



## Correlation of gene expression and contaminant concentrations in wild largescale suckers: A field-based study



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### HIGHLIGHTS

- Hepatic tissue concentrations of OCs, PCBs, and PBDEs were measured in wild largescale suckers.
- Gene expression was analyzed by microarray in the same cohort of fish.
- The expression patterns of 69 genes correlated with tissue contaminant concentration.
- Correlated genes are candidates for use in future biomarker development.

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### ABSTRACT

Toxic compounds such as organochlorine pesticides (OCs), polychlorinated biphenyls (PCBs), and polybrominated diphenyl ether flame retardants (PBDEs) have been detected in fish, birds, and aquatic mammals that live in the Columbia River or use food resources from within the river. We developed a custom microarray for largescale suckers (*Catostomus macrocheilus*) and used it to investigate the molecular effects of contaminant exposure on wild fish in the Columbia River. Using Significance Analysis of Microarrays (SAM) we identified 72 probes representing 69 unique genes with expression patterns that correlated with hepatic tissue levels of OCs, PCBs, or PBDEs. These genes were involved in many biological processes previously shown to respond to contaminant exposure, including drug and lipid metabolism, apoptosis, cellular transport, oxidative stress, and cellular chaperone function. The relation between gene expression and contaminant concentration suggests that these genes may respond to environmental contaminant exposure and are promising candidates for further field and laboratory studies to develop biomarkers for monitoring exposure of wild fish to contaminant mixtures found in the Columbia River Basin. The array developed in this study could also be a useful tool for studies involving endangered sucker species and other sucker species used in contaminant research.

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### 1. Introduction

The Columbia River Basin is home to many types of fish and wildlife, but this important river is threatened by the release of toxic compounds from a myriad of sources, including industrial and municipal discharges and agriculture. A recent report by the United States Environmental Protection Agency expressed concern

about the levels of mercury, dichlorodiphenyltrichloroethane (DDT) and its derivatives, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ether (PBDE) flame retardants in the Columbia River and the risk they present to humans, fish, and wildlife (EPA, 2009). Studies have shown bioaccumulation of hydrophobic organic compounds and heavy metals through the food web in migratory and resident fish (Feist et al., 2005; Foster et al., 2001a, 2001b; Hinck et al., 2006; Johnson et al., 2007a, 2007b; Rayne et al., 2003; Sloan et al., 2010; Webb et al., 2006), predatory birds (Anthony et al., 1993; Buck et al., 2005; Elliott et al., 2000; Henny et al., 2004, 2008, 2011), and aquatic mammals (Elliott et al., 1999; Grove and Henny, 2008; Harding et al., 1998, 1999). To fully understand which contaminants are present at different sites in the Columbia River Basin and the risks that they present, studies that incorporate a spectrum of analyses are needed. The work described here is part of a large, interdisciplinary study investigating the

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occurrence of emerging and legacy contaminants (PBDEs and other endocrine disrupting compounds) in the lower Columbia River and their food web effects (Nilsen and Morace, 2014–this issue).

Our objective was to determine the transcriptomic effects of environmental contaminants in largescale suckers (*Catostomus macrocheilus*), a species that has previously been used to assess contaminant levels in the Columbia River Basin (Hinck et al., 2006; Rayne et al., 2003). Largescale suckers are widespread and represent an intermediate level of the food web since they both feed on contaminated prey and are themselves prey for birds and other fish (Dauble, 1986). Little is known about movement patterns of largescale suckers, but as resident fish they are thought to remain within a relatively confined home range and thus would potentially accumulate the contaminant load present in their home range (Rayne et al., 2003).

Contaminant studies in the Columbia River have generally focused on a few endpoints and indicators. Physiological endpoints such as condition factor, gonad size, and plasma triglycerides were previously correlated with contaminant exposure in fish from the Columbia River (Feist et al., 2005). A negative correlation between liver and gonad contaminant burdens and proteins such as plasma testosterone and 11-ketotestosterone was also found in white sturgeon (*Acipenser transmontanus*) in the Columbia River (Feist et al., 2005; Foster et al., 2001a). Although we have gained information about contaminant effects from these studies using individual markers of exposure, the work has been limited by the few biological markers and endpoints known to respond to contaminant exposure.

Techniques that assay a broad range of molecules, such as microarray technology, offer the potential for a more global understanding of the biological effects of contaminant exposure. Microarray analyses, which simultaneously assay the expression of thousands of genes, are powerful tools for identification of biological pathways affected by contaminant exposure and for discovery of individual genes that could be used as new indicators of contaminant exposure. Microarray technology is increasingly used in aquatic toxicology (Bartosiewicz et al., 2001; Carlson et al., 2009; Garcia-Reyero et al., 2011; Popesku et al., 2010; Sellin Jeffries et al., 2012), but its application remains limited due to the lack of DNA sequence information for ecologically-relevant species. To expand the tools available for investigating the effects of complex environmental contaminant mixtures, we developed a custom microarray for largescale suckers. We used this array to identify genes with expression patterns that correlated with hepatic levels of organochlorine (OC) pesticides, PCBs, or PBDEs in wild largescale suckers collected from the lower Columbia River.

## 2. Materials and methods

### 2.1. Site descriptions

To facilitate collection of fish with a range of contaminant burdens, we chose three sites in the lower Columbia River with different levels of urbanization and contaminant point sources (see Nilsen et al. (2014–this issue) for map of study sites). The sites are as follows: Skamania (river km 225), Columbia City (river km 132), and Longview (river km 106). The Skamania site is the furthest upstream and has no known contaminant point sources nearby. It is 10 km downstream of Bonneville Dam, the lower-most dam on the Columbia River, but upstream of large urban areas, including Portland, Oregon, and Vancouver, Washington. The Columbia City site is located between Columbia City and St. Helens, Oregon. There are four environmental cleanup areas near the upstream end of this site. Other potential sources of contamination include the St. Helens wastewater treatment plant and upstream input from Lake River, which receives storm water runoff from Vancouver, Washington, via Vancouver Lake. Finally, the Longview site is furthest downstream and located directly in the Port of Longview at Longview, Washington, and across from Rainier, Oregon, the location of the now decommissioned Trojan Nuclear Power Plant. The port has

waterfront industrial property, and effluents from Three Rivers Regional and City of Rainier wastewater treatment plants also feed into the river.

