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Transcriptional responses of male fathead minnows exposed to oil sands process-affected water

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ABSTRACT

Oil sands process-affected water (OSPW) is produced by the oil sands industry in Alberta, Canada. OSPW has acute and chronic effects on aquatic organisms, but the suite of effects of OSPW, and mechanisms of effects, are not understood. The goal of this study was to use RNA sequencing (RNAseq) to quantify abundances of transcripts in livers of male fathead minnows exposed to untreated OSPW and ozone-treated OSPW to investigate sublethal effects of untreated OSPW and to determine whether ozonation imparts toxicity upon OSPW. A reference transcriptome of 25,342 contigs was constructed from RNA from livers of fathead minnows exposed to various experimental conditions. Exposure to untreated OSPW resulted in greater abundances of 104 transcripts and lesser abundances of 91 transcripts. Oxidative metabolism, oxidative stress, apoptosis, and immune function were identified as processes affected by OSPW. Exposure to ozone-treated OSPW resulted in greater abundances of 57 transcripts and lesser abundances of 75 transcripts. However, in general, putative pathways for effects of OSPW in fathead minnows exposed to untreated OSPW were not identified in minnows exposed to ozone-treated OSPW, and pathways by which ozone-treated OSPW might have effects were not identified.

1. Introduction

Oil sands process-affected water (OSPW) is a by-product of extraction of bitumen from oil sands in Alberta, Canada. OSPW is a mixture of water, residual bitumen, silts, clays, and other inorganic and organic constituents. The water-soluble organic fraction of OSPW is a toxic component of OSPW (Garcia-Garcia et al., 2011a, 2011b; Anderson et al., 2012a, 2012b; Het et al., 2012a). Naphthenic acids (NAs) are one primary persistent organic constituent of the water soluble organic fraction of OSPW. The NAs are carboxylic acids with the general formula $C_{nH2n+z}O_2$, where n indicates the number of carbons and z is the number of rings (Holowenko et al., 2002).

Effects of OSPW on aquatic organisms are not well characterized. OSPW caused endocrine disrupting effects as evidenced by lesser concentrations of testosterone (T) and estradiol (E2) in blood plasma from several species of fish (Van den Heuvel et al., 1999; Lister et al., 2008; Van den Heuvel et al., 2012) and lesser synthesis of T and E2 by gonadal explants from fish (Lister et al., 2008). In fathead minnows (*Pimephales promelas*), exposure to OSPW altered gene expression along the brain-gonad-liver (BGL) axis of males and females (He et al., 2012b), impaired reproduction, and impaired development of secondary sex characteristics (Kavanagh et al., 2011, 2012). Histopathological changes in gills and livers from yellow perch (*Perca flavescens*) and goldfish (*Carassius auratus*) exposed to OSPW have been reported (Nero et al., 2006a). Proliferation of mucous, epithelial, and chloride cells were observed in gills of yellow perch exposed to OSPW (Nero et al., 2006b). Early life stages of fathead minnows, yellow perch, and Japanese medaka (*Orizias latipes*) exposed to OSPW had greater incidences of deformities and lesser length at hatch (Peters et al., 2007; He et al., 2012a). Impairment of immune function has also been observed in fish exposed to OSPW (van den Heuvel et al., 2000; McNeill et al., 2012). In addition to effects on fish, OSPW impairs survival, growth, and development of the benthic invertebrate, the midge, *Chironomus dilutus* (Anderson et al., 2012a, 2012b).

Greater than 1 billion m^3 of OSPW is stored on-site of several companies operating in this region (Del Rio et al., 2006). Eventually

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this OSPW needs to be remediated and reclaimed as viable aquatic habitats or released to the receiving environment. For OSPW to be reclaimed, it is essential that toxicity of the water-soluble organic fraction, including NAs, be reduced. Currently this is achieved by aging OSPW in tailing ponds to allow for microbial degradation of the NAs (Holowenko et al., 2002; Del Rio et al., 2006; Han et al., 2009). However, there is a fraction of NAs with greater molecular weights and numbers of rings that are resistant to biodegradation (Martin et al., 2010). Consequently, aging is ineffective in completely abolishing the toxicity of OSPW. Concentrations of NAs in aged OSPW might gradually be diluted to a point where they are no longer sufficiently toxic to preclude development of at least rudimentary aquatic communities, but to effectively attenuate toxicity of OSPW a treatment approach that can target the water soluble organic fraction would be useful. Ozonation has been identified as a potentially effective treatment method (Gamal El-Din et al., 2011; Perez-Estrada et al., 2011). Ozone preferentially degrades those NAs in OSPW that are resistant to biodegradation (Martin et al., 2010). OSPW that has been treated with ozone is not acutely toxic towards the bacterium, *Vibrio fischeri* as measured by the Microtox® assay (Scott et al., 2008; Gamal El-Din et al., 2011), causes fewer endocrine disrupting effects (He et al., 2010, 2011, 2012b), has lesser toxicity towards *C. dilutus* (Anderson et al., 2012a) and embryos of fathead minnows (He et al., 2012b), and has lesser immunotoxic effects (Garcia-Garcia et al., 2011b).

Potential mechanisms of toxicity of OSPW have received little attention. Because NAs are surfactants OSPW might be acutely toxic because of physical disruption of membranes through a mechanism of polar narcosis (Roberts, 1991; Frank et al., 2008). However, because of the complexity of the mixture, it can be hypothesized that OSPW might exert effects via multiple mechanisms of action. Understanding the effects of OSPW on aquatic organisms is required to effectively establish methods of assessing and predicting the toxicity of OSPW. Because OSPW is a complex mixture and identities and structures of many of the organic compounds are not known, it is impossible to accurately perform targeted identification of effects. Open-format nucleic acid sequencing technologies are ideally suited to situations where the suite of effects is unknown because effects that are manifested at the cellular, tissue, or whole organism level are often due to effects on gene expression. The goal of this study was to use RNAseq to investigate effects of exposure to untreated OSPW and ozone-treated OSPW on abundances of transcripts to gain insight into effects of OSPW and further establish the usefulness of ozonation as a method for lessening toxicity of OSPW.

2. Materials and methods

2.1. Establishment of a reference transcriptome

2.1.1. RNA isolation, cDNA library construction, and high-throughput sequencing

Neither the genome nor transcriptome of the fathead minnow has been sequenced. Therefore, to determine the effects of exposure to untreated OSPW and ozone-treated OSPW on abundances of transcripts in livers from fathead minnows a reference transcriptome of the liver of this species had to be constructed. Total RNA was isolated from livers of fathead minnows using a Qiagen RNeasy Plus Mini Kit according to the manufacturer's instructions (Qiagen, Mississauga, ON, Canada) and quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The integrity of all RNA samples was determined at the National Research Council of Canada Plant Biotechnology Institute (NRC-PBI, Saskatoon, SK, Canada), by use of an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples of RNA were stored at −80 °C.

The reference transcriptome was constructed from 16 samples of RNA isolated from livers of fathead minnows. Total RNA was isolated from one sexually immature fathead minnow exposed to dechlorinated city of Saskatoon municipal tap water, untreated OSPW, or ozone-treated OSPW for three days. Untreated OSPW and ozone-treated OSPW used for this exposure were not the same as the waters used for the RNAseq experiment that is described in the next section. These three samples were sequenced on individual flow cells of an Illumina Genome Analyzer IIx, using 75-bp single end reads, at NRC-PBI. Total RNA was isolated from nine sexually maturing male fathead minnows exposed in triplicate to dechlorinated city of Saskatoon municipal tap water, untreated OSPW, or ozone-treated OSPW for 7 days. As is described below, these samples were also used for quantification of abundances of transcripts by RNAseq. Finally, total RNA was isolated from livers from four fathead minnows exposed in duplicate to dechlorinated city of Saskatoon municipal tap water maintained at either 4 °C or 20 °C for 6 months. These 13 samples were sequenced by use of an Illumina HiSeq™ 2000 sequencer at the NRC-PBI by use of 100-bp paired-end sequencing. Sequence determination and quality value calculations were performed at the NRC-PBI by use of the Illumina pipeline according to the method of the manufacturer. Raw sequence files from all 16 samples have been deposited to the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI).

