol (1.4 ±0.1 µg/L) were found at the highest average concentrations.

Atenolol, caffeine, carbamazepine, estrone, gemfibrozil, ibuprofen, meprobamate, naproxen, primidone, sulfamethoxazole, triclosan, and trimethoprim, were detected at significantly higher concentrations in PL effluent ($p < 0.05$) when compared to HTP effluent. In HTP effluent iopramide, DEET and TCEP concentrations were significantly higher ($p < 0.05$) than those detected in PL.

The seawater used for the controls and to prepare the effluent dilutions had four CECs detected at low concentrations. Detected compounds included nonylphenol (0.2 µg/L), estradiol (0.002 µg/L), estrone (0.001 µg/L), and DEET (0.002 µg/L). Some of these concentrations were close to the detection limits.

Discussion

Differential gene expression in male hornyhead turbot was found after exposure to municipal wastewater effluents. No statistically significant,

Table 3. Gene and phenotype correlations in fish exposed to control and experimental treatments including advanced-primary treated (PL; N=19) and full-secondary treated (HTP; N= 15) effluents. Parameters correlated included plasma estradiol (E2), vitellogenin (VTG), 11-ketotestosterone (11-KT), thyroxine (T4), ½ gonadal somatic index (½ GSI), liver somatic index (LSI), weight, and standard length.

Measured	Gene	PL Effluent		HTP Effluent		Measured	Gene	PL Effluent		HTP Effluent	
		Spearman R	p	Spearman R	p			Spearman R	p	Spearman R	p
E2	*VTG1*	NS	NS	0.6	0.009	11-KT	*VTG1*	-0.8	0.001	-0.6	0.018
E2	*VTG2*	0.6	0.02	0.6	0.01	11-KT	*VTG2*	-0.7	0.005	-0.6	0.027
E2	*ZP2*	0.6	0.034	0.7	0.005	11-KT	*ZP2*	-0.6	0.037	NS	NS
E2	*ZP3*	0.6	0.017	0.6	0.008	11-KT	*ZP3*	-0.6	0.022	-0.5	0.032
E2	*ERα*	0.7	0.006	0.7	0.004	11-KT	*ERα*	NS	NS	-0.6	0.022
E2	*ERβ*	0.7	0.01	0.6	0.007	11-KT	*ERβ*	-0.7	0.014	NS	NS
E2	*VDR*	0.7	0.013	0.8	0.001	11-KT	*VDR*	-0.7	0.01	-0.5	0.029