### 2.2. Fish and tissue collection

Largescale suckers were collected from the three sites by shoreline boat electrofishing on March 23, 2010 and May 4–6, 2011. In 2010, we collected four fish from Skamania and three fish from Longview and euthanized them in an overdose of MS-222 (200 mg/L) buffered with an equal amount of sodium bicarbonate. We dissected the whole liver, brain, heart, kidney, and gonad and preserved a 130 mg portion of each in RNAlater (Ambion, Austin, Texas). In 2011, we collected 6–8 fish from each site and euthanized them as described. We did not test the effects of MS-222 on gene expression in this study because all of our fish were anesthetized in the same manner, and thus all would be similarly affected by anesthetic exposure. We measured fork length to the nearest 0.5 cm and weighed fish to the nearest gram. Gonads were extracted and weighed separately to the nearest 0.1 g, and liver samples were preserved as described above. Small portions (1–2 g) of the livers were also placed into a glass jar, manually homogenized with a metal spatula, and then placed on ice for transport back to the lab where they were frozen at  $-20^{\circ}\text{C}$  until analyzed for contaminants. We calculated the gonadosomatic index (GSI) for each fish by dividing gonad weight by total fish weight and multiplying by 100.

### 2.3. Contaminant analysis

Liver samples were analyzed using high-pressure solvent extraction, column cleanup, and concentration followed by quantification by gas chromatography–mass spectrometry (GC–MS) for 58 compounds, including DDTs and other OC pesticides, PCBs, and PBDEs (Table S1). Samples were analyzed by the U.S. Geological Survey's National Water Quality Laboratory (Denver, Colorado) as described (Nilsen et al., 2014–this issue). For quality control, the lab ran method blanks, reagent spikes, and surrogate compounds. Quality control results and individual contaminant data for each fish are included in Table S2. When a contaminant was detected in the method blank only values four times greater than the blank value were reported as detections. For statistical analyses, contaminant concentration values below the method detection limit were set to zero and included in the analyses. We identified PCB congeners by their IUPAC number.

### 2.4. Microarray development

We used total RNA from the liver, brain, heart, kidney, and gonad of the seven largescale suckers (3 males and 4 females) collected in 2010 to construct a normalized cDNA library, which we sequenced using the 454 GS-FLX (Roche 454 Life Sciences, Branford, Connecticut) sequencing platform as described in Garcia-Reyero et al. (2008) and the Illumina Genome Analyzer IIx (Illumina Inc., San Diego, California) sequencing platform following the manufacturer's protocols. The assembled cDNA contigs yielded a total of 158,403 non-redundant sequences, which we annotated by conducting large-scale homology searches of the National Center for Biotechnology Information (NCBI) nr and nt databases using an in-house computational pipeline implementing the basic local alignment search tool (blastx and blastn; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). About 55,700 of the sequences queried had a hit with high sequence similarity ( $e\text{-value} \leq 10^{-5}$ ). We also characterized the assembled sequences with respect to functionally annotated genes by BLAST searching against the NCBI reference sequences (RefSeq) for human (*Homo sapiens*) and zebrafish (*Danio rerio*). We found 43,436 human homologs and 54,386 zebrafish homologs. We designed a 15K-oligonucleotide microarray with 14,708 unique probes, 50 replicate probes, and 536 control probes using the eArray service from Agilent. Replicate probes were included to ensure the consistency of expression measurements across each array, and the control probes were used to

confirm that the arrays had low background fluorescence and robust hybridization. Over 90% of the probes had an identified human or zebrafish homolog. The microarray was submitted as a platform dataset to NCBI's Gene Expression Omnibus (GEO) database. See supporting information for detailed methods of RNA extraction, cDNA library construction, sequencing, and annotation.

## 2.5. Microarray analysis

We synthesized Cyanine 3 (Cy3)-labeled cRNA from 1000 ng liver total RNA (RIN between 8.3 and 9.1) for each fish (6–8 fish per site collected in 2011) and hybridized it to the array following the Agilent One-color Microarray-Based Gene Expression Analysis (Quick Amp Labeling) protocol no. G4140-90040 v5.7. Dye incorporation and yield of the cRNA samples were measured using a NanoDrop ND-1000 (NanoDrop Technologies). The average specific activity was 9.17 pmol/ $\mu$ L. Following hybridization, we scanned the slides using the Agilent Technologies G2505B scanner at 5  $\mu$ m resolution and extracted the array data using the Agilent Feature Extraction software v9.5. We submitted the microarray data to the Gene Expression Omnibus website (GEO accession number [GSE41016](https://www.ncbi.nlm.nih.gov/geo/); <http://www.ncbi.nlm.nih.gov/geo/>), consistent with minimum information about a microarray experiment (MIAME) standard (Brazma et al., 2001).

## 2.6. Identifying significant correlations between contaminant concentration and gene expression

We used Significance Analysis of Microarrays (SAM) to identify significant correlations between contaminant concentration and gene expression values (Tusher et al., 2001). SAM was implemented using the “samr” package (Tibshirani et al., 2011) in the R statistical programming platform (R Core Team, 2012). SAM assigns a score to each probe,  $d_i$ , that represents a signal-to-noise ratio based on the change in probe-specific expression value relative to the variation in expression values:

$$d_i = \frac{r_i}{s_i + s_0},$$

where  $r_i$  is the Pearson correlation coefficient between contaminant concentration and the expression values of the  $i$ th probe,  $s_i$  is the standard error of  $r_i$ , and  $s_0$  is a small positive constant that minimizes the coefficient of variation in  $d_i$  (see Tusher et al. (2001) for details). For calculating correlation coefficients, contaminant data were first  $\log_2$ -transformed (a value of 1 was added to all concentrations so all concentrations, including zeros, could be transformed), and raw gene expression values were  $\log_2$ -transformed and then quantile normalized using the methods of Bolstad et al. (2003) as implemented in the package “preprocessCore” in R (Bolstad, 2013).

Significant correlations in SAM were identified by varying the cutoff value of a tuning parameter called “delta”, which allows the user to balance the number of significant probes with the false discovery rate (FDR). Delta was calculated as the difference between the observed value of  $d_i$  and the expected value of  $d_i$ . Both the FDR and the expected value of  $d_i$  were estimated based on random permutations of the gene expression data (Tusher et al., 2001). We conducted SAM for each contaminant that had a minimum of four data points above the method detection limit (i.e. the contaminant was detected in at least four of the 21 fish analyzed). Nineteen contaminants met these criteria. All 21 fish (detects and non-detects) were analyzed using SAM for these 19 contaminants. For each analysis, we used 1000 permutations of the gene expression data set. The delta cutoff value for each contaminant was determined by constraining the FDR below 28% and the median number of false-positive probes below 6, which allowed a reasonable number of probes to be called significant while minimizing the number of false-positive probes. For each contaminant with significant probes,

we present the delta cutoff value, the resulting number of significant probes, the median number of false-positive probes, and the FDR (Table 1). For each probe significantly correlated with a contaminant, we searched the PubMed database on the National Center for Biotechnology Information website ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) to determine the functional category for its human homolog. There were no significant relations between the concentrations of individual contaminants and any morphometric characteristic (i.e., fork length, mass, GSI) as determined by simple correlation analysis.