2.1.2. De novo assembly and annotation of the reference transcriptome

Contigs (contiguous, overlapping sequences assembled from individual sequencing reads) for the reference transcriptome were assembled by use of CLC genomics workbench v5.0 (CLC Bio, Aarhus, Denmark). The algorithm for de novo assembly in the CLC genomics workbench uses de Bruijn graphs. Prior to assembly all sequences were filtered by eliminating sequences of poor quality, including filtering sequences with greater than 2 unknown nucleotides. Sequences shorter than 50 bp were excluded from the assembly. Conflicts among bases in assembled contigs were resolved by voting for the base with the maximum number of occurrences among reads being assembled into that contig. The minimum contig length was set at 200 bp. Contigs comprising the reference transcriptome were annotated by use of Blast2GO v2.5.0 software (Conesa et al., 2005). Identities of contigs were determined by use of BLASTX against sequences in the NCBI non-redundant protein database. A minimum E-value of 10^{-5} and a minimum coverage length of ≥33 amino acids were used as the threshold for determination of sequence identities.

2.2. Effect of OSPW and ozone-treated OSPW on abundances of transcripts

2.2.1. Description of OSPW

The OSPW that was used in the exposure for the RNAseq study was collected in February 2010 from the West-In-Pit (WIP), an active settling pond on the site of Syncrude Canada Ltd. (Fort McMurray, AB, Canada). The WIP-OSPW is fresh, untreated process water from the main bitumen extraction plant, characteristics of which have been described previously (Han et al., 2009). Total concentration of NAs, as operationally determined by ultra-pressure liquid chromatography high-resolution mass spectrometry (UPLC-HRMS) was 19.7 mg/L (Wang, 2011). Ozonation of OSPW was conducted at the University of Alberta (Edmonton, AB, Canada) by using a semi-batch ozonation system and following a standard protocol as described elsewhere (Gamal El-Din et al., 2011; Wang, 2011). Ozonation was continued until approximately 90% degradation of parent NAs, as determined by the remaining sum response of all UPLC-HRMS peak area corresponding to NAs, was achieved. The total concentration of NAs in the ozone-treated OSPW was 1.9 mg/L. The effect of ozonation on the distribution of the NAs is described in Wang (2011).

2.2.2. Experimental exposure

Fish were exposed in the Aquatic Toxicology Research Facility (ATRF) in the University of Saskatchewan's Toxicology Centre and the

protocol was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use (protocol # 20080093). Exposures were conducted in 25 L aquaria containing 20 L of city of Saskatoon municipal tap water, undiluted untreated OSPW, or undiluted ozone-treated OSPW. The water temperature in each tank was 23 ± 1 °C and a 16 h day: 8 h night photoperiod was used. Exposures were conducted in duplicate aquaria and consisted of six male and six female fathead minnows randomly assigned to the two tanks from a stock maintained within the ATRF. The exposure was continued for 7 days and half the volume of water was changed daily. Minnows were fed approximately 2% body weight of frozen blood worms once daily. At the end of the exposure minnows were netted and immediately anesthetized with 150 mg/L MS-222. Livers were collected from each fish and frozen at −80 °C until needed for RNAseq.

2.2.3. RNAseq, functional annotation of differentially expressed transcripts, and statistics

To avoid sex-specific effects on abundances of transcripts three randomly selected male minnows from each exposure were used for the RNAseq. Total RNA was isolated and quantified and construction of cDNA libraries for sequencing was conducted as described above. Samples were sequenced on an Illumina HiSeq™ 2000 sequencer at the NRC-PBI by use of 100-bp paired-end sequencing. Samples were also used in construction of the reference transcriptome. Reads were filtered (as described above) and were mapped to the reference transcriptome using CLC genomics workbench v5.0. Abundances of transcripts were determined by using the RPKM method (Reads Per Kilobase of transcript per Million mapped reads) (Mortazavi et al., 2008). Fold changes in abundances of transcripts in livers of fathead minnows exposed to untreated OSPW or ozone-treated OSPW were determined relative to the freshwater control.

The following criteria were used for the RNAseq analysis. Only when a minimum of 5 reads from each of the three samples in at least one of the two treatments being compared were successfully mapped to the reference transcriptome was that contig used in the RNAseq. For any contig, if reads were present in each of the three samples of a condition then it did not matter if reads were present in any of the three samples from the other condition. In some instances reads were present in all three samples of one condition but in neither of the three reads form the other condition. In these instances the change in abundance of transcripts was infinite but a value of >23.3-fold was assigned because this was the greatest change in abundance of transcripts that was determined from instances where both conditions had reads that mapped to the reference transcriptome. Data were normalized by using quantile normalization and statistical analysis of RNAseq data was performed using Baggerley's test (Baggerly et al., 2003) by use of CLC Genomics Workbench 5.0. To correct for multiple comparisons a false discovery rate of 5% was used and corrected p-values <0.05 were considered statistically significant. Only contigs whose abundance was significantly ($p<0.05$) 1.5-fold greater or lesser than in the control were treated as being biologically relevant. Contigs whose expression was significantly altered by exposure to either untreated OSPW or ozone-treated OSPW, relative to the freshwater control, were functionally annotated using gene ontology (GO) terms and Kyoto Encyclopedia of Genes and genomes (KEGG) mapping using Blast2GO v2.5.0 software (Conesa et al., 2005).

2.2.4. Quantitative real time PCR and statistics

Quantitative real-time PCR (qPCR) was performed on a subset of genes to determine if quantification of abundances of transcripts by RNAseq was reliable. RNA samples used for qPCR included those used for the RNAseq experiment and 3 additional samples of RNA isolated from livers of male minnows. The 6 samples analyzed in the qPCR experiment were comprised of 3 samples from each of the duplicate exposure tanks and there were no significant differences in abundance of transcripts between fish from different tanks (data not shown). Purified RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). Purified RNA samples were stored at −80 °C. First-strand cDNA synthesis was synthesized from 1 μg of RNA using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. The cDNA samples were stored at −80 °C.

The qPCR was performed in 96-well PCR plates by use of an ABI 7300 Real-Time PCR System (Applied Biosystems). Sequences of gene-specific PCR primers are shown (Table 1). A separate 45 μL PCR reaction mixture consisting of Quantitect SYBR Green PCR master mix (Qiagen), cDNA, gene-specific primers, and nuclease free water was prepared for each cDNA sample and primer pair. A final reaction volume of 20 μL was transferred to each well and reactions were performed in duplicate. The PCR reaction mixture was denatured at 95 °C for 10 min before the first PCR cycle. The thermal cycle profile was as follows: denature for 10 s at 95 °C and extension for 1 min at 60 °C for a total of 40 PCR cycles. The qPCR cycle was followed by a dissociation step to validate amplification of a single product. Abundances of transcripts of target genes were normalized to abundance of transcripts of ribosomal protein L8 (*rpl8*) and quantified according to the Mean Normalized Expression (MNE) method of Simon (2003).

3. Results and discussion

3.1. Assembly and annotation of the reference transcriptome

The reference transcriptome was developed so RNAseq could be conducted. A total of 877,829,873 reads from the 16 samples used for construction of the reference transcriptome were *de novo* assembled into 62,103 contigs of 200 bp or greater. The maximal contig size was 20,818 bp and the mean contig size was 1,250 bp. The N50 value, which is the length for which 50% of the sequences in the assembly are in a contig of this length or greater, was 2,095 bp. Annotation of 62,103 contigs against the NCBI non-redundant (nr) protein database using BLASTX identified 25,342 contigs with an e-value of $\leq 10^{-5}$. These contigs were used as the reference transcriptome.

3.2. Effects of untreated OSPW

Neither untreated OSPW nor ozone-treated OSPW caused lethality to fathead minnows during the 7-day exposure, and there were no obvious signs of distress such as an inability to maintain body position in the water column or changes in feeding behavior. Therefore, changes in abundances of transcripts in livers of fathead minnows exposed to untreated OSPW or ozone-treated OSPW might not be indicative of acute toxicity but might be indicators of sublethal effects. A greater number of transcripts were of significantly greater or lesser abundance in livers of fathead minnows exposed to untreated OSPW compared to ozone-treated OSPW. Exposure to untreated OSPW resulted in greater abundances of 104 transcripts (Table S1) and lesser abundances of 91 transcripts (Table S2). Exposure to ozone-treated OSPW resulted in greater abundances of 57 transcripts (Table S3) and lesser abundances of 75 transcripts (Table S4). In general, there was very little overlap between the two exposures in transcripts that were of greater or lesser abundances. This leads to the conclusion that the dissolved organic fraction of OSPW is driving many of the transcriptional responses because ozonation decreases concentrations of dissolved organic compounds, including NAs, in OSPW (Scott et al., 2008; Gamal El-Din et al., 2011; Perez-Estrada et al., 2011). This result also supports other studies that demonstrate that ozonation attenuates effects of OSPW (Scott et al., 2008; He et al., 2010; Garcia-Garcia et al., 2011a,b; He et al., 2011; Anderson et al., 2012a; He et al., 2012a,b). The GO terms and KEGG assignments of the transcripts that were of greater of lesser abundances in livers of fathead minnows exposed to untreated OSPW were analyzed

Table 1
Sequences of primers used for qPCR.