E2	*PPARα*	0.8	0.003	NS	NS	11-KT	*AR*	-0.6	0.033	NS	NS
E2	*CYP1A*	0.6	0.02	NS	NS	11-KT	*TR*	NS	NS	-0.7	0.006
E2	*CYP3A*	-0.8	0.001	NS	NS	11-KT	*CYP3A*	0.6	0.029	0.6	0.007
E2	*HSD11B1*	-0.8	0.002	0.6	0.02	11-KT	*SHBG*	0.6	0.031	NS	NS
E2	*TR*	-0.8	0.003	NS	NS	11-KT	*HAMP*	NS	NS	-0.9	<0.0001
E2	*MT1*	-0.7	0.007	NS	NS	11-KT	*MT1*	0.6	0.022	NS	NS
E2	*HAMP*	NS	NS	0.5	0.049	11-KT	*GSTα*	-0.6	0.031	NS	NS
E2	*SHBG*	NS	NS	-0.7	0.003	11-KT	*HSD11B1*	0.6	0.037	NS	NS
VTG	*VTG1*	0.8	0.001	0.7	0.005	T4	*TR*	0.6	0.039	NS	NS
VTG	*VTG2*	0.9	<0.001	0.7	0.006	T4	*PPARα*	-0.7	0.003	NS	NS
VTG	*ZP2*	0.6	0.031	0.6	0.016	T4	*VDR*	-0.7	0.006	NS	NS
VTG	*ZP3*	0.7	0.006	0.7	0.006	T4	*ERα*	-0.7	0.006	NS	NS
VTG	*ERα*	NS	NS	0.7	0.002	T4	*ERβ*	-0.7	0.005	NS	NS
VTG	*ERβ*	0.6	0.037	0.6	0.027	T4	*CYP3A*	0.6	0.029	NS	NS
VTG	*VDR*	0.7	0.011	0.7	0.005	T4	*CYP1A*	-0.6	0.022	NS	NS
VTG	*PPARα*	0.6	0.05	NS	NS	T4	*SHBG*	0.6	0.035	NS	NS
VTG	*CYP3A*	-0.8	0.003	NS	NS	T4	*HSD11B1*	0.8	0.002	NS	NS
VTG	*TR*	-0.7	0.009	-0.6	0.017	T4	*MT1*	0.7	0.003	NS	NS
VTG	*MT1*	-0.6	0.044	NS	NS	LSI	*MT1*	NS	NS	0.6	0.008
VTG	*HAMP*	NS	NS	0.6	0.007	LSI	*AHR*	0.7	0.015	NS	NS
VTG	*SHBG*	NS	NS	-0.7	0.002	Weight	*VTG1*	-0.7	0.009	NS	NS
VTG	*HSD11B1*	-0.6	0.031	0.6	0.026	Weight	*VTG2*	-0.7	0.015	NS	NS
VTG	*GSTα*	0.7	0.015	NS	NS	Standard Le	*GAPDH*	NS	NS	0.6	0.027
½ GSI	*VTG1*	NS	NS	-0.5	0.047						
½ GSI	*VTG2*	NS	NS	-0.5	0.043						
½ GSI	*ZP2*	-0.8	0.003	NS	NS						
½ GSI	*ZP3*	-0.6	0.021	NS	NS						
½ GSI	*ERα*	-0.7	0.008	NS	NS						
½ GSI	*ERβ*	-0.6	0.047	NS	NS						
½ GSI	*VDR*	-0.7	0.015	NS	NS						
½ GSI	*PPARα*	-0.7	0.006	NS	NS						
½ GSI	*CYP1A*	-0.7	0.01	-0.5	0.037						
½ GSI	*MT1*	NS	NS	0.5	0.036						
½ GSI	*GSTα*	-0.7	0.004	NS	NS						
½ GSI	*AHR*	NS	NS	-0.5	0.029						
½ GSI	*HSP90*	NS	NS	0.7	0.004						
½ GSI	*HSD11B1*	0.6	0.044	NS	NS						