## 2.7. Real-time PCR

Real-time PCR was used to validate the expression values determined by microarray. We designed primers for sequences corresponding to three genes (carboxylesterase 2 [CES2], cytochrome b<sub>5</sub> reductase 3 [CYB5R3], and transmembrane emp24 protein transport domain containing 1 [TMED1]) with expression patterns that correlated with contaminant concentration and for ribosomal protein L8 (RPL8), which served as an endogenous control (Table S3). Real-time PCR experiments were performed using the Applied Biosystems (Carlsbad, California) StepOne Real-time PCR System with Power SYBR Green labeling per manufacturer's instructions. Gene expression in 12 fish (four from each site) with a range of contaminant concentrations was analyzed. We calculated  $\log_2$  fold change between fish for both microarray and real-time PCR gene expression data, using a fish with low contaminant concentration as the control. We used Pearson correlation to test the significance of the relation between the microarray and real-time PCR results for each gene. See supporting information for detailed real-time PCR methods.

## 3. Results and discussion

### 3.1. Contaminant concentrations in the liver of largescale suckers from the lower Columbia River

In the 21 adult largescale suckers (see Table 2 for information on size and sexual maturity as indicated by GSI) collected from three sites in the lower Columbia River, we detected two DDT metabolites, six other OC pesticides (*cis*-chlordane, DCPA, dieldrin, hexachlorobenzene, *cis*-nonachlor, and *trans*-nonachlor), twelve PCB congeners, and four PBDE congeners (Fig. 1, Tables S2, S4). We also detected two current use pesticide/herbicides (chlorpyrifos and oxyfluorfen) and one personal care product (triclosan) once each in three separate fish (Tables S2, S4). There was substantial individual variation between fish including those collected at the same site, which suggests largescale suckers may have a larger home range than previously thought and demonstrates the importance of sampling multiple fish for environmental contaminant monitoring (Fig. 1).

**Table 1**

Results from SAM analysis of gene expression and contaminants data. FDR, false discovery rate.

Contaminant	Delta cutoff	# of significant probes	Median # of false positives	FDR (%)
Cis-chlordane	0.13	5	0.9	18
DCPA	0	3	0	0
PCB 170	0.19	17	2.7	16
PCB 174	0.21	23	5.6	24
PCB 177	0.23	26	5.2	20
PCB 180	0.13	7	1.9	27
PCB 183	0.24	13	3.6	28
PCB 187	0.12	4	1.0	26
PBDE 100	0.06	2	0	0
PBDE 154	0.17	9	2.0	22

**Table 2**  
Length, mass, sex, and gonadosomatic index (GSI) of largescale suckers collected for contaminant and microarray analysis.

Site	Sex	N	Fork length (mm)		Mass (g)		GSI	
			Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
Skamania	Female	6	440 (56)	330–485	1117 (343)	463–1436	8.2 (3.8)	0.9–10.6
	Male	2	393 (4)	390–395	733 (19)	719–746	0.5 (0.1)	0.4–0.6
Columbia City	Female	5	420 (45)	375–490	902 (204)	679–1220	3.4 (3.6)	0.7–8.0
	Male	1	425	–	972	–	6.1	–
Longview	Female	6	472 (32)	420–510	1219 (360)	762–1739	5.0 (6.0)	1.0–13.5
	Male	1	430	–	890	–	1.0	–

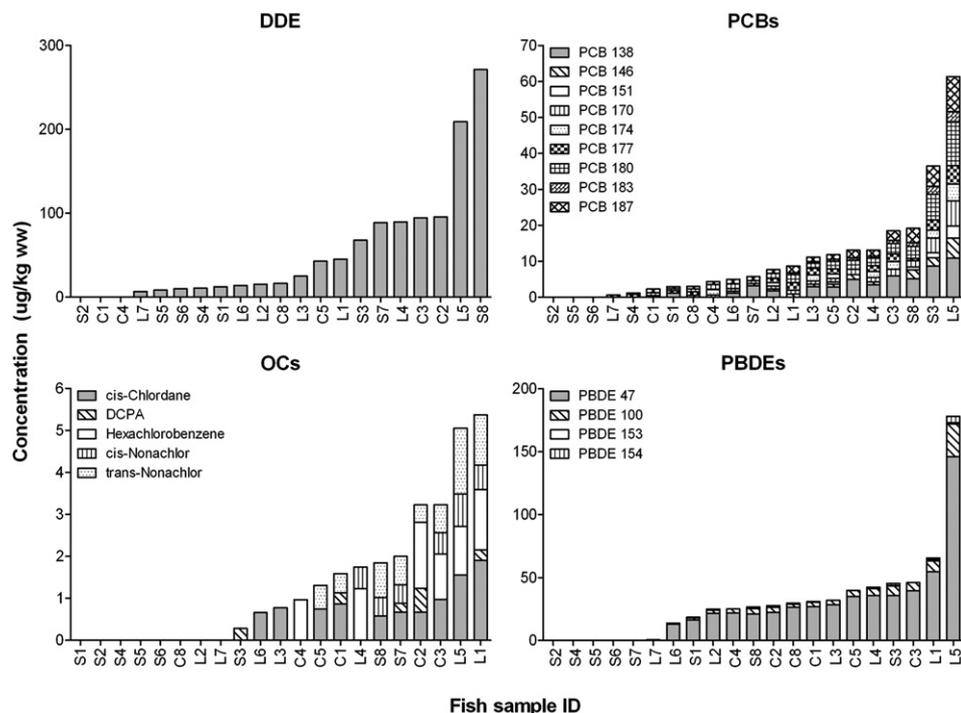
Previous studies have detected other contaminants in the lower Columbia River besides those measured in this study, including dioxins and furans, heavy metals, polycyclic aromatic hydrocarbons, and pharmaceuticals (USGS, 1996, 2006). It is certain that the largescale suckers we collected were exposed to many other compounds besides those measured. However, for the purposes of this study, we focused on PBDEs and other endocrine disrupting compounds that have been frequently detected in fish from the lower Columbia River (Feist et al., 2005; Foster et al., 2001a, 2001b; Hinck et al., 2006; Johnson et al., 2007a, 2007b; Sloan et al., 2010) because they are known to accumulate in fish tissue and are of ecological concern (EPA, 2009).