Gene name	Forward primer	Reverse primer	Efficiency
Apoptosis-inducing factor 3 (*aif-3*)	GCGTATCATGCTGGACTTCA	TTAAACTGGCCCACTCCATC	1.92
Glutathione synthase (*gs*)	CGGTCCAGAACTACCTGCTC	ACACCACGTCTTTGCCTTTC	1.88
Glutaredoxin reductase (*gr*)	GTCCTACTGTGCCGATTGGT	GTCCAGGCGTTTATGCAGTT	1.85
Cytochrome P450 1A (*cyp1a*)	CCTGCAGGGAGAACTGAG	TCGACGTACAGTGAGGGA	1.85
Cytochrome P450 2k6 (*cyp2k6*)	AACATGTTTCCTTGGCTTCG	GATTCCTCGTCCTTCTGCTG	1.94
Cytochrome P450 2AD2 (*cyp2ad2*)	ATTTGGACGGTCAACGAAAC	AGCCTAAGAGTGGGCAGTGA	1.93
Glutathione-s-transferase (mitochondrial) (*gst-m*)	TCTGGTGCGCTCTTTGAATA	TCTCGGGCTAAAAGTTGGTG	1.93
Multidrug resistance associated protein-2 (*mrp-2*)	CAGGGCTCCAAAGACACCTA	GGCCATCATTGGTCAAAATC	1.93
Ribosomal protein L8	CTCCGTCTTCAAAGCCCATGT	TCCTTCACGATCCCCTTGATG	2.01

to gain insight into sublethal effects of OSPW. These processes are discussed below.

3.2.1. Biotransformation/detoxification pathways

Exposure to untreated OSPW affected abundances of transcripts of enzymes that metabolize endogenous and exogenous substrates. Abundances of transcripts of the cytochrome P450 (CYP) genes *cyp1a*, *cyp2j28*, *cyp2ad2*, *cyp2k6*, and *cyp2k19* were greater (Table 2). Cytochrome P450s are phase-I enzymes that catalyze the oxidative metabolism of endogenous and exogenous substrates. Greater CYP1A enzyme activity (ethoxyresorufin *O*-dethylase, EROD) has been quantified in fish exposed to sediment from OSPW tailing ponds and bitumen (Colavecchia et al., 2004, 2006, 2007; McNeill et al., 2012) and abundance of transcripts of *cyp1a* was greater in primary cultures of rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to OSPW (Gagné et al., 2012). However, abundance of transcripts of *cyp1a* was not greater in embryos of fathead minnows exposed to the OSPW used in this study (He et al., 2012a). Members of the CYP2 subfamily metabolize a variety of endogenous and exogenous substrates (Buhler and Wang-Buhler, 1998; Scarborough et al., 1999; Thibaut et al., 2002; Oleksiak et al., 2003), including T and the synthetic estrogen mimic nonylphenol. This study is the first to demonstrate greater abundance of transcripts of a *cyp2* in fish exposed to OSPW. In addition to greater abundances of transcripts of CYPs, abundances of transcripts of aldehyde oxidase I (*ao1*), an aldehyde dehydrogenase-2 like protein (*aldh2*), monoamine oxidase (*moa*), and cytosolic epoxide hydrolase (*eh*) were greater (Table 2).

Table 2
Fold-changes in abundances of transcripts of genes involved in the metabolism of xenobiotic compounds. Gene names, abbreviations, fold-change in abundance of transcripts determined by RNAseq, and gene ontology numbers of biological processes are given.

Transcript	Fold change (RNAseq)	GO ID
Cytochrome P450 1A (*cyp1a*)	2.1	0008152
Cytochrome P450 2k19 (*cyp2k19*)	11.7	005114
Cytochrome P450 2k6 (*cyp2k6*)	10.1	005114
Cytochrome P450 2AD2 (*cyp2ad2*)	2.7	005114
Cytochrome P450 2J28 (*cyp2j28*)	2.2	005114
Uridine diphospho-glucuronosyltransferase 2a3 (*ugt2a3*)	6.3	0008152
Uridine diphospho-glucuronosyltransferase 5F1 (*ugt5f1*)	−4.3	0008152
Sulfotransferase 1,3 (*sult1-3*)	1.8	0006805
Glutathione-s-transferase (mitochondrial) (*gst-m*)	4.5	0016740
Glutathione-s-transferase (cytosolic) (*gst-c*)	>23.3	0016740
Multidrug resistance associated protein-2 (*mrp-2*)	3.3	0006855
Aldehyde oxidase 1 (*ao*)	3.1	0055114
Aldehyde dehydrogenase (*aldh*)	3.6	0006805
Monoamine oxidase (*moa*)	3.2	0055114
Epoxide hydrolase (*eh*)	2.0	0006805
Basic transcription element binding protein 1 (*bteb-1*)	−3.7	0006351
Nuclear factor (erythroid-derived 2)-like 2 (*nrf2*)	1.8	0034599

Epoxide hydrolases catalyze the hydration of chemically reactive epoxides that are formed by the P450 cataylzed metabolism of substrates (Fretland and Omiecinski, 2000; Shimada and Fujii-Kuriyama, 2004). Epoxides might have been formed by the biotransformation of organics by CYP enzymes. Expression of *MOA* is upregulated by E2 (Sarabia and Liehr, 1998) and some organic compounds in OSPW have structures similar to E2 (Rowland et al., 2011). It is not known if enzyme products of these transcripts biotransform organic compounds in OSPW and what effects this might have on the toxicity of OSPW.

Biotransformation and detoxification of chemicals are also dependent on Phase II and Phase III reactions. Phase II enzymes include glutathione-*S*-transferase (GST) that catalyzes conjugation of reduced glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH) to reactive electrophiles of endogenous and exogenous compounds, uridine diphosphoglucuronosyl transferases (UGT) that transfer a glucuronic acid moiety to substrates, and sulfotransferases (SULT) that conjugate sulfate groups to substrates (Xu et al., 2005). Abundances of transcripts of microsomal GST (*gst-m*) and cytosolic GST (*gst-c*) were greater in livers of fathead minnows exposed to untreated OSPW (Table 2). This is consistent with greater abundance of transcripts of *gst* in rainbow trout hepatocytes exposed to OSPW (Gagné et al., 2012). Abundances of transcripts of uridine diphospho-glucuronosyltransferase 2A3 (*ugt2a3*), and sulfotransferase 1 isoform 3 (*sult1,3*) were greater in livers of fathead minnows exposed to untreated OSPW (Table 2). Abundance of transcripts of *ugt5f1*, another member of the UGT family of proteins, was lesser (Table 2) suggesting that expression of the *UGT* family of genes might be differentially regulated in livers of fathead minnows exposed to untreated OSPW. Greater abundance of transcripts of *sult 1,3* is interesting because promoter regions of *SULT* genes contain estrogen responsive elements and estrogenic compounds have been identified in OSPW (He et al., 2011; Rowland et al., 2011). Expression of SULT1,3 in male fish might be protective against estrogenic compounds (Assem et al., 2006). Substrates of Phases I and II metabolism are excreted from cells by multidrug resistance associated protein (MRP) (Borst et al., 1999). Abundance of transcripts of *mrp2* was greater in livers from fathead minnows exposed to untreated OSPW (Table 2). Greater abundance of transcripts of MRP was quantified in rainbow trout hepatocytes exposed to OSPW (Gagné et al., 2012).