changes were found in physiological responses (i.e., plasma hormone and VTG concentrations). Gene expression responses were observed in fish exposed to chemically enhanced advanced-primary (PL) and full-secondary treated (HTP) effluents, even at environmentally realistic concentrations with PL effluent. Exposure to effluent was associated with the differential expression of genes involved in diverse functions such as reproduction, metabolism, stress and immune responses. Gene expression patterns differed among effluents and concentrations. Some gene expression responses were significantly correlated with plasma hormones, VTG concentrations, LSI, ½ GSI, and fish weight.

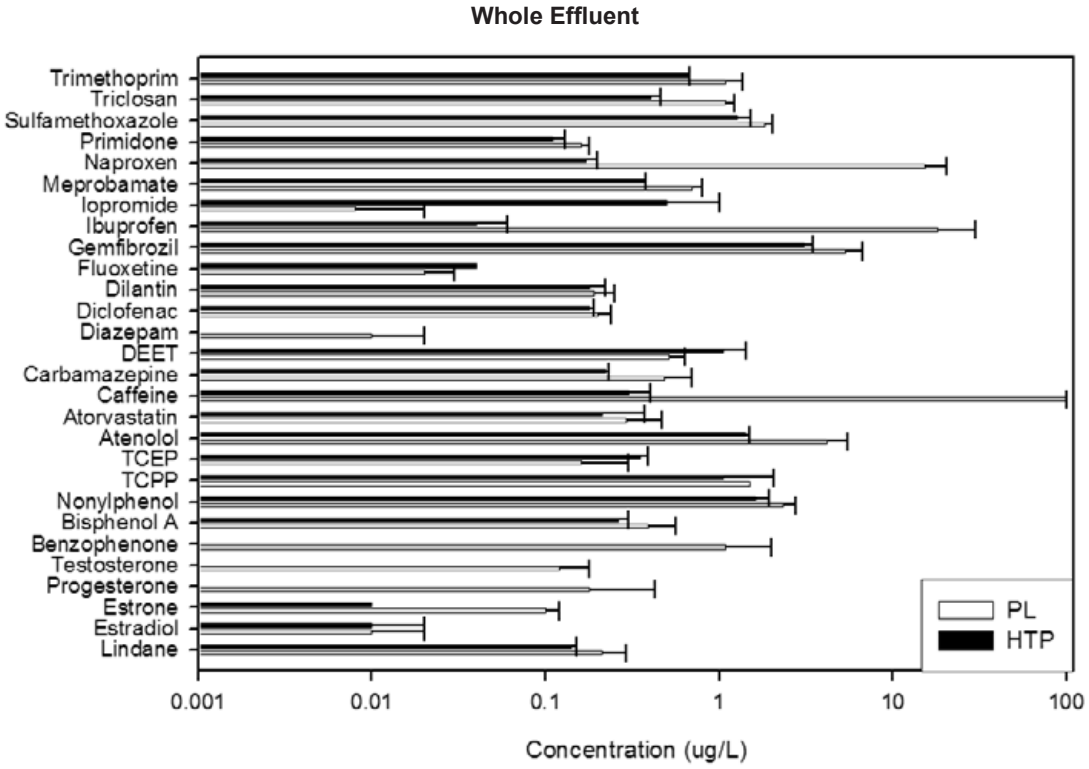

Figure 3. Average concentrations (N = 3) for compounds detected in advanced-primary treated effluent (PL) and full-secondary treated effluent (HTP). Reporting limits for each compound are provided in Table SI-2. Data for the chemical concentrations found in the fish tanks are shown in Figure SI-1.

The absence of clear physiological response patterns in exposed hornyhead turbot males could be due to different factors. For instance, one factor could be minor reproductive state differences, although similar fish sizes were targeted during collection to minimize differences in fish age and reproductive status (Reyes *et al*. 2012). Another factor could be that the two week exposure to 5% effluent concentrations was not long enough and / or strong enough to cause consistent physiological changes, yet still enough to elicit changes in more sensitive responses such as gene expression.

Several expected correlations between gene expression and concentrations of plasma hormones and VTG were found. For example, there were strong and positive correlations between plasma VTG concentrations and the differential gene expression of VTG genes. There were also positive correlations between plasma VTG concentrations and the differential gene expression of *ZP*, *ER* and other nuclear receptor transcripts. These types of relationships have been previously found in fish injected with E2 (Davis *et al*. 2007). These expected correlations of physiological characteristics and gene expression responses further validate the gene expression data.

An unexpected negative correlation between male weight and VTG gene expression in fish exposed to PL effluent was also found. Other studies have suggested that VTG protein production in male fish may divert resources from other important biological processes (Davis *et al*. 2007). Decreased weight was found in males of other fish species with high levels of plasma VTG after exposure to estrogenic compounds (Sohoni *et al*. 2001). In the current study, fish weight was measured after exposure, but not before exposure, so weight changes could not be determined. Thus, it is not possible to determine the significance of the relationship between weight and *VTG* gene expression in male hornyhead turbot. However, our preliminary data also point to a negative correlation between *VTG* transcripts and weight in field collected fish for this species (unpublished data), which suggests that this may be an important relationship that merits further study.

The correlations between gene expression and physiological responses suggest that some of the genes differentially expressed could serve as exposure indicators in hornyhead turbot. Similar transcript responses have been observed in other fish species. For example, the data from this study showed that increasing plasma E2 levels were

associated with the down-regulation of *CYP3A*. *CYP3A* plays an important role in the detoxification of xenobiotic compounds (Herwaarden *et al*. 2007) and in steroid hormone clearance (Miranda *et al*. 1989). Another gene involved in detoxification (*GSTα*) had a positive correlation with plasma VTG concentrations. *GSTα* has been found to be involved in stress responses and in detoxification activities (Pinto *et al*. 2010).