### 3.2. Identification of genes correlated with contaminant concentration

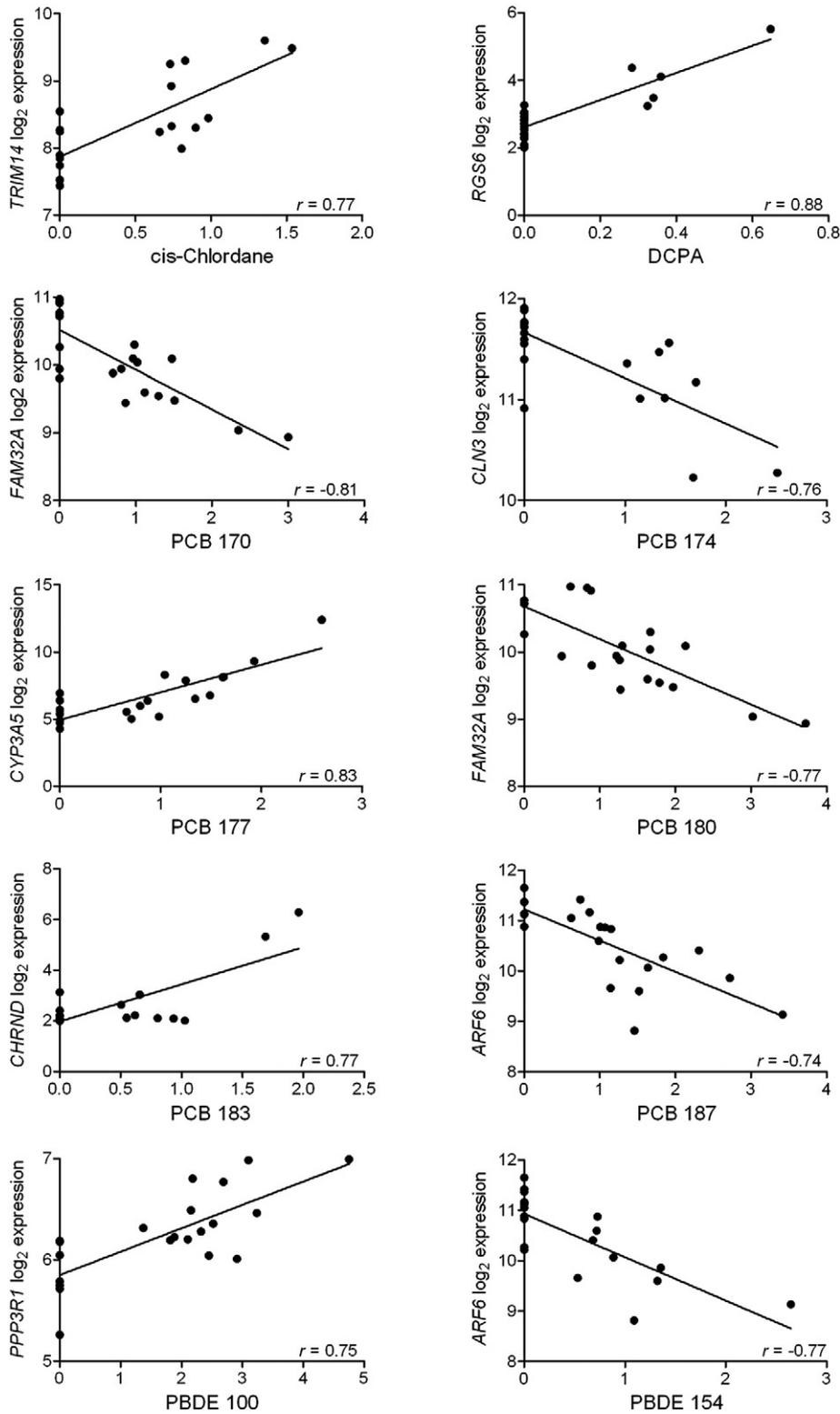
Because we measured both liver contaminant concentrations and gene expression individually in the same 21 fish, we were able to directly test for genes with expression patterns that correlated with concentrations of specific contaminants. The expression of 72 probes correlated significantly with liver contaminant concentrations of individual OCs (*cis*-chlordane or DCPA), PCBs (congeners 170, 174, 177, 180, 183, or 187), or PBDEs (congeners 100 or 154; Fig. 2, Tables 3–5). Twenty-three probes correlated with multiple

PCB and PBDE congeners. These findings were likely due to the high level of correlation between concentrations of these contaminants ( $r = 0.73$ – $0.99$ ) and suggest that these data show general relations between contaminant concentration and gene expression and not responses to specific congeners or contaminant classes. There was no overlap between probes significantly correlated with OCs and the other two contaminant classes (PCBs and PBDEs). This is consistent with the low degree of association between concentrations of OCs and other contaminants ( $r = -0.16$ – $0.62$ ). Nine contaminants analyzed did not correlate significantly with any probes, based on SAM, which could be due to a variety of factors including confounding effects from other contaminants or environmental variables that were not measured. For those contaminants that correlated with expression of specific probes, we cannot exclude the possibility that the relation is not causal but rather due to a correlated contaminant or other variable that we did or did not measure.

Evidence from laboratory studies will be required to identify direct contaminant/gene effects, and a combination of field and laboratory studies may allow us to identify genes that are activated or suppressed by particular classes of contaminants. Our study measured only a select group of chemicals and was not confirmed by laboratory studies, but we did see different gene expression patterns that correlated with OCs



**Fig. 1.** Liver tissue concentration of DDE, OCs (not including DDTs), PCBs, and PBDEs in largescale suckers collected from three sites on the lower Columbia River arranged from lowest to highest contaminant concentration. Fish sample IDs on the x-axis include the first letter of the site name where the fish was collected (S = Skamania, C = Columbia City, and L = Longview) and a number to distinguish individual fish from the same site. Only contaminants used for comparison with gene expression are shown. ww, wet weight.



**Fig. 2.** Contaminant and gene expression correlation examples. The gene with the highest Pearson correlation coefficient ( $r$ ) and lowest  $q$ -value for each contaminant is shown. Contaminant values are expressed as  $\log_2(x + 1)$   $\mu\text{g}/\text{kg}$  wet weight, and each data set is fit with a linear regression.

versus PCBs or PBDEs suggesting that different classes of contaminants may generate a different gene response and that this can be differentiated in the field. Specific gene responses could be useful for identifying which toxic compound classes are present in an ecosystem. Contaminant research is hindered by the sheer number of contaminants in our

lakes, rivers, and oceans, and it can be cost-prohibitive to measure the majority of compounds suspected to be present in an ecosystem. Screening a panel of contaminant-responsive genes may be a quick and cost-effective way to determine the main contaminant classes present in an ecosystem and thus focus the contaminant identification process.

**Table 3**  
Human gene homologues, functional categories, Pearson correlation coefficients ( $r_i$ ), denominator of  $d_i$  ( $s_i + s_0$ ), and  $q$ -values for probes correlated with organochlorine contaminants cis-chlordane (CC) and DCPA. The  $q$ -value is defined as the lowest false discovery rate (FDR) at which the gene is called significant (Storey, 2002). HS, *Homo sapiens*.