Greater abundances of transcripts that encode enzymes which biotransform endogenous and exogenous compounds provide insight into the interactions between organic compounds in OSPW and intracellular receptor proteins. Because expression of *CYP1A* is regulated by the binding of dioxins and dioxin-like compounds, including PAHs, to the aryl-hydrocarbon receptor (AhR) (Whitlock, 1999), agonists of the AhR might be present in OSPW. Presence of PAHs is characteristic of OSPW contamination however it is thought that PAHs are associated with the sediment phase of tailings ponds and that concentrations in water are small (Colavecchia et al., 2004, 2006, 2007). When assayed

in the H4IIE-*luc* transactivation assay (Sanderson et al., 1996), untreated OSPW did not exhibit AhR agonist properties (data not shown) indicating that either concentrations in untreated OSPW of agonists of the AhR are very small or that any agonists have a very weak affinity for the AhR. Identities of these compounds are not known, but several NAs in OSPW do not bind the AhR (Scarlett et al., 2012). Although the AhR can regulate expression of *GST*, *UGT*, *SULT*, and *MRP2*, in mammals expression of these genes can also be regulated by the constitutive androstane receptor (CAR) and pregnane-x-receptor (PXR) (Yueh et al., 2003; Sugatani et al., 2005; Xu et al., 2005; Chen et al., 2007; Tolson and Wang, 2010). Although a CAR has not been identified in teleost fishes (Mathäs et al., 2012), these results indicate that compounds in OSPW induce expression of genes that encode biotransformation enzymes via activation of nuclear receptors other than AhR. In addition to the activation of receptor proteins, some of the effects of OSPW might be independent of the activation of receptor proteins. Abundance of transcripts of the basic transcription element binding protein (*bteb*), a transcription factor that represses constitutive and inducible transcription of numerous CYP enzymes, including CYP1A1 (Kaczynski et al., 2002), was lesser in livers of fathead minnows exposed to OSPW (Table 2). This might explain the greater abundance of transcripts of *cyp1a*. Also, metabolites of substrates generated by CYPs activate the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor that regulates expression of *UGT*, *GST*, *MRP2*, *MOA*, and *AlDH* by binding to antioxidant response elements in promoters of these genes (Thimmulappa et al., 2002). Abundance of transcripts of *nrf2* was greater in livers of fathead minnows exposed to OSPW (Table 2). Overall, there appear to be multiple mechanisms by which untreated OSPW might cause greater abundances of transcripts of enzymes that metabolize endogenous and exogenous substrates.

3.2.2. Oxidative stress

Exposure to untreated OSPW might have caused oxidative stress in livers of fathead minnows. A minimal concentration of reactive oxygen species (ROS) that is required for normal cellular function is maintained by antioxidant defense enzymes and a pool of antioxidants. Concentrations of GSH, the most abundant antioxidant in eukaryotic cells, are maintained in part by the actions of glutathione synthase (GS), glutathione reductase (GR), and glutathione peroxidase (GPx) (Circu and Aw, 2010). Abundances of transcripts of *gs*, *gr*, and *gpx* were greater in livers from fathead minnows exposed to untreated OSPW (Table 3). When GSH is utilized to reduce ROS a molecule of NADPH supplied from the pentose phosphate pathway is used to reduce the resulting disulfide-oxidized GSH. Abundances of transcripts of enzymes of the pentose phosphate pathway, including transketolase (*tk*), 6-phosphate dehydrogenase (*6-pgdh*), and glucose-6-phosphate dehydrogenase (*g6pdh*) were greater in livers of fathead minnows exposed to untreated OSPW (Table 3). Of these transcripts, only abundance of *6-pgdh* was greater (2.7-fold) in livers from fathead minnows exposed to ozone-treated OSPW. Expression of G6PDH, TK, 6-PGDH, GS, GPx, and GR is regulated by the Nrf2 signaling pathway (Thimmulappa et al., 2002) that is activated by metabolites from CYP catalyzed metabolism of organic compounds. These metabolites might have originated from metabolism of organic compounds in OSPW by CYP enzymes. These results are consistent with a study where concentrations of ROS were greater in embryos of fathead minnows exposed to the same untreated OSPW used in this study (He et al., 2012b).

Oxidative stress can disrupt redox conditions required for proper folding of proteins. Consequently, the glutaredoxin and thioredoxin systems, which are important for proper folding of proteins, are important in the response to oxidative stress (Berndt et al., 2008). The thioredoxin system is comprised of NADPH, thioredoxin (Trx), thioredoxin reductase (TrxR), and protein disulfide isomerases (PDI) (Wilkinson and Gilbert, 2004). Abundances of transcripts of thioredoxin (*trx*), thioredoxin reductase (*trxr*), protein disulfide isomerase related protein P5 (*pdip5*) precursor, and protein disulfide isomerase family A member 3 (*pdia3*) precursor were greater in livers of fathead minnows exposed to untreated OSPW (Table 3). The glutaredoxin system consists of GSH, NADPH, and GR (Circu and Aw, 2010). In addition to the 3.2-fold greater abundance of transcripts of *gr*, abundance of transcripts of glutaredoxin related protein 5 (*grx5*) was greater in livers of fathead minnows exposed to untreated OSPW (Table 3). The function of Grx5 is unknown, but loss of function in yeast leads to oxidative damage and sensitization to ROS (Rodríguez-Manzaneque et al., 2002).

Sources of oxidative stress in fathead minnows exposed to untreated OSPW are not known. Oxidative metabolism by CYPs and oxidases has been linked to greater generation of ROS (Bondy and Naderi, 1994; Lushchak, 2011). Another source of ROS is complexes I and III of the respiratory chain of mitochondria (Ott et al., 2007). Abundances of transcripts of the NADH dehydrogenase 1 beta subcomplex subunit 3 (*nadh1β-3*) and the precursor of acyl carrier protein (*acyl*), which are components of complex I, were greater in livers of fathead minnows exposed to untreated OSPW (Table 4). Abundances of transcripts of cytochrome b-c1 complex subunit 9 (*cypbc1-9*), cytochrome b561 domain 2 (*cypb561d2*), and cytochrome b5a (*cypb5a*), components of complex III, were greater in livers of fathead minnows exposed to untreated OSPW (Table 4). Effects of untreated OSPW mitochondrial function are not known, but mitochondria might be important targets of OSPW. Abundances NADH dehydrogenase 1 beta subcomplex 2 (*nadh1β-2*) and NADH dehydrogenase 1 alpha subcomplex subunit 5 (*nadh1α-5*) of the mitochondrial electron transport chain were greater by 1.5- and 1.6-fold, respectively, in livers from fathead minnows exposed to ozone-treated OSPW. Although this indicates that exposure to ozone-treated OSPW might cause oxidative stress through effects on the respiratory chain of mitochondria, abundances of transcripts of other markers of oxidative stress were not greater.

Table 3
Fold-changes in abundances of transcripts of genes involved in the response to oxidative stress. Gene names, abbreviations, fold-change in abundance of transcripts determined by RNAseq, and gene ontology numbers of biological processes are given.

Transcript	Fold change (RNAseq)	GO ID
Glutathione synthase (*gs*)	3.1	0006979
Glutathione reductase (*gr*)	3.2	0045454
Glutathione peroxidase (*gpx*)	1.7	0006749
Transketolase (*tk*)	2.4	0009052
6-phosphogluconate dehydrogenase (*6-pgdh*)	10.1	0009051
Glucose-6-phosphate dehydrogenase (*g6pdh*)	2.7	0006098
Thioredoxin (*trx*)	2.5	001535
Thioredoxin reductase 3 (*trxr3*)	2.7	001535
Protein disulfide isomerase precursor 5 (*pdi p5*)	2.2	001535
Protein disulfide isomerase A3 (*pdi a3*)	1.5	001535
Glutaredoxin related protein 5 (*grx5*)	1.7	0045454

Table 4
Fold-changes in abundances of transcripts of genes of the mitochondrial electron transport chain. Gene names, abbreviations, fold-change in abundance of transcripts determined by RNAseq, and gene ontology numbers of biological processes are given.

