Gene expression of fathead minnows (*Pimephales promelas*) exposed to two types of treated municipal wastewater effluents

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Abstract

Contaminants of emerging concern (CECs) in treated municipal effluents have the potential to adversely impact exposed organisms prompting elevated public concern. Using transcriptomic tools, changes in gene expression and cellular pathways in the liver of male fathead minnows (*Pimephales promelas*) exposed to 5% concentrations of full-secondary or advanced-primary treated municipal wastewater effluents containing CECs were investigated. Gene expression changes were associated with apical endpoints (plasma vitellogenin and changes in secondary sexual characteristics). Of 32 effluent CECs analyzed, 28 were detected including pharmaceuticals, personal care products, hormones, and industrial compounds. Exposure to both effluents produced significantly higher levels of plasma VTG and changes in secondary sexual characteristics (e.g., ovipositor development). Transcript patterns differed between effluents, with <10% agreement in the detected response (e.g., altered production of transcripts involved in xenobiotic detoxification, oxidative stress, and apoptosis were observed following exposure to both effluents). Exposure to advanced-primary treatment effluent caused changes in transcription of genes involved in metabolic pathways (e.g., lipid transport and steroid metabolism). Exposure to full-secondary treatment effluent affected transcripts involved in signaling pathways (e.g., focal adhesion assembly and extracellular matrix). The results suggest a potential association between some transcriptomic changes and physiological responses following effluent exposure. This study identified responses in pathways not previously implicated in exposure to complex chemical mixtures containing CECs, which are consistent with effluent exposure (e.g., oxidative stress) in addition to other pathway responses specific to the effluent type.

Introduction

Indications that contaminants of emerging concern (CECs) in treated municipal effluents have the potential to adversely impact exposed aquatic organisms have elevated public concern (Kolpin *et*

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al. 2004). The term CECs is used to identify unregulated and unmonitored compounds, which have been recently "discovered" in natural waters and have the potential to affect aquatic life at environmental concentrations. CECs in the context of this paper include pharmaceuticals and personal care products, hormones, industrial and commercial compounds and current use pesticides. CECs can be found in effluent, receiving waters, sediments, and tissues below levels known to be acutely toxic (Maruya *et al.* 2011, Vidal-Dorsch *et al.* 2012), but within concentrations that can produce sublethal effects, including endocrine disruption. Subtle responses can result in altered reproduction (Gros *et al.* 2007; Burkhardt-Holm *et al.* 2008; Vajda *et al.* 2008, 2011; Björkblom *et al.* 2009; Garcia-Reyero *et al.* 2011), as shown with ethinylestradiol, when chronic exposure resulted in fathead minnow population declines in a Canadian experimental lake where researches dosed with 5 ng/L over 3 years (a concentration occurring in some environments; Kidd *et al.* 2007).

The biological effects of CECs present in environmental chemical mixtures can be diverse, subtle, and undetectable with traditional toxicity tools, thus non-traditional tools are being studied to investigate their effects in exposed organisms (Folmar *et al.* 2001). Genomic technologies (e.g., microarrays) have been recently applied to investigate transcriptome changes in response to pollutant exposure since gene expression alterations can result in regulatory processes preceding physiological and toxicological responses (Garcia-Reyero *et al*. 2009). Microarray based gene expression analysis is a sensitive tool to investigate a wide range of biological responses in fish exposed to contaminant mixtures (Garcia-Reyero *et al.* 2009b,c). Yet, the usefulness of this technology in assessments is challenged to some extent by a paucity of information linking gene expression changes to physiological effects, population impacts and the lack of clear directives for decision makers

This study investigated biological responses following exposure to CECs and other municipal wastewater effluent constituents. Using microarrays and quantitative real time polymerase chain reaction (qPCR), changes in liver gene expression patterns and their association with physiological responses (changes in plasma vitellogenin (VTG) and in secondary sexual characteristics) in exposed fish were investigated. Two laboratory experiments were conducted: in one, fish were exposed to chemically enhanced advanced-primary treatment effluent; in the other, fish were exposed to full-secondary treatment effluent. In both experiments, sexually mature male fathead minnows (*Pimephales promelas*) were exposed to 5% concentrations of effluent. Two types of effluent containing varied chemical mixtures were used to investigate the usefulness of the microarray techniques to assess exposure responses.

Methods

Experimental Design

Laboratory experiments were conducted using effluents from the Point Loma Wastewater Treatment Plant (PL), a publically owned advanced-primary wastewater treatment plant in San Diego, CA, and the Hyperion Treatment Plant (HTP), also a publically owned full secondary wastewater treatment plant in Playa Del Rey, CA. Fathead minnows were exposed to negative controls (synthetic moderately hard water), positive controls (4 µg/L estradiol), and 5% effluent. The 5% effluent treatment was chosen for this study in order to elicit multiple biological responses. The high positive control concentration was selected to link the results from the present study with previous experiments(Vidal-Dorsch *et al.* 2010). The exposures lasted 14 days and included three replicate tanks per treatment.

Fathead minnows were selected because of their well-studied responses to pollutants, including gene expression responses (Garcia-Reyero *et al.* 2006; Garcia-Reyero *et al.* 2009b,c). The fish used for this study were sexually mature adults with developed secondary sexual characteristics. Fish were provided in two batches (one for each experiment) by the USEPA Certified Aquatic Research Facility of the Office of Research and Development (Cincinnati, OH). Both experiments were conducted at agency laboratories (PL and HTP).