Contaminant	Probe name	HS gene symbol	HS gene ID	HS gene name	Functional category	$r_i$	$s_i + s_0$	$q$ -value (%)
CC	LSS_UF_15469	TRIM14	9830	Tripartite motif containing 14	Unknown	0.77	0.29	0
	LSS_UF_20914	VWA9	81556	von Willebrand factor A domain containing 9	Unknown	-0.76	0.29	0
	LSS_UF_19792	SERINC1	57515	Serine incorporator 1	Lipid metabolism	0.74	0.30	0
	LSS_UF_20005	SCPEP1	59342	Serine carboxypeptidase 1	Protein degradation	0.73	0.30	0
	LSS_UF_16665	SCMH1	22955	Sex comb on midleg homolog 1 ( <i>Drosophila</i> )	Development	0.72	0.30	23
DCPA	LSS_UF_15293	RGS6	9628	Regulator of G-protein signaling 6	Signaling	0.88	0.22	0
	LSS_UF_19631	PCBP4	57060	Poly(rC) binding protein 4	Apoptosis	0.83	0.24	0
	LSS_UF_11937	ILF3	3609	Interleukin enhancer binding factor 3, 90 kDa	Transcription	-0.80	0.25	0

### 3.3. Real-time PCR confirmation of gene expression correlations

We used real-time PCR to validate the expression values obtained from microarray for three genes with expression patterns that correlated with contaminant concentration (*CES2*, *CYB5R3*, and *TMED1*) in 12 largescale suckers from our microarray analysis. Expression values determined by microarray and real-time PCR for *CES2* and *CYB5R3* were highly correlated ( $r = 0.95$ – $0.97$ ,  $p < 0.0001$ ; Fig. 3). Values for *TMED1* showed a weaker but still significant correlation ( $r = 0.65$ ,  $p = 0.03$ ). These results confirm the gene expression patterns observed by microarray.

### 3.4. Functional categories associated with significant genes

The significantly correlated probes represented 69 unique genes (plus one unknown) from a variety of functional groups (Tables 3–5). Besides genes with protein products that specifically function in drug metabolism, we also identified genes involved in a variety of other key functional categories known to respond to contaminant exposure, including various metabolic pathways, apoptosis, transport, cellular chaperones, and oxidative stress.

#### 3.4.1. Drug metabolism

We identified multiple genes that are specifically involved in drug metabolism, including *CYP3A5*, *CYB5R3*, and *CES2* (Table 4). The *CYP3A5* gene belongs to the family of cytochrome p450 enzymes, and *CYP3A5* and a related gene, *CYP3A4*, encode important drug metabolizing enzymes in the human liver (Deenen et al., 2011). Examples of pollution-associated upregulation within this gene family include a *CYP3A* gene in wild male turbot (*Pleuronichthys verticalis*) from polluted sites along the Southern California coast (Baker et al., 2009) and *CYP3A4* in fathead minnows (*Pimephales promelas*) caged in rivers near highly agricultural areas (Sellin Jeffries et al., 2012). Another *CYP3A* gene, *cyp3a65*, was induced in zebrafish by treatment with municipal effluent and other xenobiotics (Bresolin et al., 2005; Lister et al., 2009; Tseng et al., 2005). These studies indicate that *CYP3A* genes play a key role in drug metabolism in fish, supporting our observation that *CYP3A5* expression was upregulated in largescale suckers with higher contaminant concentrations.

Carboxylesterases, including *CES2*, are another gene family known for their role in xenobiotic metabolism (Wheelock et al., 2005). Studies in fish showed that a variety of environmental toxicants inhibit carboxylesterase activity (Denton et al., 2003; Güngördü et al., 2012; Ru et al., 2003; Wheelock et al., 2005), and we found that the expression of carboxylesterase *CES2* correlated negatively with PCB concentration (Table 4). Finally, studies have shown that toxicant exposure also alters the activity of NADH cytochrome  $b_5$  reductase, which is encoded by the gene *CYB5R3*, but its usefulness as a biomarker has been unclear with some studies showing an increase in hepatic cytochrome  $b_5$  reductase while others show no change or a decrease in enzyme activity (Van der Oost et al., 2003). Our study showed a negative correlation between *CYB5R3* expression and PCB liver tissue concentration (Table 4). Identification of genes known to be involved in drug metabolism suggests that

our analysis is indeed selecting for genes with expression patterns that relate to tissue contaminant concentration in a biologically-relevant manner.

#### 3.4.2. Other metabolic pathways

Expression of multiple genes involved in metabolism of lipids and single genes involved in other metabolic pathways such as vitamin A, catecholamine, glutathione, and protein metabolism and the electron transport chain correlated with liver contaminant concentration. Previous studies of wild fish or fish exposed to complex contaminant mixtures found in nature have shown differential expression of genes involved in lipid metabolism (Falciani et al., 2008; Garcia-Reyero et al., 2009; Moens et al., 2007; Sellin Jeffries et al., 2012) and, in some cases, changes in expression of genes involved in energy generation via the electron transport chain (Falciani et al., 2008; Moens et al., 2007). One metabolic gene of particular interest identified in our study is retinol binding protein 2 (*RBP2*), which is involved in vitamin A metabolism. It has specifically been shown to be upregulated in fathead minnows caged near sewage treatment plants (Garcia-Reyero et al., 2009) and downregulated in zebrafish embryos exposed to polluted sediments (Kosmehl et al., 2012). Similar to Kosmehl et al. (2012), we observed a decrease in *RBP2* expression with increasing contaminant concentration (Table 4). The gene expression response (up- vs. downregulation) may be affected by multiple factors including duration or mode of exposure or the composition of the contaminant mixture, resulting in different responses in different studies.

#### 3.4.3. Apoptosis

We observed a decrease in expression of pro-apoptotic genes as PCB and PBDE concentration increased (Tables 4–5), and a pro-apoptotic gene (*PCBP4*) increased in expression with increasing DCPA tissue concentration (Table 3). Microarray studies in wild European flounder (*Platichthys flesus*) collected from polluted sites and flounder exposed to toxicants such as PCB mixtures in the laboratory have also revealed induction of both pro- and anti-apoptotic pathways (Falciani et al., 2008; Leaver et al., 2010; Williams et al., 2008). Hook et al. (2006) showed upregulation of pro-apoptotic genes and downregulation of anti-apoptotic genes in rainbow trout (*Oncorhynchus mykiss*) exposed to PBDE 47, and Garcia-Reyero et al. (2009) found differential expression of genes involved in apoptosis in fathead minnows caged near sewage treatment plants.