Transcript	Fold change (RNAseq)	GO ID
NADH dehydrogenase 1 beta subcomplex subunit 3 like (*nadh1β-3*)	1.8	0008137
Acyl carrier (mitochondrial precursor) (*acyl-carr*)	1.5	0006120
Cytochrome b-c1 complex subunit 9 (*cypbc1-9*)	1.5	0006120
Cytochrome b561 domain 2 (*cypb561d2*)	3.3	0016021
Cytochrome b5a (*cypb5a*)	8.8	0022900

3.2.3. Apoptosis

Exposure to untreated OSPW might have caused apoptosis in livers of fathead minnows. Caspase-independent apoptosis is stimulated when apoptosis inducing factors (AIFs) are cleaved from the inner mitochondrial membrane and translocate to the nucleus where they mediate chromatin condensation and degradation (Candé et al., 2002; Delavallée et al., 2011). Induction of apoptosis by untreated OSPW is supported by a study where abundances of transcripts of genes involved in apoptosis were greater in embryos of fathead minnows exposed to the same untreated OSPW used in this study (He et al., 2012a). Abundances of transcripts of apoptosis-inducing factor 3 (*aif3*) and apoptosis inducing factor mitochondrial accociated-2 (*aif m2*) were greater in livers of fathead minnows exposed to untreated OSPW (Table 5). Multiple intrinsic stimuli, including oxidative stress, cause release of AIF and a role for AIF in scavenging free radicals has been proposed (Klein et al., 2002). Release of AIF from the mitochondria is stimulated by poly [ADP-ribose] polymerase (PARP) (Yu et al., 2006). Abundance of transcripts of *parp-14* was greater in livers of fathead minnows exposed to untreated OSPW (Table 5). Abundance of transcripts of other indicators of apoptosis, including programmed cell death 4a (*pdcd4a*) and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (*bnip3*) were greater and lesser, respectively, in livers of fathead minnows exposed to OSPW (Table 5). Expression of BNIP3 is repressed as a mechanism to control apoptosis, and this repression is mediated by the forkhead box transcription factor O3A (FOX03A) (Chinnadurai et al., 2008). Abundance of transcripts of *foxo3a* was lesser in livers from fathead minnows exposed to OSPW (Table 5).

Autophagy, a process that results in programmed cell death and is up-regulated in response to stressors such as oxidative stress (Lee et al., 2012), might have been stimulated in fathead minnows exposed to untreated OSPW. Abundances of transcripts of the DNA damage-regulated autophagy modulator protein 2 (*dram2*), a key mediator of autophagy, were greater in livers of fathead minnows exposed to untreated OSPW (Table 5). During autophagy, acid proteases such as cathepsins degrade cellular components. In livers of fathead minnows exposed to untreated OSPW abundance of transcripts of *cathepsin b* was greater than in controls (Table 5). Cathepsins also catalyze cleavage of AIFs from the inner mitochondrial membrane (Delavallée et al., 2011).

3.2.4. Immune system

Exposure to OSPW might have affected immune responses in fathead minnows. Abundances of transcripts of a novel protein similar to vertebrate complement component 8 beta polypeptide (*c8β*), complement C1q like protein 4 precursor C, complement C3 (*c3*), complement C3-H1-like (*c3-h1*), and complement C4-2 (*c4-2*) were lesser in livers from fathead minnows exposed to untreated OSPW (Table 6). Studies that have reported impaired immune responses in organisms exposed to OSPW. There were tissue and dose- and time-dependent changes in expression of mRNA of the pro-inflammatory genes in mice exposed to the organic fraction of OSPW (Garcia-Garcia et al., 2011a, 2011b). Rainbow trout exposed to oil sands affected waters had greater incidences of fin erosion, a decrease in counts of leukocytes, thrombocytes, and granulocytes, and antibodies against *Aeromonos salmonicida* (McNeill et al., 2012). Yellow perch exposed to oil sands affected water had greater incidence of fin erosion and viral lesions (van den Heuvel et al., 2000). In general, ozonation of OSPW attenuated the effects on expression of genes of the complement system. However, the abundance of transcript of the s subcomponent of complement component 1 (*c1-s*) was significantly greater by 3.6-fold. The complement system plays a crucial role in the innate and the adaptive immune responses (reviewed in Dunkelberger and Song, 2010). This study is the first to report effects of OSPW on the complement system.

Table 5
Fold-changes in abundances of transcripts of genes involved in apoptosis. Gene names, abbreviations, fold-change in abundance of transcripts determined by RNAseq, and gene ontology numbers of biological processes are given.

Transcript	Fold change (RNAseq)	GO ID
Apoptosis-inducing factor 3 (*aif-3*)	4.3	0042981
Apoptosis-inducing factor mitochondrial associated-2 (*aif m2*)	4.1	0006917
Poly [ADP-ribose] polymerase (*parp*)	4.8	0006355
Programmed cell death 4a (*pdcd4a*)	1.5	0045785
DNA damage-regulated autophagy modulator protein 2 (*dram2*)	>23.3	0006914
Cathepsin b (*cthpb*)	1.5	0006508
BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (*bnip3*)	−1.8	0006917
Forkhead box transcription factor O3A (*foxo3a*)	−3.3	0006917

Table 6
Fold-changes in abundances of transcripts of genes of the complement immune system. Gene names, abbreviations, fold-change in abundance of transcripts determined by RNAseq, and gene ontology numbers of biological processes are given.

Transcript	Fold change (RNAseq)	GO ID
Complement 8 beta polypeptide (*c8β*)	−2.1	0045087
Complement c1q like protein 4 precursor (*c1q4c*)	−19.7	0048523
Complement C3 (*c3*)	−7.6	0042221
Complement C3-H1-like (*c3-h1*)	−2.1	0045087
Complement C4-2 (*c4-2*)	−2.0	0006955

3.3. RNAseq vs qPCR

A subset of transcripts that were determined to be of greater abundances by RNAseq were quantified by qPCR. Both methods report abundances of transcripts as fold-change relative to the control but the calculation and normalization methods are not the same. The RNA-Seq expression values were calculated on the basis of RPKM (Mortazavi et al., 2008), and qPCR fold-change values were calculated by use of the MNE method and incorporated reference gene (Simon, 2003). For transcripts quantified by both methods, directions of change were always the same and the magnitude of the fold-change in abundance was very similar (Table 7). Correlation between the fold changes measured by RNAseq and qPCR was only 0.05 when the entire dataset was analyzed. However, when the fold-change in abundance of transcripts of *cyp2k6* was omitted the correlation was 0.76. The data support previous studies where RNAseq was an effective method for determining changes in abundances of transcripts (Marioni et al., 2008).

4. Conclusions — proposed mechanism of sublethal toxicity of untreated OSPW

Based on abundances of transcripts in livers of fathead minnows exposed to untreated OSPW, and the fact that ozonation of OSPW attenuated these effects, a mechanism of sublethal toxicity involving

Table 7
A comparison of fold-changes in abundances of transcripts as determined by RNAseq and qPCR. Gene names and abbreviations are given.

Transcript	Fold change (RNAseq)	Fold change (qPCR)
Apoptosis-inducing factor 3 (*aif-3*)	4.3	4.1
Glutathione synthase (*gs*)	3.1	2.1
Glutathione reductase (*gr*)	1.7	2.8
Cytochrome P450 1A (*cyp1a*)	2.2	1.5
Cytochrome P450 2k6 (*cyp2k6*)	10.1	2.1
Cytochrome P450 2AD2 (*cyp2ad2*)	2.7	3.4
Glutathione-s-transferase (mitochondrial) (*gst-m*)	4.5	4.6
Multidrug resistance associated protein-2 (*mrp-2*)	3.3	4.4