Exposures

Three replicates were used for each experimental treatment; all replicates contained male and female fish (three of each) placed in a single tank (a total of 36 males). Only male fish were used for analysis. The tanks consisted of a 12 L glass aquarium containing 10 L of moderately hard water or exposure solution. The moderately hard water used for controls and dilutions was prepared by each participating laboratory following USEPA recommendations (Russom *et al.* 1997, USEPA 2002a).

Fish were acclimated for one week prior to exposure. In the tanks the fish experienced a regime of 50% daily renewal and a photoperiod of 16 hours light:8 hours dark. The fish were fed frozen adult brine shrimp twice a day (approximately 0.1 g of food per gram of fish weight). Water quality (dissolved oxygen, conductivity, pH, temperature, and total ammonia (NH_3)) was recorded three times a week. Water quality averages during acclimation were within acceptable limits: dissolved oxygen 7.6 ±0.4 mg/L (mean ±standard deviation), conductivity 340.3 ±33.2 µS, pH 7.4 ±0.1, temperature 25 ±1°C, and NH_3 1.5 ±1.3 mg/L (USEPA 2002b). Following acclimation, the fish tanks were randomly assigned to exposure treatments.

Throughout exposures fish remained in their original acclimation tanks for 14 days and received daily renewals of exposure solutions. Solution renewals consisted of removing 5 L of water from each tank and adding 5 L of experimental treatment solution or water. Negative controls received only water during renewals using methods and equipment similar to those used for other experimental treatments. Photoperiod, temperature, and feeding regimes were kept similar to acclimation conditions. During exposure, water quality averages were: dissolved oxygen 7.3 ±0.04 mg/L, conductivity 346.8 ±17.4 µS, and pH 7.6 ±0.4, with NH_3 at 3.5 ±1.4 mg/L in the HTP effluent and 2.0 ±0.6 in the PL effluent. During exposure fish percent survival ranged between 88 and 100% in both experiments, except in estradiol controls where it was 56 and 67% in the PL and HTP experiments, respectively. The fish size in the PL experiment ranged from 5.3 to 5.7 cm and 7.3 to 7.5 cm in the HTP experiment.

Information regarding the effluents used, experimental treatments preparation, and chemical analysis is shown in the Supplemental Information (SI; ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2013AnnualReport/ar11_137_151SI.pdf). A suite of 32 CECs was analyzed in whole effluents and negative controls to characterize the exposures. Chemical measurements from the exposure tanks are shown in Table SI-1.

Biological Indicators

Several physiological responses were investigated including changes in secondary sexual characteristics, plasma VTG concentrations and other measures of general fish condition (the methods are described in SI).

Total ribonucleic acid (RNA) from liver was used for gene expression analysis. The RNA was extracted from each liver using RNA STAT-60 reagent (Tel-Test, Friendswood, TX), reconstituted in RNASecure (Ambion, Austin, TX), and DNase-treated with Turbo DNA-free (Ambion). In all cases, manufacturer protocols were followed. RNA quantity for microarray analysis was measured using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE), and RNA quality was evaluated using the Agilent 2100 BioAnalyzer with the RNA 6000 Nanochip. RNA integrity values were >8.0 for all samples. The total RNA extracted was transcribed into cDNA, as previously described (Poynton *et al.* 2008a,b). cDNA synthesis, cRNA labeling, amplification, and hybridization were conducted using the Agilent Quick Amplification Kit and following Agilent protocols (Agilent, Santa Clara, CA). The liver samples were subsequently analyzed with microarrays designed in the Denslow laboratory (GEO accession no. GPL9248). Additional gene expression analysis methods, including differential expression calculation, normalization, and analysis, are described in SI.

Quantitative real time polymerase chain reaction (qPCR) analysis was carried out to validate microarray data. Four genes including vitellogenin (*VTG*), estrogen receptor alpha (*ESR1*); Cytochrome P4501A (*CYP1A*), and Retinol binding Protein (*RBP*) were quantified by qPCR and normalized to 18S ribosomal RNA (*18S*; probes shown in Table SI-2). The *VTG*, *ESR1* and *RBP* genes are known to be potential targets for endocrine-disrupting compounds (Levine and Oris 1999, Filby and Tyler 2005 , Gunnarsson *et al.* 2007, Sabo-Attwood *et al.* 2007, Garcia-Reyero *et al.* 2009a, Filby *et al.* 2010). The *CYP1A* gene was chosen to investigate xenobiotic metabolism and detoxification (Levine and Oris 1999, Van den Heuvel 2006).

Statistical Analysis

Biological responses in fish exposed to effluent were compared to their respective negative (water only) controls. Chemical concentrations are described as averages and standard deviation. Statistical tests were conducted with JMP V8. Differences in chemical and biological measurements were analyzed by effluent type using one-way analysis of variance (ANOVA; $p < 0.05$), followed by a comparison to water controls using Dunnet's test. The chemical concentrations were corrected

for blank contamination. When contamination was found, resulting values were adjusted by subtracting the concentration found in the blanks (4% of the samples were adjusted for ibuprofen, nonylphenol and estradiol).