#### 3.4.4. Transport

We identified many genes involved in various forms of cellular transport. Among these were two genes from the ATP-binding cassette family of transporters (*ABCG5* and *ABCG8*) and two solute carrier family members (*SLC39A13* and *SLC46A3*; Table 4). Other genes within sub-family G of the ABC family of transporters are known to be involved in drug transport. The *ABCG5* and *ABCG8* genes encode sterol transporters (Annilo et al., 2006), suggesting that they may not directly be involved in drug transport but rather affect lipid transport. Falciani et al. (2008) found differential expression (both up- and downregulation) of lipid transporters in wild European flounder collected from polluted waters,

**Table 4**

Human homologues, Pearson correlation coefficients ( $r_i$ ), denominator of  $d_i$  ( $s_i + s_0$ ), and  $q$ -values for probes correlated with PCBs organized by functional category. The  $q$ -value is defined as the lowest false discovery rate (FDR) at which the gene is called significant (Storey, 2002). HS, *Homo sapiens*.

Functional category	Probe name	HS gene symbol	HS gene ID	HS gene name	Correlated PCB	$r_i$	$s_i + s_0$	$q$ -value (%)	
Apoptosis	LSS_UF_10449	CASP8	841	Caspase 8, apoptosis-related cysteine peptidase family with sequence similarity 32, member A	177	-0.67	0.30	20	
					174	-0.67	0.32	24	
	LSS_UF_17434	FAM32A	26017		170	-0.81	0.37	0	
					180	-0.77	0.35	0	
					187	-0.71	0.32	26	
					174	-0.69	0.31	24	
	LSS_UF_17946	CDIP1	29965	Cell death-inducing p53 target 1	174	-0.64	0.32	24	
					170	-0.70	0.40	18	
	LSS_UF_18469	WVVOX	51741	WW domain containing oxidoreductase	180	-0.69	0.37	27	
					174	-0.67	0.32	24	
Blood coagulation	LSS_UF_13910	TFPI	7035	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	183	0.73	0.30	28	
Catecholamine metabolism	LSS_UF_17426	MOXD1	26002	Monoxygenase, DBH-like 1	177	-0.80	0.26	0	
					174	-0.65	0.32	24	
Cell adhesion	LSS_UF_22407	HAPLN3	145864	Hyaluronan and proteoglycan link protein 3	183	0.72	0.31	28	
					177	0.71	0.29	20	
Cell communication	LSS_UF_11485	GJA1	2697	Gap junction protein, alpha 1, 43 kDa	183	0.73	0.30	28	
Cell growth	LSS_UF_12553	OCM2	4951	Oncomodulin 2	177	-0.66	0.30	20	
Cell survival	LSS_UF_22085	CYB5D2	124936	Cytochrome b <sub>5</sub> domain containing 2	177	-0.67	0.30	20	
Chaperone	LSS_UF_10469	SERPINH1	871	Serpine peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	183	0.71	0.31	28	
	LSS_UF_17678	HSPB7	27129	Heat shock 27 kDa protein family, member 7 (cardiovascular)	177	-0.71	0.29	12	
					170	-0.67	0.41	18	
Development	LSS_UF_17362	RTTN	25914	Rotatin	174	-0.64	0.32	24	
DNA methylation	LSS_UF_10983	DNMT3B	1789	DNA (cytosine-5-)-methyltransferase 3 beta	177	-0.67	0.30	20	
DNA replication	LSS_UF_12471	NFIX	4784	Nuclear factor I/X (CCAAT-binding transcription factor)	187	-0.71	0.32	26	
					180	-0.69	0.37	27	
					170	-0.68	0.41	18	
Drug metabolism	LSS_UF_10874	CYP3A5	1577	Cytochrome P450, family 3, subfamily A, polypeptide 5	177	0.83	0.25	0	
					183	0.71	0.31	28	
	LSS_UF_10942	CYB5R3	1727	Cytochrome b <sub>5</sub> reductase 3	180	-0.74	0.36	0	
					170	-0.73	0.39	18	
	LSS_UF_14747	CES2	8824	Carboxylesterase 2	187	-0.70	0.32	26	
					174	-0.67	0.32	24	
	LSS_UF_REP_20011	ABP1	26	Amiloride binding protein 1 (amine oxidase copper-containing)	177	-0.77	0.27	0	
					174	-0.71	0.31	24	
					170	-0.75	0.39	18	
					180	-0.68	0.37	27	
Electron transport chain	LSS_UF_10718	COX5B	1329	Cytochrome c oxidase subunit Vb	174	-0.64	0.32	24	
Glutathione metabolism	LSS_UF_10997	DPEP1	1800	Dipeptidase 1 (renal)	170	-0.71	0.40	18	
					174	-0.68	0.32	24	
	LSS_UF_10998	DPEP1	1800	Dipeptidase 1 (renal)	170	-0.67	0.41	18	
					174	-0.65	0.32	24	
Homeostasis	LSS_UF_10049	AGT	183	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	177	0.70	0.29	20	
Immune response	LSS_UF_11982	IRF6	3664	Interferon regulatory factor 6	177	-0.67	0.30	20	
Lipid metabolism	LSS_UF_18005	PLA2G3	50487	Phospholipase A2, group III	170	-0.73	0.39	18	
					180	-0.69	0.37	27	
	LSS_UF_19111	ACER3	55331	Alkaline ceramidase 3	174	-0.66	0.32	24	
	LSS_UF_REP_20019	ACADS	35	Acyl-CoA dehydrogenase, C-2 to C-3 short chain	177	-0.68	0.30	20	
	Protein degradation	LSS_UF_14096	UBE2G1	7326	Ubiquitin-conjugating enzyme E2G 1	177	-0.67	0.30	20
	LSS_UF_18578	KCTD5	54442	Potassium channel tetramerization domain containing 5	174	-0.64	0.32	24	
Protein metabolism	LSS_UF_REP_20028	ACP1	52	Acid phosphatase 1, soluble	174	-0.65	0.32	24	
Protein processing	LSS_UF_15784	USPL1	10208	Ubiquitin specific peptidase like 1	177	0.75	0.28	20	
LSS_UF_18245	ZDHHC3	51304	Zinc finger, DHHC-type containing 3	174	-0.74	0.30	24		
				177	-0.66	0.30	20		
Reproduction	LSS_UF_14297	ZP3	7784	Zona pellucida glycoprotein 3 (sperm receptor)	183	0.76	0.29	28	
RNA processing	LSS_UF_15588	THRAP3	9967	Thyroid hormone receptor associated protein 3	177	0.71	0.29	20	
Signaling	LSS_UF_19628	TWSG1	57045	Twisted gastrulation homolog 1 (Drosophila)	170	-0.69	0.40	18	
	LSS_UF_21791	ARHGAP12	94134	Rho GTPase activating protein 12	183	0.73	0.30	28	
	LSS_UF_22542	RASGEF1B	153020	RasGEF domain family, member 1B	183	0.71	0.31	28	
	LSS_UF_REP_20046	ACVRL1	94	Activin A receptor type II-like 1	183	0.72	0.31	28	
	Synapse organization	LSS_UF_17456	LRRTM2	26045	Leucine rich repeat transmembrane neuronal 2	177	-0.66	0.30	20
Translation	LSS_UF_24061	CSDE1	7812	Cold shock domain containing E1, RNA-binding	170	-0.68	0.41	18	
Transport	LSS_UF_10156	ARF6	382	ADP-ribosylation factor 6	170	-0.76	0.39	18	
					187	-0.74	0.31	26	
	LSS_UF_10614	CHRND	1144	Cholinergic receptor, nicotinic, delta	183	0.77	0.29	28	
				177	0.73	0.28	20		