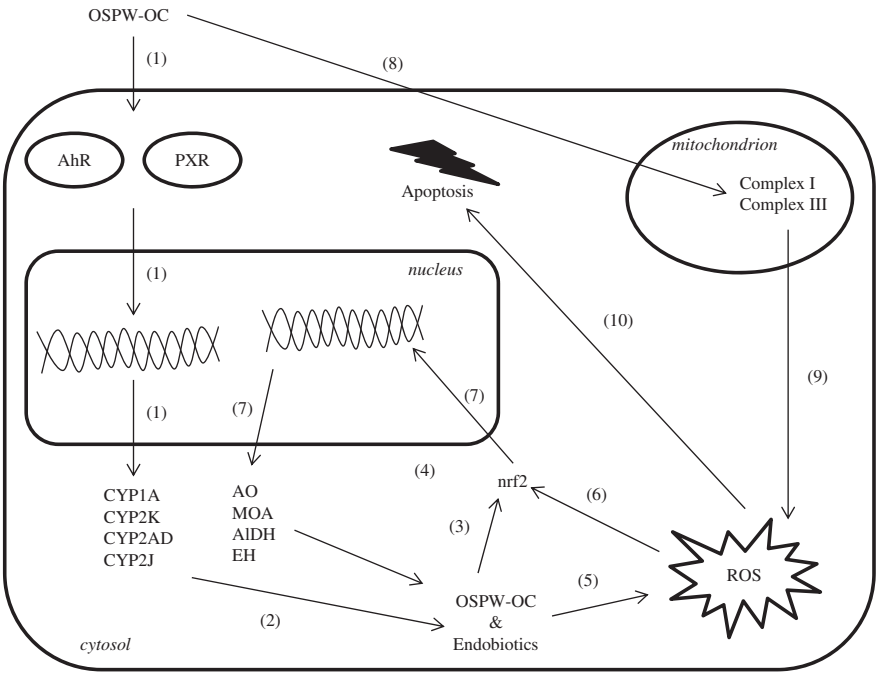

Fig. 1. Schematic illustration of a proposed mechanism of sublethal effects of untreated OSPW. The mechanism is inferred from abundances of transcripts in livers of fathead minnows exposed to untreated OSPW. Sublethal effects of untreated OSPW might be due to apoptosis (step 10) that is caused by reactive oxygen species (ROS) generated by either oxidative metabolism of organic compounds in OSPW (steps 1–7) or by effects on respiration at complex I and complex III of the electron transport chain of the mitochondria (steps 8–9). In steps 1–7, compounds in the organic fraction of OSPW act via the AhR or PXR to stimulate expression of cytochrome P450 enzymes. These enzymes generate reactive oxygen species as they metabolize organic compounds in OSPW and endogenous compounds. Metabolites of phase I metabolism activate the nuclear factor (erythroid-derived 2)-like 2 (NRF2) that stimulates expression of non-P450 enzymes that metabolize organic compounds in OSPW and generate ROS. The ROS might also be generated by effects of OSPW on complex I and complex III of the electron transport chain.

ROS and apoptosis is proposed (Fig. 1). Oxidative stress caused by greater P450-mediated oxidative metabolism of organic compounds in the untreated OSPW, and possibly by the metabolism of endogenous substrates such as hormones, lead to oxidative stress via production of ROS. In addition, greater rates of respiration might lead to greater levels of ROS. Despite the cellular response to oxidative stress, ROS might cause induction of caspase-independent apoptosis and autophagy. In addition, OSPW might exert chronic toxicity due to effects on the immune system. As a note of caution, the mechanism of sublethal toxicity proposed here is based only on transcriptional responses and additional studies to explore the role of these sublethal effects in the mechanism of toxicity of OSPW are required. However, the results support the mechanism of effects of untreated OSPW to embryos of fathead minnows proposed in a recent study that used the same untreated OSPW used in this study (He et al., 2012a). Studies are required to identify the chemicals in the dissolved organic fraction of OSPW that cause these sublethal effects. Chemicals responsible for acute toxicity have long been assumed to be NAs but there is no direct evidence to support this hypothesis. Rather, evidence to date suggests only that the dissolved organic acid fraction, which is a very complex mixture of chemicals, is responsible for toxicity of OSPW.

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Appendix A. Supplementary data

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Table S1. Fold-change in abundances of transcripts in livers of fathead minnows exposed to untreated OSPW.

Gene ID	Acc #	Fold Change
thioredoxin reductase 3 [Danio rerio]	NP_898895	2.5
thioredoxin [Danio rerio]	NP_001002461	2.9
sulfotransferase 1 isoform 3 [Squalius cephalus]	ABJ98761	1.8
programmed cell death 4a [Danio rerio]	NP_998153	1.5
UDP-glucuronosyltransferase 2A3-like [Danio rerio]	XP_002666938	6.3
apoptosis-inducing factor 3-like [Xenopus (Silurana) tropicalis]	XP_002936104	4.3
cytochrome P450 2K1-like [Danio rerio]	XP_692555	11.7
multidrug resistance-associated protein member 2 [Danio rerio]	ADX66438	3.3
microsomal glutathione s-transferase 3	ABD67515	4.5
glutathione synthetase [Cyprinus carpio]	AEM44786	3.1
glutathione s-transferase [Pimephales promelas]	ABV30909	> 23.2
glutathione reductase, mitochondrial [Danio rerio]	NP_001018390	3.2
glutathione peroxidase 3 (plasma) precursor [Danio rerio]	NP_001131027	1.7
epoxide hydrolase 2, cytoplasmic [Danio rerio]	NP_001008642	2
cytochrome P450 2AD2 (arachidonic acid epoxygenase) [Danio rerio]	NP_694486	2.7
cytochrome P450 CYP2K6 [Danio rerio]	AAK97022	10.1
cytochrome P450 1a [Gobiocypris rarus]	ABV01348	2.1
cytochrome P450 2j28 [Danio rerio]	AAH45948	2.2
apoptosis-inducing factor, mitochondrion-associated 2 [Danio rerio]	NP_001186939	4.1
zinc transporter 5 [Danio rerio]	NP_001002322	2.2
zinc finger protein 71-like [Danio rerio]	XP_002667859.1	2.5
cyclic amp-responsive element-binding protein 3-like protein 3 [Danio rerio]	XP_003198009	2.4
unnamed protein product [Trypanosoma congolense IL3000]	CCD15513	1.9
uncharacterized protein LOC100216204 [Xenopus (Silurana) tropicalis]	NP_001135645	2.1
uncharacterized protein C1orf31 homolog isoform 1 [Danio rerio]	XP_001923280	3.1
transmembrane emp24 domain-containing protein 2-like	XP_003455198	1.6
translocating chain-associated membrane protein 1 [Danio rerio]	NP_705955	3.2
transketolase-like protein 2 [Danio rerio]	NP_932336	2.4
trafficking protein particle complex subunit 4 [Danio rerio]	NP_957058	2.7
surfeit gene 4, like [Danio rerio]	NP_001002470	1.9
stress-associated endoplasmic reticulum protein 1 [Danio rerio]	NP_001002573	1.8
small EDRK-rich factor 2 [Danio rerio]	NP_001153807	1.8
Si:ch211-93f2.1 protein [Danio rerio]	AAI55643	2.4
selenoprotein s [Danio rerio]	AAI18686	1.7
putative arginase 2C precursor [Cyprinus carpio]	CAI38847	2.1
protein YIPF3 [Danio rerio]	NP_956201	1.9
protein slowmo homolog 2 [Danio rerio]	NP_956028	2.8
protein disulfide isomerase-related protein P5 precursor [Danio rerio]	AAK71636	2.2
protein disulfide isomerase family A, member 3 precursor [Danio rerio]	NP_001186666	1.5
prostaglandin reductase 1 [Danio rerio]	NP_001008651	3.3
proline synthase co-transcribed bacterial homolog protein [Danio rerio]	NP_001119881	1.6
integrin beta-2-like [Danio rerio]	XP_686012	2.2
hypothetical protein LOC100333442 [Danio rerio]	XP_002667924	23.3
fatty acid 2-hydroxylase-like [Oreochromis niloticus]	XP_003439633	1.6
cytochrome b-c1 complex subunit 9-like [Oreochromis niloticus]	XP_003453904	1.5
a-kinase anchor protein 7 isoform gamma-like [Anolis carolinensis]	XP_003215759	2.4
amine oxidase [flavin-containing] A-like [Danio rerio]	XP_686310	2.1