Microarray image and data processing was conducted with Agilent's feature extraction software V 9.5 (Agilent) and analyzed using JMP Genomics V5 (SAS, Cary, NC), as previously described (Martyniuk *et al.* 2011). Data were subjected to a log2 transformation followed by median normalization, followed by one-way ANOVA. Transcripts that were altered by more than 1.5-fold in either direction and had a p value <0.05 were further investigated. Subsequent analysis of gene expression data included functional enrichment analysis of gene ontology (GO) terms and biological pathways. In order to include as many probes as possible for pathway analysis, a false discovery rate was not corrected for. Functional enrichment analysis for GO was performed by Fisher's exact test using JMP Genomics V5 ($p < 0.05$). All data resulting from the microarray analysis were added to the NCBI's Gene Expression Omnibus (GEO) database (GSE: 29350).

PathwayStudio™ (V9.0) operating with the ResNet 9.0 database (Elsevier, Inc) was used to further analyze the gene expression data. Further descriptions of this analysis can be found in the SI.

Results and Discussion

Chemical Measurements

A wide range of CECs, including industrial and commercial compounds, hormones, pharmaceuticals, and personal care products, were detected in effluents. Some CECs were observed in both PL and HTP effluents, but many differed in occurrence or concentration by effluent type (Table 1). Of the 32 analytes investigated, 26 were detected in PL whole effluent, and 25 were detected in HTP whole effluent. Twenty-three analytes were detected in both effluents, although concentrations were generally higher in PL effluent.

Atenolol, benzophenone, butylated hydroxyanisole, caffeine, estradiol, estrone, gemfibrozil, ibuprofen, meprobamate, naproxen, primidone, progesterone, sulfamethoxazole, testosterone, triclosan, and trimethoprim were detected at significantly higher ($p < 0.05$) concentrations in PL effluent when compared to HTP effluent. Their average concentrations in the PL effluent ranged from 0.003 (±0.001) µg/L to 94 (±0.001) µg/L. In the HTP effluent, only fluoxetine (0.04 ±0.001 µg/L) was significantly higher ($p < 0.05$) than the concentration in PL effluent. Some PL compounds were detected in the whole effluent, but not in the 5% concentration possibly due to their loss in the exposure system or to the analytical precision of the measurement when working with low concentrations. Previously, Gagne´ *et al.* (2011) found that some of the compounds (e.g., caffeine, ibuprofen, naproxen, gemfibrozil, sulfamethoxazole, carbamazepine, nonylphenol) detected in effluent used in this study can influence oxidative metabolism in trout liver cells and lead to oxidative damage at similar or lower concentrations than those found in this study.

A few CECs were detected in the moderately hard water used for the controls and to prepare effluent dilutions. In the PL experiment, nonylphenol and triclosan were detected at average concentrations of 1.7 and 0.05 µg/L, respectively; in the HTP experiment, they were detected at 0.4 and 0.05 µg/L, respectively. In addition, bisphenol A (0.08 µg/L) was also detected in the HTP experiment. While these compounds were very low in the control water, they could have played an additive role during effluent exposures.

Physiological Responses

Physiological changes were found in fish exposed to both effluents when compared to negative controls. Fish exposed to effluent had significantly higher plasma VTG concentrations ($p < 0.05$). In fish exposed to PL effluent, plasma VTG averaged 726.3 µg/ml, while fish exposed to HTP effluent averaged 146.4 µg/ml (Figure 1). Fish exposed to PL effluent had significantly higher VTG ($p < 0.05$) when compared to fish exposed to HTP effluent. VTG levels in negative controls were below detection limits (1.2 µg/ml), except for one male with 12 µg/ml VTG. Plasma VTG in E2-exposed fish averaged 26 mg/ml in the PL experiment and 12.3 mg/ml in the HTP experiment.

Significantly higher vitellogenin synthesis (compared to controls) was likely induced by PL or HTP effluent compounds. For example, the estrone concentration in this study (330 ng/L) could induce plasma VTG, as demonstrated in previous studies (Thorpe *et al.* 2007). Furthermore, calculated E2 equivalents in whole PL effluent were 20 ng/L (unpublished data). The estrogenicity level is within

Table 1. Concentration of contaminants of emerging concern present in whole effluent samples from Point Loma (PL) and Hyperion Treatment Plant (HTP). Chemical names, common uses, and reporting limits (RL) are grouped by contaminant type. Three samples were used to calculate mean values (Mean) and standard deviation (StdDev) for each chemical. Chemical concentrations measured in the tanks with 5% effluent concentration are shown in Table SI-1.