Gene expression patterns in hornyhead turbot were distinct to each effluent; only one of the differentially expressed genes was found in common between PL and HTP exposed fish. Although sample-size was insufficient to correlate gene expression and effluent chemistry concentrations, the data indicated that the PL effluent had higher concentrations than HTP for several chemicals known to affect the estrogenic system. We observed greater differential gene expression in the *VTG* and ZP genes from hornyhead turbot after exposure to this effluent. It is also possible that after exposure to the more treated effluent (HTP), hornyhead turbot males exhibited gene expression responses that were more representative of narcotic responses (e.g., *HSP90* and *GST*). Chemicals with this mode of action were found at higher concentrations in the HTP effluent such as iopramide (Sanderson and Thomsen 2009).

Expression of the immune response gene (*HAMP*) was down-regulated in fish exposed to both effluents. Genes involved in immunological processes have been found to be down-regulated after exposure to wastewater effluent in fish living downstream of municipal treatment plants (Liney *et al*. 2006, Garcia-Reyero *et al*. 2009, Müller *et al*. 2009). Further studies are needed to determine if fish living near outfall discharge areas possess impaired immune systems (e.g., increased parasite load or infection). A previous study found that estuarine fish (*Gillichthys mirabilis*) that had a higher degree of gonad parasite infection also had down-regulation of genes involved in immune functions when compared to fish with a lower degree of infection (Vidal-Dorsch *et al*. 2011).

Fish exposed to PL effluent had changes in the expression of *ER* related genes (e.g., *VTG* or *ZP*). These transcripts were down-regulated rather than up-regulated as has been observed in other studies conducted with marine fish exposed to effluents containing estrogenic compounds (Raimondo *et al*. 2009). There are three potential explanations for the down-regulation of *ER* genes. First, the transcription of mRNAs was decreased due to inhibition of either *ER1* or *ER2* by compounds present in the effluent. A second explanation for the down-regulation of ER related genes could be a reduction in E2 biosynthesis. A third explanation is the potential activation of other anti-estrogenic feedback loops activated by effluent compounds or by a lack of exposure to field stimulation. With the data obtained from this study it is difficult to determine which of the three explanations applies to the exposed hornyhead turbot.

Fish exposed to PL effluent also showed down-regulation in transcripts encoding proteins involved in metabolic and stress processes. Other studies have found that exposure to sunscreen and cosmetic components such as benzophenone can alter the expression of genes involved in metabolism (Bernheim and Falk 1983). *FXRα*, a gene involved in RNA processing was down-regulated in fish exposed to 0.5% PL effluent. Long term down-regulation of this gene could affect the ability of fish to renew and repair RNA, induce programmed cell death, and affect fish growth (Bellacosa and Moss 2003, Devin and Rigoulet 2007). The down-regulation of *FXR* in hornyhead turbot may also suggest an oxidative stress response after exposure to PL effluent (Honda *et al*. 2001). Other CECs found in PL effluent, such as caffeine, are also known for their ability to alter fish metabolism by acting as an inhibitor of primary amine oxidase at concentrations in the millimolar and micromolar range (Olivieri *et al*. 2011). Caffeine concentrations in PL whole effluent were on average close to 4 µg/L.

Different compounds detected in the effluent could be partially responsible for some of the gene expression responses observed in fish exposed to PL effluent. For example, hornyhead turbot males exposed to PL effluent had differential expression of genes involved in the metabolism of steroids. Compounds like ibuprofen and triclosan were detected in PL effluent; these chemicals have been found to affect steroid metabolism and produce effects such as male fish vitellogenesis and delayed egg hatching (Raut and Angus 2010, Han *et al*. 2010). Other chemicals detected in PL effluent such as nonylphenols have also been found to alter fish steroidogenesis (Arukwe *et al*. 2000, Vajda *et al*. 2008, James 2011).

Fish exposed to HTP effluent showed down-regulation of transcripts involved in metabolic and immunity processes. *GSTα* (involved in xenobiotic detoxification) and *HAMP* (involved in cellular iron ion homeostasis) were differentially expressed after

exposure to HTP effluent. GSTs detoxify endogenous and exogenous substances (drugs, pesticides, and other pollutants) through their conjugation to glutathione. Alterations of *GSTα* can affect the fish detoxification potential, as well as cellular redox potential, and eicosanoid pathways (Ward *et al*. 2006). In addition, the up-regulation of *GSTα* and *HSP90* could suggest that the fish were stressed following exposure to the effluent (Wang *et al*. 2005).