(continued on next page)

Table 4 (continued)

Functional category	Probe name	HS gene symbol	HS gene ID	HS gene name	Correlated PCB	$r_i$	$s_i + s_0$	q-value (%)
	LSS_UF_10647	CLN3	1201	Ceroid-lipofuscinosis, neuronal 3	174	-0.76	0.30	24
	LSS_UF_13796	SYNPR	132204	Synaptoporin	177	-0.68	0.30	20
	LSS_UF_16332	TMED1	11018	Transmembrane emp24 protein transport domain containing 1	183	0.73	0.30	28
					177	0.71	0.29	20
	LSS_UF_20153	ABCG5	64240	ATP-binding cassette, sub-family G (WHITE), member 5	174	-0.65	0.32	24
	LSS_UF_20155	ABCG8	64241	ATP-binding cassette, sub-family G (WHITE), member 8	170	-0.71	0.40	18
	LSS_UF_20156	ABCG8	64241	ATP-binding cassette, sub-family G (WHITE), member 8	174	-0.66	0.32	24
	LSS_UF_21618	SLC39A13	91252	Solute carrier family 39 (zinc transporter), member 13	177	0.71	0.29	20
	LSS_UF_22336	TMEM37	140738	Transmembrane protein 37	170	-0.67	0.41	18
	LSS_UF_23096	SLC46A3	283537	Solute carrier family 46, member 3	177	-0.72	0.29	12
					174	-0.71	0.31	24
					170	-0.68	0.40	18
Vitamin A metabolism	LSS_UF_13210	RBP2	5948	Retinol binding protein 2, cellular	177	-0.83	0.26	0
					174	-0.66	0.32	24
Unknown	LSS_UF_18074	GLOD4	51031	Glyoxalase domain containing 4	177	-0.72	0.29	12
					170	-0.69	0.40	18
	LSS_UF_19859	DIP2C	22982	DIP2 disco-interacting protein 2 homolog C (Drosophila)	183	0.73	0.30	28
	LSS_UF_20471	C17orf62	79415	Chromosome 17 open reading frame 62	183	0.72	0.31	28
	LSS_UF_21501	Unknown			177	-0.69	0.29	20
	LSS_UF_21577	C15orf57	90416	Chromosome 15 open reading frame 57	177	-0.66	0.30	20
	LSS_UF_22154	TATDN3	128387	TatD DNase domain containing 3	170	-0.68	0.40	18

and others have shown enrichment of genes involved in steroid biosynthesis and steroid receptor signaling in the liver of fathead minnows caged near sewage treatment plants or in rivers with high agricultural inputs (Garcia-Reyero et al., 2009; Sellin Jeffries et al., 2012).

Expression of the solute carriers *SLC39A13* and *SLC46A3* correlated with PCB concentration in largescale suckers. Exposure of embryonic zebrafish to a variety of environmental toxicants, including PCBs, caused differential regulation of multiple members of the solute carrier family, suggesting that these transporters do respond to contaminant exposure in fish (Yang et al., 2007). *SLC39A3*, another *SLC39* family member, was specifically upregulated in zebrafish exposed to polluted sediments containing various heavy metals and organic pollutants (Kosmehl et al., 2012). *SLC39A13* is a zinc transporter, and upregulation of *SLC39A13* in largescale suckers from the Columbia River could indicate a response to trace element concentrations that are correlated to PCB concentrations rather than a direct response to PCB exposure.

### 3.4.5. Oxidative stress and chaperones

Genes involved in oxidative stress and encoding cellular chaperones have been repeatedly identified in toxicogenomic studies of wild fish from polluted sites and fish exposed to toxicants in the laboratory (Falciani et al., 2008; Hook et al., 2006; Kosmehl et al., 2012; Moran et al., 2007; Williams et al., 2003, 2008; Yang et al., 2007). Expression of two genes involved in the oxidative stress response (sirtuin 3 [*SIRT3*] and paraoxonase 2 [*PON2*]) decreased with increasing PBDE tissue

concentrations (Table 5). Williams et al., 2003 showed an increase in *PON2* in European flounder from a polluted estuary, and other studies (Falciani et al., 2008; Yang et al., 2007) have typically found activation of oxidative stress genes in fish exposed to contaminants. It is unclear why *SIRT3* and *PON2* decreased with increasing contaminant concentration, but it could be due to the length of exposure or specific chemical mixtures to which our fish were exposed. In the case of cellular chaperone proteins, some studies (Kosmehl et al., 2012; Yang et al., 2007) have observed upregulation of genes encoding these proteins in fish exposed to contaminants while others (Falciani et al., 2008) showed downregulation. Taken together, these results suggest that gene expression profiles can vary depending on the species, length of exposure, and type of chemical exposure. We identified one chaperone (*SERPINH1*) that correlated positively with PCB concentration and another chaperone (*HSPB7*) that correlated negatively with PCB concentration (Table 4).

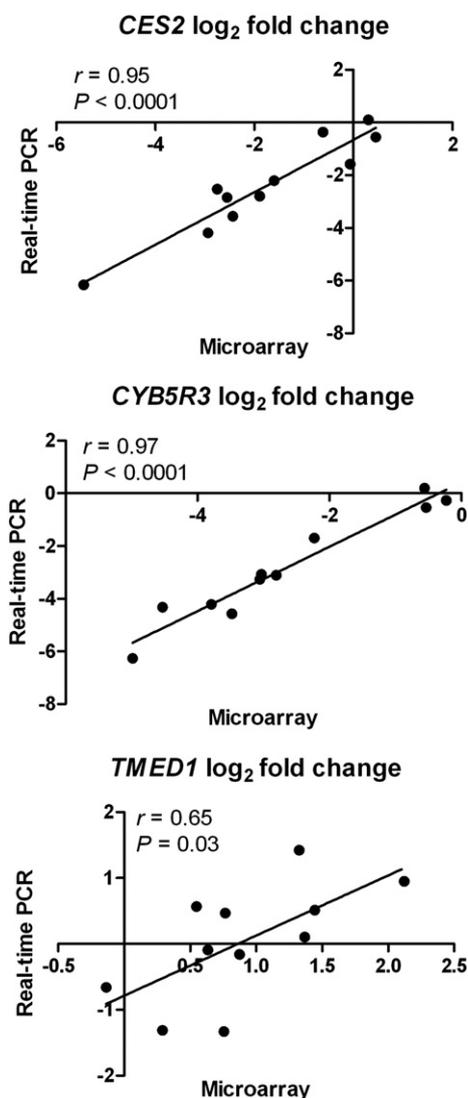
Our study was not designed to provide a comprehensive view of biological pathways affected by specific contaminants as could be accomplished in a laboratory study with controlled exposures. Instead, we conducted a field-based exploratory analysis of largescale suckers and identified contaminant-responsive genes from a variety of functional categories. Our approach allowed us to quickly screen for both known and novel contaminant-responsive genes and is a preliminary step in discovering gene indicators in wild fish exposed to contaminant mixtures in the lower Columbia River.