Chemical Name by Type	Common Use	RL	PL		HTP	
			Mean (µg/L)	Std Dev	Mean (µg/L)	Std Dev
Pharmaceuticals and Personal Care Products						
Triclosan	Antibacterial	0.02	1.27	0.21	0.43	0.04
Sulfamethoxazole	Antibiotic	0.01	2.1	0.2	1.53	0.12
Trimethoprim	Antibiotic	0.01	0.87	0.05	0.66	0.04
Fluoxetine	Antidepressant	0.01	0.01	0.001	0.04	0.002
Meprobamate	Antidepressant	0.01	0.67	0.01	0.37	0.02
Carbamazepine	Antiepileptic	0.01	0.32	0.03	0.28	0.07
Dilantin	Antiepileptic	0.02	0.16	0.03	0.15	0.04
Primidone	Antiepileptic	0.01	0.16	0.01	0.11	0.01
Diclofenac	Anti-inflammatory	0.01	0.2	0.03	0.19	0.02
Ibuprofen[1]	Anti-inflammatory	0.02	20.97	1.76	0.02	0.01
Naproxen	Anti-inflammatory	0.01	16.07	2	0.13	0.02
Atenolol	Beta blocker	0.02	3.77	0.4	1.3	0.1
Atorvastatin	Cholesterol regulator	0.01	0.19	0.02	0.15	0.01
Musk Ketone	Fragrance	0.5	ND	-	ND	-
Gemfibrozil	Lipid regulator	0.01	4.71	0.38	2.87	0.23
Caffeine	Stimulant	0.1	93.67	17.04	0.11	0.01
Diazepam	Tranquilizer	0.01	ND	-	ND	-
Iopromide	X-ray contrast medium	0.2	ND	-	0.48	0.35
DEET	Insect repellant	0.02	0.74	0.04	0.8	0.16
Current-Use Pesticides						
Atrazine	Herbicide	0.01	ND	-	0.01	0
Hormones						
Estradiol[1]	Hormone	0.0005	0.003	0.001	ND	-
Estrone	Hormone	0.0002	0.033	0.006	0.001	0.001
Progesterone	Hormone	0.0005	0.035	0.002	0.001	0.001
Testosterone	Hormone	0.0005	0.077	0.014	ND	-
Ethinylestradiol	Synthetic hormone	0.001	ND	-	ND	-
Industrial/Commercial Compounds						
Tris(1-chloro-2-propyl) phosphate (TCPP)	Flame retardant	2	ND	-	ND	-
Tris(2-chloroethyl) phosphate (TCEP)	Flame retardant	0.2	0.24	0.02	0.35	0.05
Benzophenone	UV blocker	1	1.37	0.29	ND	-
Bisphenol A	Plasticizer	0.1	0.61	0.24	0.32	0.08
Nonylphenol[1]	Surfactant	0.5	2.28	0.58	2.46	0.52
Octylphenol	Surfactant	0.5	0.76	0.48	0.35	0.18
BHA	Antioxidant	0.02	0.2	0.04	0.12	0.01

ND= Not detected.

[1]= Values adjusted for blank contamination.

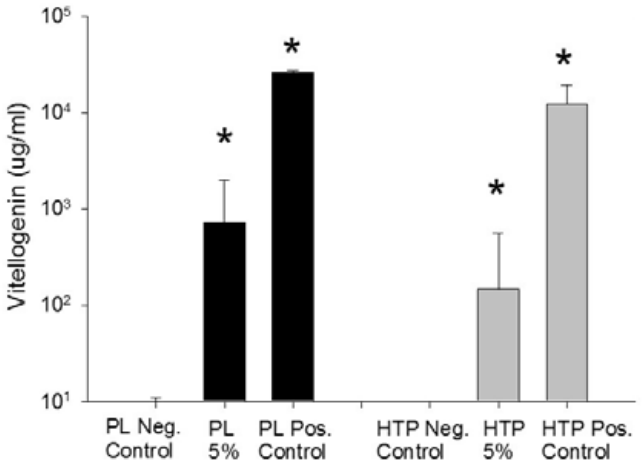

Figure 1. Average plasma vitellogenin concentrations for fathead minnows exposed to advanced-primary effluent (PL) or full-secondary effluent (HTP). Respective findings are reported for negative control (Neg. Control), 5% effluent concentration (5%) and positive control (Pos. Control) treatment levels. Asterisks represent significant differences relative to respective negative controls. Error bars represent the 95% confidence interval.

the range found to induce VTG production, even in mixtures (Brian *et al.* 2005, Liao *et al.* 2008).

Fathead minnows exposed to 0.5% PL or 0.5% HTP effluent (environmentally realistic concentrations) contained significantly higher plasma VTG concentrations when compared to controls (Figure SI-1). These results should be used with the understanding that this study was conducted with a model fish species exposed to 5% dilution of municipal wastewater effluents. Native species living in effluent discharge areas may be less sensitive (Bay *et al.* 2012), as indicated by hornyhead turbot (*Pleuronichthys verticalis*) data, and in the zone of initial dilution (ZID) for PL and HTP effluents, it is likely that concentrations are closer to <1% than to 5%. Furthermore, environmental conditions such as temperature and salinity might also influence the contaminant responses in marine fish exposed to effluent, making them less sensitive than some freshwater fish (Meina *et al.* 2013). The concentration tested is generally relevant for wastewater treatment plants, where effluent concentration in the ZID can be as high as 100% (Kolpin *et al*. 2004).

Fish condition as measured by LSI and ½ GSI was not affected by effluent exposure (additional information in SI). Similarly, other studies conducted with fathead minnows have found no significant changes in organosomatic indices following exposure to estrogens (Shappell *et al.* 2010).

Changes in secondary sexual characteristics were observed in some of the effluent-exposed fish and none were observed in fish exposed to the negative controls (Figure 2). Of the fish exposed to PL effluent, 25% lost their nuptial tubercles and dorsal nape pads, and 75% developed ovipositors. In fish exposed to the HTP effluent, 9% lost their tubercles and dorsal nape pads and 27% developed ovipositors. In the fish exposed to positive controls (E2), 33% lost their dorsal pads, 33% lost their tubercles, and 100% developed ovipositors. Changes in secondary sexual characteristics are a hallmark of exposure to estrogenic substances and have been observed in fathead minnows following exposure to 44 ng E2/L (Shappell *et al.* 2010).