poly [ADP-ribose] polymerase 14, partial [Danio rerio]	XP_691115	4.8
phosphogluconate dehydrogenase [Danio rerio]	NP_998717	10.1
phosphatidylinositol synthase [Danio rerio]	AAT68039	1.7
Period homolog 3 (Drosophila) [Danio rerio]	AAI62472	2.4
ORF2-encoded protein [Danio rerio]	BAE46429	> 23.2
nuclear factor (erythroid-derived 2)-like 2 [Danio rerio]	AAH45852	1.8
novel protein similar to vertebrate aldehyde oxidase 1 (AOX1) [Danio rerio]	CAK04754	3.1
novel protein similar to trafficking protein particle complex 5 (TRAPPC5) [Danio rerio]	NP_001002482	2
novel protein similar to procollagen-proline, 2-oxoglutarate 4-dioxygenase, alpha polypeptide III (P4HA3) [Danio rerio]	CAM56657	4.6
novel protein similar to human and mouse cytochrome b-561 domain containing 2 (CYB561D2) [Danio rerio]	CAK11032	3.3
novel protein similar to H.sapiens MYO1B, myosin IB (MYO1B) [Danio rerio]	CAX15478	2.7
novel protein similar to H.sapiens DPP4, dipeptidyl-peptidase 4 (DPP4) [Danio rerio]	NP_001154809	3.4
novel protein similar to E-cadherin (cdh1) [Danio rerio]	CAD60789	2
novel protein similar to aldehyde dehydrogenase 2, like (aldh2l) [Danio rerio]	CAM13323	3.6
novel protein [Danio rerio]	CAI11845	3.9
novel protein (zgc:100861) [Danio rerio]	CAM14040	4.4
novel NACHT domain containing protein [Danio rerio]	CAP09448	> 23.2
neutral alpha-glucosidase AB-like [Danio rerio]	XP_002664506	2.1
NADH dehydrogenase 1 beta subcomplex subunit 3-like [Oreochromis niloticus]	XP_003457472	1.8
myeloid protein-1 [Cyprinus carpio]	BAB16024	2.8
myelin gene regulatory factor-like [Danio rerio]	XP_002667695	3.3
monoamine oxidase [Cyprinus carpio]	BAH02786	3.2
mitochondrial inner membrane protein	NP_001001401	1.8
metaxin-2 [Danio rerio]	NP_997740	3
insulin-like growth factor binding protein-1 [Cyprinus carpio]	ACV72066	3.5
IgE Fc receptor high affinity I gamma polypeptide [Ctenopharyngodon idella]	AEF32116	2.4
hypoxia-inducible factor 3 alpha [Megalobrama amblycephala]	ADF50045	2.3
hypothetical protein LOC569467 [Danio rerio]	XP_697952	5.1
hypothetical protein LOC100710874 [Oreochromis niloticus]	XP_003453997	> 23.2
hypothetical protein LOC100126232 [Danio rerio]	XP_001922722	2.9
hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-coenzyme A hydratase, alpha subunit a [Danio rerio]	NP_001098746	> 23.2
guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 [Danio rerio]	NP_001013515	3.8
guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2	NP_001001818	1.7
GTPase IMAP family member 4-like [Danio rerio]	XP_001339168	21.8
glutaredoxin-related protein 5, mitochondrial [Danio rerio]	NP_998186	1.7
glutamate carboxypeptidase 2 [Danio rerio]	NP_95657100	2
glucose-6-phosphate dehydrogenase [Danio rerio]	XP_697820	2.7
galectin-3-binding protein A precursor [Danio rerio]	NP_001035130	4.9
fatty acyl- hydrolase medium chain-like [Danio rerio]	AAI54654	2
F11 receptor [Danio rerio]	NP_001004667	1.7
ezr protein [Danio rerio]	AAH78311	6.1
erythrocyte membrane protein band 4.1 like 5 [Danio rerio]	NP_956383	2
endonuclease/exonuclease/phosphatase family domain-containing protein 1 [Danio rerio]	NP_991322522	3
elongation of very long chain fatty acids protein 2 (Elovl2) [Danio rerio]	AAH95785	3.9
early growth response 1 [Danio rerio]	NP_571323	7.9
Dnajb11 protein [Danio rerio]	AAH66411	2.8
DNA damage-regulated autophagy modulator protein 2 [Danio rerio]	NP_001002135	> 23.2
cytochrome b5a [Danio rerio]	AAI54825	8.8

C-X-C motif chemokine 10 precursor [Anoplopoma fimbria]	ACQ59055	2.2
C-Myc-binding protein [Salmo salar]	ACI69041	6.5
cathepsin b [Danio rerio]	AAH44517	1.5
calcium activated nucleotidase 1b [Danio rerio]	NP_001003595	3.5
ATP-binding cassette sub-family D member 3 [Danio rerio]	NP_998647	2.1
aspartoacylase-2B [Danio rerio]	NP_001002347	2.5
apolipoprotein A-IV [Danio rerio]	AAH93239	3.5
adipose triglyceride lipase [Larimichthys crocea]	ADY89608	3
acyl carrier mitochondrial precursor [Osmerus mordax]	ACO09677	1.5

Table S2. Fold-change in abundances of transcripts in livers of fathead minnows exposed to untreated OSPW.

Description	Accession #	Fold Change
5'-3' exoribonuclease 2 [Danio rerio]	NP_001001944	-1.8
vertebrate complement component 8, beta polypeptide (C8B) [Danio rerio]	NP_001243652	-2.1
BTEB transcription factor [Pimephales promelas]	ABO28528	-3.7
HCLS1-associated protein X-1 [Danio rerio]	NP_001002337	-1.9
MAP kinase-interacting serine/threonine kinase 2 [Danio rerio]	CAQ14030	-2.2
phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha) [Danio rerio]	CAQ14607	-1.5
Zgc:103710 protein [Danio rerio]	AAI22312	-2.7
5'-3' exoribonuclease 2 [Danio rerio]	AAH47182	-2
acetylserotonin O-methyltransferase-like [Danio rerio]	CAQ14999	-1.8
actn3a [Danio rerio]	ACZ28497	-2.7
acyl-Coenzyme A dehydrogenase family, member 11 [Danio rerio]	NP_956472	-1.6
alpha-2,8-sialyltransferase 8F [Danio rerio]	XP_683398	-3
alpha-amylase [Ctenopharyngodon idella]	ACX35465	-2.1
apolipoprotein b-100-like	NP_001025233	-4.6
ATH1, acid trehalase-like 1 (yeast) [Danio rerio]	NP_001071193	-2.3
ATPase, Na+/K+ transporting, alpha 1b polypeptide [Danio rerio]	NP_571765	-2.7
BCL2/adenovirus E1B 19kDa interacting protein 3b [Danio rerio]	NP_997858	-1.8
breast cancer anti-estrogen resistance protein 3-like [Oreochromis niloticus]	XP_003457816	-2.5
chromobox homolog 3a (HP1 gamma homolog, Drosophila) [Danio rerio]	NP_001038867	-1.9
complement c1q-like protein 4 precursor	AAI59112	-19.7
complement C3 [Ctenopharyngodon idella]	AAQ74974	-7.6
complement C3-H1-like [Danio rerio]	NP_001008582	-2.1
complement C4-2 [Cyprinus carpio]	BAB03285	-2
cytosolic malate dehydrogenase [Ctenopharyngodon idella]	ACC94161	-1.6
deiodinase, iodothyronine, type II [Danio rerio]	NP_997954	-6.3
eukaryotic translation initiation factor 4A isoform 1B [Danio rerio]	NP_958918	-1.6
F9 protein [Danio rerio]	AAH94987	-2.5
family with sequence similarity 102, member B, b [Danio rerio]	NP_001038309	-3
fetuin long form [Cyprinus carpio]	AAO74862	-2
fibroblast growth factor receptor 2 isoform 1 precursor [Danio rerio]	NP_001229933	-2
fibroblast growth factor receptor 4 [Danio rerio]	AAI71704	-3
fibronectin 1b [Danio rerio]	AAI34006	-2.7
forkhead box O3A [Danio rerio]	NP_001009988	-3.3
GDNF family receptor alpha 3 [Danio rerio]	NP_001243565	-2.2
glycine N-methyltransferase [Danio rerio]	NP_997981	-1.9
hypothetical protein LOC100536798 [Danio rerio]	XP_003199642	-3.7
lancl1 protein, partial [Danio rerio]	AAH61705	-5.2
leucine-rich repeat-containing protein 68 [Danio rerio]	XP_003200541	-1.8
LOC100149074 protein [Danio rerio]	AAI33148	-2.3
LOC571260 protein [Danio rerio]	AAI22307	-1.5
lon peptidase 2, peroxisomal [Danio rerio]	NP_001008573	-1.8
low density lipoprotein receptor [Danio rerio]	NP_001025454	-2.1
lysine (K)-specific demethylase 6B, b [Danio rerio]	NP_001025349	-3.7
methionine adenosyltransferase alpha [Danio rerio]	NP_956165	-1.6
monocarboxylate transporter 10 [Danio rerio]	NP_001073497	-2.2
natriuretic peptide receptor B [Anguilla japonica]	P55202	-2.3
neural adhesion molecule L1.2 [Danio rerio]	AAI62464	-2.6