Table 5

Human gene homologues, Pearson correlation coefficients ( $r_i$ ), denominator of  $d_i$  ( $s_i + s_0$ ), and q-values for probes correlated with PBDEs. The q-value is defined as the lowest false discovery rate (FDR) at which the gene is called significant (Storey, 2002).

HS, *Homo sapiens*.

Contaminant	Probe name	HS gene symbol	HS gene ID	HS gene name	Functional category	$r_i$	$s_i + s_0$	q-value (%)
PBDE 100	LSS_UF_12888	PPP3R1	5534	Protein phosphatase 3, regulatory subunit B, alpha	Signaling	0.75	0.39	0
	LSS_UF_18245	ZDHHC3	51304	Zinc finger, DHHC-type containing 3	Protein processing	-0.74	0.39	0
PBDE 154	LSS_UF_10156	ARF6	382	ADP-ribosylation factor 6	Transport	-0.77	0.38	22
	LSS_UF_17434	FAM32A	26017	Family with sequence similarity 32, member A	Apoptosis	-0.72	0.39	22
	LSS_UF_17620	SEC22A	26984	SEC22 vesicle trafficking protein homolog A ( <i>S. cerevisiae</i> )	Transport	-0.72	0.39	22
	LSS_UF_17071	SIRT3	23410	Sirtuin 3	Oxidative stress	-0.72	0.40	22
	LSS_UF_19628	TWGS1	57045	Twisted gastrulation homolog 1 (Drosophila)	Signaling	-0.71	0.40	22
	LSS_UF_20584	ECHDC3	79746	Enoyl CoA hydratase domain containing 3	Unknown	-0.71	0.40	22
	LSS_UF_22336	TMEM37	140738	Transmembrane protein 37	Transport	-0.71	0.40	22
	LSS_UF_20273	KLC2	64837	Kinesin light chain 2	Transport	-0.71	0.40	22
	LSS_UF_12825	PON2	5445	Paraoxonase 2	Oxidative stress	-0.70	0.40	22



**Fig. 3.** Real-time PCR confirmation of microarray results. Gene expression for 11 largescale suckers, as measured by real-time PCR and microarray, was expressed as the log<sub>2</sub> fold change relative to a fish with a low contaminant concentration. Pearson correlation ( $r$ ) was used to determine the significance of the linear relation.

### 3.5. Field versus laboratory toxicology studies and their role in biomarker identification

Controlled laboratory studies have identified many genes that respond to toxicant exposure. We identified some of these same genes but did not detect changes in the expression of other genes for potentially multiple reasons. First, the sensitivity of our analysis may not be adequate to detect subtle gene–environment interactions due to restricted sample sizes. Studies using large samples will be required to further validate our list of genes correlated to contaminant concentration and to tease out more subtle gene expression changes. Second, wild fish are exposed daily to complex mixtures of contaminants from many sources, and although each individual compound may be at relatively low levels, the combined effects due to the interactions between many contaminants could be profound and distinctly different from the effects of the individual contaminants at higher concentrations. For example, Leaver et al. (2010) showed that European flounder chronically exposed to polluted sediments did not have expected transcriptional alterations in common environmental biomarkers. They could evoke the expected transcriptional response, however, by treating with a single contaminant, suggesting that biomarkers developed from

laboratory exposures with single compounds may not be effective in identifying biological effects of contaminant exposure in a complex environment. In a separate study, Garcia-Reyero et al. (2011) compared the gene expression profiles of fathead minnows exposed to estrogenic wastewater treatment plant effluent with those exposed to a single estrogen or androgen. Although similar in some respects, the overall gene expression profile of fish exposed to effluent was different from that of fish exposed to a single compound again demonstrating that complex contaminant mixtures differ in their biological effects from single contaminants.

Another difference between the exposure of laboratory and wild fish to contaminants is the duration of the exposure. Wild fish are often exposed to contaminant mixtures for many years. Chronic exposure of wild fish to contaminants may have different effects than short-term exposure of laboratory cells or animals to toxic compounds. Thus, some biological effects of contaminants may be attenuated after long-term exposure, and others could be augmented.

Because laboratory studies do not necessarily mimic the environmental factors and conditions that wild fish experience, it is imperative that field studies be incorporated into the process of biomarker development for contaminant monitoring in at-risk watersheds. Here, we developed a tool for contaminant research that is well-suited to field studies since our array targeted an ecologically-relevant fish species that can be used for contaminant monitoring in the wild. Moreover, just as the Atlantic salmon (*Salmo salar*) array developed by Koop et al. (2008) cross-hybridizes with a wide variety of other salmonid species, our array may be useful for other sucker species used for contaminant research such as the white sucker (*Catostomus commersonii*) and for endangered sucker species such as the shortnose sucker (*Chasmistes brevirostris*) and the Lost River sucker (*Deltistes luxatus*).

### 3.6. Conclusions

We have demonstrated how microarrays can be used to identify genes with expression patterns that correlate with contaminant exposure. The genes identified in this study reflect the contaminant exposure response at only three sites in the Columbia River and in one tissue type. Future work should focus on describing the gene expression profiles in a variety of different tissues from large numbers of largescale suckers at many sites in the Columbia River to identify genes with expression profiles that correlate consistently with contaminant concentration. Specifically, identification of gene expression biomarkers in tissues that can be collected in a non-lethal manner would be highly useful for monitoring the biological effects of contaminant exposure. Concurrent collection of other biological information such as condition factor and reproductive health would also provide insight into the link between the gene response to contaminants and other measurable indicators of fish health and fitness. Laboratory studies could then be used to confirm that the gene responses observed in the field are indeed due to contaminant exposure and not other environmental factors. Contaminant-responsive genes discovered in field studies and then confirmed in the laboratory will have excellent potential as biomarkers for monitoring of contaminant exposure in largescale suckers across the Columbia Basin, and a similar model of discovery and validation using the array developed in this study could be used to identify biomarkers of contaminant exposure in other sucker species from other at-risk watersheds.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2013.08.034>.

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