Additional studies have found similar physiological changes in other fish species exposed to municipal wastewater effluent (Parrott 2005). Male fish feminization (ovipositor presence) was observed after exposure to diverse effluent concentrations, including receiving waters and effluent concentrations lower than those used in this study. For example, exposure to wastewater-dominated streams altered sex ratios and induced VTG production in fish, among other effects (Vajda *et al.* 2008). Another study found that chronic exposure of fish to effluent concentrations ranging between 20 and 78% resulted in feminization, genotoxic and immunotoxic effects (Liney *et al.* 2006).

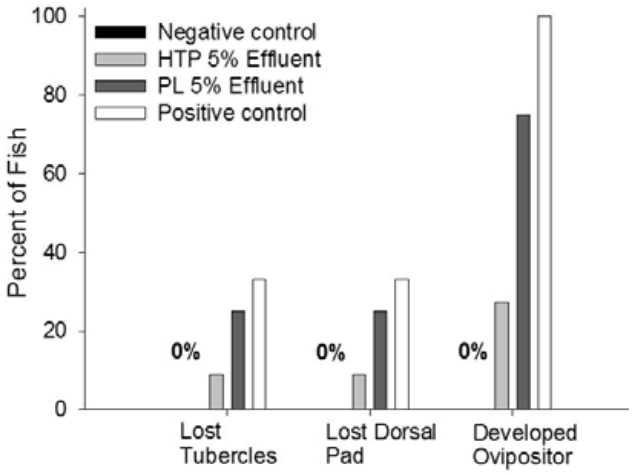

Figure 2. Percent of male fathead minnows with changes in secondary sexual characteristics. No responses were observed in negative control fish (0%).

Gene Expression Responses

Statistical analysis revealed a large number of transcripts to be significantly regulated (relative to negative controls) among fish exposed to 5% effluent. Fish exposed to PL effluent had 1,892 significantly altered transcripts (745 increased and 1,147 reduced), while fish exposed to HTP effluent had 2,776 significantly regulated transcripts (757 increased and 2,019 reduced). For further evaluation, the transcripts were ordered according to fold-expression levels in PL effluent followed by their expression levels in HTP effluent. This evaluation revealed that both effluents elicited some similar transcriptomic responses, but most responses differed (Figure SI-2; a partial list of transcripts with the highest magnitude of expression is shown in Table SI-3). The enriched GO categories for biological processes were distinctive to each effluent type. Fish exposed to PL effluent showed alterations in lipid metabolism (G0:0006629), Ras protein signal transduction (GO:0007265), and regulation of GTPase activity (GO:0043087), among others. Fish exposed to HTP effluent showed alterations in regulation of cell growth (GO:0001558), chromatin assembly or disassembly (GO:0006333), and ion transport (GO:0006811), among others (Table 2).

Similarities in Transcriptional Responses to the Two Effluent Types

Only 332 genes were altered in common between the two effluents. Using PathwayStudio™ to identify gene sets of importance, a high percentage of these genes were observed to be involved in the oxidative stress pathway, which was the most significantly enriched (Figure SI-3). Increased transcript levels of aryl hydrocarbon receptor nuclear translocator (*ARNTL*) and Cyclooxygenase-1 (*COX1*) were also noted, perhaps indicating the activation of a cellular defense response against oxidative stress. These genes are known to promote an apoptosis cascade, and some of the transcripts differentially expressed following exposure to both effluents predict such a response. For example, the connective tissue growth factor (*CTGF*) and Cytochrome c, somatic (*CYCS*) transcripts decreased. Inhibition of *CTGF* has been associated with oxidative stress (Honda *et al.* 2001), and with blocking *TGF* effects (Blom *et al.* 2002). *CYCS* regulation in fish could be a response to DNA damage and cellular stress (Li *et al.* 2000). Other studies have found oxidative stress and apoptosis responses caused by pharmaceuticals found in municipal effluent (Thibaut *et al.* 2006). Different types of pharmaceutical and personal care products (PPCPs) have been found to increase oxidative metabolism in liver cells. For instance, caffeine, naproxen, sulfamethoxazole and nonylphenol can accelerate oxidative metabolism in fish liver cells and lead to oxidative damage at concentrations found in municipal wastewater effluent (Gagne´ *et al.* 2011).

Additional gene sets that were commonly targeted following exposure to PL and HTP effluents included xenobiotic detoxification, signaling and immune responses, as well as endocrine function (Table SI-3). Important transcripts included in these sets were the aryl hydrocarbon receptor (*AHR*), myosin, light chain kinase, and cytochrome C, among others. It is well established that genes belonging to the cytochrome P450 family are controlled by nuclear receptors, including *AHR* (Fujii-Kuriyama and Mimura 2005). Additional transcripts such as *CYP2C* were up-regulated in both exposures. From other studies, it is known that CYPs metabolize pharmaceuticals like caffeine, ibuprofen, naproxen, gemfibrozil, diclofenac and carbamazepine detected in this study (Thibaut *et al.* 2006). Perhaps, *CYP2C* and other CYPs were up-regulated to cope with these effluent compounds.