Exposure to the NH_3 treatment elicited some gene expression changes that were similar to those observed in fish exposed to effluent. NH_3 is known to induce stress responses in fish by affecting signaling pathways. For example, a study that exposed fish to ammonium bicarbonate (NH_4HCO_3) observed elevated concentrations of plasma cortisol, possibly affecting signaling processes (Nawata and Wood 2009). Ammonia has been found to cause reproductive effects such as reduction in fathead minnow fecundity (Armstrong *et al*. 2012). Further study is necessary to better understand the potential gene expression responses of this constituent in effluent-exposed organisms.

It is also plausible that effluent chemicals not measured in this study contributed to the observed gene responses. For example, compounds such as lipid lowering agents (e.g., colofibrate) can affect fish metabolic and signaling pathways by inducing oxidative stress (Corcoran *et al*. 2010). Although 31 compounds were investigated, this study could not analyze all effluent constituents. A considerable advantage of using gene expression data is to investigate responses to all constituents present in the effluent chemical mixture including those unmeasured or unknown.

Results may indicate that estrogenic compound concentrations needed to be higher than those measured in the present study are needed to elicit hornyhead turbot physiological responses. The estradiol equivalent value (EEQ) in PL was 20.3 ng/L and 11.2 ng/L HTP whole effluent. It is conceivable that the levels of estrogenicity in the effluent are lower than those found in the hornyhead turbot marine environment where diverse types of estrogenic chemical compounds are present (e.g., legacy DDTs). Xenoestrogen concentrations in PL or HTP effluents may be below the threshold needed to elicit a transcriptional response (Cheek *et al*. 1998), despite the fact that the PL EEQ value is similar to those of other municipal wastewater effluents (Körner *et al*. 2001, Gadd *et al*. 2010).

On the other hand, the findings of the present study could indicate that hornyhead turbot are less sensitive to estrogen-like substances than some freshwater fish species. EEQs values over 5 ng/L of E2 have been found to cause male vitellogenesis in freshwater species such as Japanese medaka (*Oryzias latipes*; Kitamura *et al*. 2009, Bulloch *et al*. 2010). Reduced sensitivity compared to freshwater species has been observed in other marine fishes. For instance, a concentration of 200 ng/L of E2 was necessary to elicit estrogenic responses in sheepshead minnow (*Cyprinodon variegatus*), while a lower concentration of 25 ng/L generated estrogenic responses in fathead minnow (Brian *et al*. 2005, Raimondo *et al*. 2009).

The results of this study have contributed information to understand the unexpected elevated estradiol concentrations found in field-collected male hornyhead turbot. A previous study suggested that high E2 levels could represent a natural condition for males of this species (Rempel *et al*. 2006). However, our results illustrated that plasma E2 concentrations in male controls acclimated in the laboratory were much lower than those found in field collected males. Average plasma E2 concentrations in control males exposed to effluent during the current study ranged from 6-60 pg/ml, while concentrations in field males ranged from 200 to 800 pg/ml (Bay *et al*. 2012, Forsgren *et al*. 2012). The results of the current study suggest that elevated plasma estradiol concentrations in field males are not likely caused by effluent exposure, but could result from a combination of compounds currently discharged and other stressors present in the fish environment (e.g., DDTs). However, the results of this study are limited to a 14 day exposure, which does not mimic exposure conditions of field fish. It is possible that longer exposures or exposure at different life stages (e.g., larva/embryo) could produce different results.

Despite the biological responses observed in the present laboratory study, data from our previous field studies did not show evidence of adverse biological effects in field collected hornyhead turbot. For example, gonadal abnormalities (such as intersex), or changes in reproductive behavior in male hornyhead turbot collected near the PL and HTP outfall discharge areas were not found (Forsgren *et al*. 2012). Furthermore, according to our recent field studies, the population for this species appears stable (Bay *et al*. 2012). These results suggest that, despite field and laboratory results indicating that hornyhead

turbot is exposed to estrogenic substances, we have not identified an adverse outcome pathway effect resulting from effluent exposure in this species.

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Supplemental Information

Supplemental Information is available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2013AnnualReport/ar13_153_168SI.pdf.