Other common regulated transcripts are involved in cellular stress and apoptosis (e.g., *HSTF2*, *DAD1* and *TIAL1*), immune response (*ITK* and *NFAT1)* and endocrine function (e.g., *NCOA7*, *OGFR*, *TGFB*). Previous studies exposing salmonids to effluent also identified some of the same genes differentially expressed in this study such as those associated with detoxification, metabolism, signaling and immunity (Cuklev *et al.* 2012, Osachoff *et al.* 2012).

Differences in Transcriptional Responses to the Two Effluent Types

Despite some commonalities, the majority of transcripts changed were only affected following exposure to one effluent type, but not the other. Pathway analysis results revealed that PL effluent exposure affected genes involved in metabolic pathways (e.g., lipid transport, reactive oxygen species (ROS), catabolism, and steroid metabolism; Figure 3, abbreviations in SI). Exposure to HTP effluent altered pathways involved in cellular processes and signal transduction (e.g., CI transport, focal adhesion assembly and the extracellular matrix). Additional pathways not described in this paper were also significantly enriched (Table SI-4).

Table 2. Functional enrichment analysis for biological processes regulated by exposure to 5% effluent (p-value <0.05), ordered by GO ID. DR = number of genes differentially regulated on arrays Not DR = number of genes not differentially regulated on arrays.

Source	GO ID	GO Biological Process	DR	Not DR	Fisher p-value
PL					
	GO:0001558	regulation of cell growth	10	26	0.0107
	GO:0006118	electron transport	40	185	0.0229
	GO:0006446	regulation of translational initiation	5	9	0.0142
	GO:0006486	protein amino acid glycosylation	8	26	0.0391
	GO:0006506	GPI anchor biosynthetic process	6	16	0.03
	GO:0006512	ubiquitin cycle	36	130	0.0012
	GO:0006629	lipid metabolic process	15	43	0.0048
	GO:0006783	heme biosynthetic process	8	26	0.0391
	GO:0006810	transport	109	512	0.0007
	GO:0006888	ER to Golgi vesicle-mediated transport	4	6	0.0163
	GO:0007160	cell-matrix adhesion	7	18	0.0191
	GO:0007265	Ras protein signal transduction	4	5	0.0109
	GO:0008150	biological process	372	2251	0.0408
	GO:0008152	metabolic process	85	325	<0.00001
	GO:0008299	isoprenoid biosynthetic process	5	7	0.007
	GO:0008654	phospholipid biosynthetic process	5	10	0.019
	GO:0015992	proton transport	5	12	0.0313
	GO:0043087	regulation of GTPase activity	6	16	0.03
	GO:0046847	filopodium formation	4	8	0.0311
	GO:0048015	phosphoinositide-mediated signaling	3	5	0.0384
	GO:0051258	protein polymerization	4	5	0.0109
HTP					
	GO:0001558	regulation of cell growth	9	27	0.0154
	GO:0006333	chromatin assembly or disassembly	7	26	0.0485
	GO:0006364	rrna processing	5	15	0.0366
	GO:0006418	trna aminoacylation for protein translation	11	34	0.0079
	GO:0006811	ion transport	30	141	0.0106
	GO:0006812	cation transport	8	26	0.026
	GO:0006816	calcium ion transport	25	120	0.0183
	GO:0006865	amino acid transport	6	15	0.014
	GO:0006916	anti-apoptosis	6	17	0.0214
	GO:0007017	microtubule-based process	7	14	0.0037
	GO:0007018	microtubule-based movement	10	37	0.0309
	GO:0007156	homophilic cell adhesion	12	34	0.0025
	GO:0007186	g-protein coupled receptor protein signaling pathway	28	147	0.0366
	GO:0007229	integrin-mediated signaling pathway	7	24	0.041
	GO:0009416	response to light stimulus	4	5	0.007
	GO:0016042	lipid catabolic process	5	13	0.0245
	GO:0019538	protein metabolic process	6	13	0.0086
	GO:0043010	camera-type eye development	3	6	0.0382
	GO:0044267	cellular protein metabolic process	4	9	0.0272
	GO:0045449	regulation of transcription	40	221	0.0311
	GO:0048015	phosphoinositide-mediated signaling	3	5	0.0278
	GO:0051258	protein polymerization	3	5	0.0278

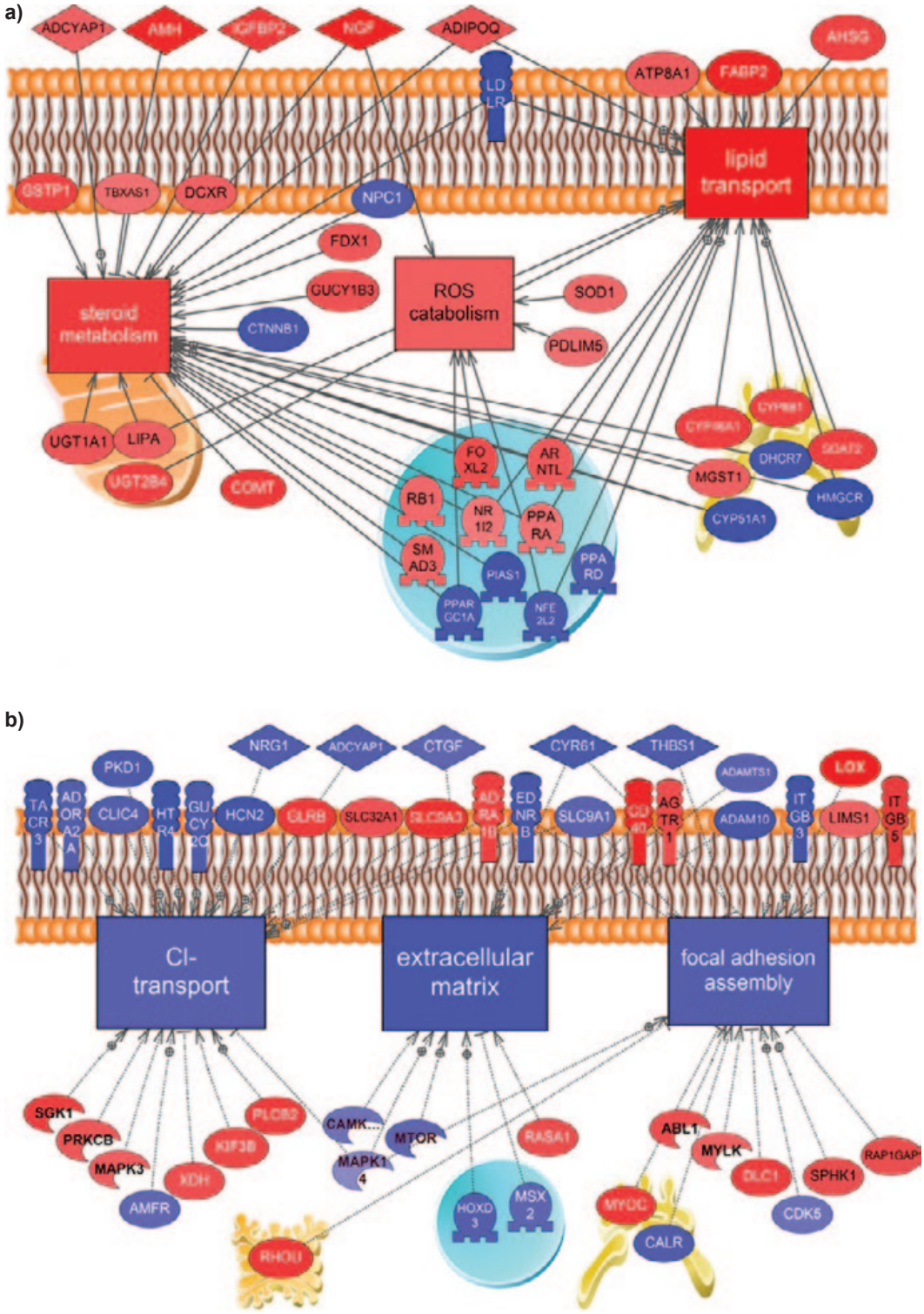

Figure 3. Pathway analyses of transcripts differentially regulated by the two effluents. Pathway analysis of PL effluent (a). The majority of transcripts were associated with metabolic pathways like steroid metabolism, ROS catabolism, lipid transport and xenobiotic metabolism (clearance). Most enriched pathways following exposure to HTP effluent were involved in intracellular signals associated with Cl-transport, extracellular matrix and focal adhesion assembly (b). Blue indicates down-regulation, and red indicates up-regulation of gene expression. The darker the color intensity the stronger a process or sub-unit is impacted by the treatment. Symbols represent protein ⬭, transcription factor, membrane receptor, ligand ◇, cell process ▭, and kinases. The abbreviations used are the official symbols located in http://www.ncbi.nlm.nih.gov/gene/. Further details provided in SI.

Exposure to PL effluent caused changes in the expression of 1,594 genes unique to this exposure, with a main focus on genes involved in metabolism. There is considerable evidence in the literature that compounds such as plasticizers, used in flexible polyurethane, or gemfibrozil can alter metabolic pathways by regulating the peroxisome proliferator-activated receptor (*PPARα*) and the retinoic acid receptor (*RAR*), and both of these were differentially regulated following exposure to PL effluent (Table SI-3; Wintz *et al.* 2006, Skolness *et al.* 2012). Genes involved in steroid metabolism such as the pregnane-X receptor and certain CYPs were also affected in PL effluent exposed fish, and these have previously been shown to be altered by estrogenic compounds, such as nonylphenol (Acevedo *et al.* 2005). The ROS pathway genes can be affected by natural and anthropogenic causes, but exposure to environmental contaminants can increase ROS and cause oxidative stress in fish (Livingstone *et al.* 2000).

Exposure to HTP effluent caused differential gene expression in 1,478 genes not affected following exposure to PL effluent. Altered transcripts were mostly involved in transport and signaling pathways. For example, the Cl ion transport pathway was repressed. Other studies have shown that metals can alter this pathway by affecting ion transport and osmoregulation and by blocking substance transport across membranes (Poopal *et al.* 2013). Arsenic, cadmium, copper, lead, and zinc were detected in PL and HTP effluents (Table SI-5). Other pathways affected by exposure to HTP effluent included focal adhesion assembly and extracellular matrix (ECM), which are required for cell growth (Hornberger *et al.* 2006). Fish exposure to priority contaminants such as dioxins can impact genes involved with these pathways by repressing genes related to ECM composition and metabolism (Andreasen *et al.* 2006).

The qPCR results confirmed microarray results. For example, both the qPCR and the microarray have shown transcript levels of estrogen receptor alpha (*ESR1*) were significantly increased in fish exposed to HTP effluent when compared to negative controls, but not in fish exposed to PL effluent (Table SI-6). Similarly, both assays showed that retinol binding protein (*RBP*) mRNA levels were significantly higher in fish exposed to PL effluent, but not in fish exposed to HTP effluent. Microarray and qPCR results indicated that *CYP1A* transcripts were not significantly different following exposure to either effluent.

Overall, a mixture of chemicals, rather than a single compound, present in the effluent were the likely cause of the responses found in this study, as individual chemicals were present at low concentrations. It is known that chemicals with similar modes of action can be additive by targeting the same nuclear receptors (Thorpe *et al.* 2003). For example, exposure to low levels of individual estrogenic chemicals in a mixture can produce additive effects in fathead minnows (Harrisa *et al.* 2009). It is also plausible that effluent chemicals not measured in this study contributed to the responses. Effluent mixtures can be very complex; in addition to parent chemicals they may also contain metabolites that could have estrogenic activities. For example, benzophenone metabolites can induce VTG production in male fish and alter secondary sex characteristics (Weisbrod *et al.* 2007). A substantial advantage of the gene expression data is that it provided information to investigate responses to all constituents in the chemical mixture, even to those unmeasured or unknown.

Linking Physiological Responses, Gene Expression and Effluent Characteristics

Two effluent mixtures which produced distinctive overall patterns of response at the gene expression level were used. Yet, relatively similar responses were observed at the organ and individual levels and these physiological responses seemed to follow a dose response pattern in which the PL effluent with higher chemical concentrations elicited a higher magnitude of gene expression response.

Chemical analyses indicated that advanced-primary treatment effluent had significantly higher concentrations for 78% of the chemicals detected in both effluents. Biodegradation and sorption processes used during secondary treatment are known to partially remove some CECs, and may account in some measure for the lower concentrations observed in HTP effluent (Drewes *et al.* 2008). The higher CEC concentrations in PL effluent can be partially responsible for the response differences. Previous studies found that fish exposure to effluents with a lower degree of treatment had a higher magnitude of effect physiologically and genetically, than fish exposed to more treated effluent (Pottinger *et al.* 2011).

Factors besides treatment level could have influenced the composition and concentration of effluent constituents. For example, the composition of CECs in PL and HTP influent (i.e., raw sewage)

was not examined; its characteristics could have a strong bearing on chemical composition regardless of treatment level. A comparison of available data for metals and ammonia shows considerable differences between PL and HTP influents (Table SI-5).

Exposure to effluent compounds could be responsible for an association between fish gene expression and physiological responses. A significant positive correlation between the expression of the *VTG* gene and plasma VTG concentrations ($r = 0.73$; $p = 0.001$) was found, suggesting that the inducer of plasma VTG was in the treatments used for the experiments. Different types of estrogenic compounds were detected in PL and HTP effluents including pharmaceuticals, industrial compounds and hormones (Vidal-Dorsch *et al.* 2012).

The results of this study suggest the potential activation of an adverse outcome pathway associated with effluent xenoestrogens exposure (Figure SI-4). The outcome pathway represents a linkage of molecular responses and an adverse outcome at a biological level relevant to risk assessment (e.g., reproduction; Ankley *et al.* 2010). For example, these results showed that exposure to effluent altered endpoints associated with estrogenic responses. Increased mRNA levels of estrogen receptor genes may indicate an increase of estradiol or estrogen-like compounds in fish plasma (Bowman *et al.* 2000). An abundance of estrogen or estrogen-like chemicals can trigger VTG production and changes in secondary sexual characteristics as observed in this study (Miller *et al.* 2007). Such changes could lead to reproduction impacts. This type of information could be used by managers to determine whether a follow up study is needed to investigate potential adverse reproductive effects.

In summary, gene expression analysis provided novel information describing different responses resulting from exposure to PL and HTP effluents. However, additional studies should be conducted to determine if the genes and pathways activated following exposure represent responses typical to these effluent types. In addition, continuing examinations of how effluent mixtures alter changes in signaling and metabolic pathways are planned.

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Acknowledgements

Partial research funding and assistance was provided by the City of San Diego Public Utilities Department (Wastewater Branch and the Environmental Monitoring and Technical Services Division) and the City of Los Angeles (Environmental Monitoring Division); the authors particularly thank Tim Stebbins, Curtis Cash, and Stan Asato for their support. In addition, the authors thank Shane Snyder, Brett Vanderford, Rebecca Trenholm, and Janie Zeigler from Southern Nevada Water Authority for assistance; Jim Lazorchak and the staff of the Aquatic Research Facility (ORD, USEPA) for providing fish; and former SCCWRP staff members Monica Mays, Diana Young, Richie LeClair, and Roxana Zepeda for their contributions to this study. The authors also thank Karlene Miller for her editorial suggestions, which improved this manuscript.

Supplemental Information

Supplemental Information is available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2013AnnualReport/ar11_137_151SI.pdf.