novel alpha globin [Danio rerio]	CAE48989	-11.3
novel protein (zgc:101116) [Danio rerio]	CAK05291	-2.8
novel protein [Danio rerio]	CAM56517	-1.6
novel protein containing multiple sushi domains (SCR repeat) [Danio rerio]	CAP09611	-2.3
novel protein similar to H.sapiens C1orf57, chromosome 1 open reading frame 57 (C1orf57, zgc:92420) [Danio rerio]	CAX12587	-3.7
similar to H.sapiens DENND2D, DENN/MADD domain containing 2D (DENND2D) [Danio rerio]	CAX14499	-2.6
novel protein similar to vertebrate apolipoprotein B (including Ag(x) antigen) (APOB) [Danio rerio]	CAN88170	-3.4
novel protein similar to vertebrate arrestin domain containing 3 (ARRDC3) [Danio rerio]	NP_001073498	-3.1
novel protein similar to vertebrate EF hand calcium binding protein 2 (EFCBP2, zgc:112232) [Danio rerio]	CAM56479	-5.2
novel protein similar to vertebrate fibulin 5 (FBLN5, zgc:103575) [Danio rerio]	CAX14183	-2.1
novel protein similar to vertebrate IQ motif containing GTPase activating protein 2 (IQGAP2) [Danio rerio]	NP_001121812	-3.5
novel protein similar to vertebrate metastasis suppressor 1 (MTSS1) [Danio rerio]	NP_001116724	-2.2
nuclear receptor subfamily 1, group D, member 2 (NR1D2) [Danio rerio]	XP_691607	-6.3
period 1-like protein [Danio rerio]	NP_997604	-2.7
Phf23a protein [Danio rerio]	NP_001013517	-3.8
complement C4-like [Danio rerio]	XP_001334640	-1.8
ectonucleotide pyrophosphatase/phosphodiesterase family member 2-like [Oreochromis niloticus]	XP_003458635	-2.2
elongation factor 2 [Danio rerio]	XP_697966	-3.2
hypothetical protein LOC100149918 [Danio rerio]	XP_001923568	-4.7
hypothetical protein LOC100333416 [Danio rerio]	XP_002661139	-2.5
hypothetical protein LOC100333442 [Danio rerio]	XP_002667924	-7.1
hypothetical protein LOC555305 [Danio rerio]	XP_682868	-8.8
kinesin-like protein KIF21A [Oreochromis niloticus]	XP_003455423	-2
leucine-rich repeat-containing protein 68-like [Danio rerio]	XP_002665462	-1.7
monocarboxylate transporter 7-like [Danio rerio]	XP_690266	-2
protein Jumonji [Danio rerio]	XP_001345885	-2
similar to OSJNBa0059D20.6 [Strongylocentrotus purpuratus]	XP_786277	-19.2
tetratricopeptide repeat protein 39B-like [Oreochromis niloticus]	XP_003454695	-2.4
tribbles homolog 1-like [Oreochromis niloticus]	XP_003444195	-2.9
zinc finger and BTB domain-containing protein 16-A-like [Danio rerio]	XP_698274	-4.4
secernin-2 [Danio rerio]	NP_001025287	-3.6
secreted modular calcium-binding 1 [Danio rerio]	NP_001188322	-3.1
selenoprotein P, plasma, 1a [Danio rerio]	AAI55822	-2.2
Slco1c1 protein [Danio rerio]	AAI63443	-4.9
steryl-sulfatase	AAH49491	-1.9
transcription elongation factor A (SII), 3 [Danio rerio]	NP_991246	-1.8
transducer of ERBB2, 1b [Danio rerio]	AAH71356	-1.5
UDP glucuronosyltransferase 5 family polypeptide f1 [Danio rerio]	NP_001170971	-4.3
uncharacterized protein LOC790940 [Danio rerio]	NP_001073554	-1.9
uncharacterized protein Zgc:175187 protein [Danio rerio]	NP_001107947	-9.1
unnamed protein product [Tetraodon nigroviridis]	CAG12468	-4.3
upf0498 protein kiaa1191 homolog [Danio rerio]	NP_001035083	-1.9
yth domain-containing protein 1	NP_0010075411	-1.8
Zgc:153813 protein [Danio rerio]	AAI54741	-1.5
Zgc:92880 protein [Danio rerio]	NP_001003431	-9.8

Table S3. Fold-change in abundances of transcripts in livers of fathead minnows exposed to ozone-treated OSPW.

Description	Accession #	Fold Change
ORF2-encoded protein [Danio rerio]	BAE46429	> 23.2
translocon-associated protein subunit alpha precursor [Danio rerio]	NP_958484	1.8
3-beta-hydroxysteroid-Delta(8),Delta(7)-isomerase [Danio rerio]	NP_001002328	6.1
6-phosphogluconate dehydrogenase, decarboxylating isoform 2 [Danio rerio]	NP_998717	2.7
abhydrolase domain containing 4 [Danio rerio]	NP_001017613	1.6
acid phosphatase 5b, tartrate resistant [Danio rerio]	NP_001002452	1.6
activating transcription factor 4 [Danio rerio]	NP_001096662	1.7
arylsulfatase B precursor [Salmo salar]	NP_001167123	3.1
cadherin 1, epithelial precursor [Danio rerio]	NP_571895	1.6
calreticulin like precursor [Danio rerio]	NP_958873	18.8
cathepsin Z [Cyprinus carpio]	AAX51298	1.6
CCAAT/enhancer binding protein (C/EBP), delta [Danio rerio]	AAH56512	1.8
coatomer subunit epsilon [Danio rerio]	NP_001007365	1.6
complement component 1, s subcomponent-like precursor [Danio rerio]	NP_001107921	3.6
cytosolic Fe-S cluster assembly factor nubp2 [Danio rerio]	NP_001032191	> 23.2
dynein light chain 1, cytoplasmic [Danio rerio]	NP_998189	3.1
ectonucleotide pyrophosphatase/phosphodiesterase family member 6 precursor [Danio rerio]	NP_001013545	1.9
endothelial differentiation-related factor 1 homolog [Danio rerio]	NP_957039	1.6
eukaryotic translation elongation factor 2, partial [Nyctibius bracteatus]	ACF26932	2.6
glyceraldehyde 3-phosphate dehydrogenase [Danio rerio]	NP_998259	4.9
hypothetical protein LOC100710874 [Oreochromis niloticus]	XP_003453997	1.8
serine/threonine-protein kinase SIK2 [Danio rerio]	XP_696325	1.7
interferon regulatory factor 6 [Danio rerio]	NP_956892	3.1
isopentenyl-diphosphate Delta-isomerase 1 [Danio rerio]	NP_001020646	2.8
LOC796833 protein [Danio rerio]	AAI55670	1.6
meteorin-like protein precursor [Danio rerio]	NP_998150	2.4
mitochondrial import inner membrane translocase subunit Tim8 B [Danio rerio]	NP_001122251	2.2
NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2, 8kDa [Danio rerio]	NP_957085	1.6
nicastrin [Danio rerio]	AAI62515	1.5
novel protein similar to vertebrate cell division cycle associated 7 (CDCA7, zgc:110113) [Danio rerio]	CAQ13464	9.8
novel protein similar to vertebrate glyceronephosphate O-acyltransferase (GNPAT) [Danio rerio]	CAX13824	1.5
novel protein similar to vertebrate homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (HERPUD1) [Danio rerio]	CAK03951	3.2
phosphatidylinositol-glycan biosynthesis class F protein [Danio rerio]	NP_991208	2.7
ATP-dependent Clp protease ATP-binding subunit clpX-like, mitochondrial-like [Danio rerio]	XP_002667004	1.7
coagulation factor IX-like [Danio rerio]	XP_001343728	1.7
interferon-induced guanylate-binding protein 1 [Danio rerio]	XP_001920654	2.6
kelch domain-containing protein 2-like [Oreochromis niloticus]	XP_003459599	2.0

leucine-rich repeat protein 1-like [Oreochromis niloticus]	XP_003453288	3.0
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5-like [Oreochromis niloticus]	XP_003455688	1.5
nocturnin [Danio rerio]	XP_700794	8.9
putative nuclease HARBI1-like [Amphimedon queenslandica]	XP_003391006	3.3
sodium/potassium/calcium exchanger 6-like [Danio rerio]	XP_689091	1.9
zinc transporter 6-like [Oreochromis niloticus]	XP_003455736	3.3
selenoprotein t precursor	Q802F2	1.5
serine/threonine-protein phosphatase 2A catalytic subunit beta isoform [Oryctolagus cuniculus]	NP_001095177	1.9
signal recognition particle 72 kDa protein [Danio rerio]	XP_002664308	1.8
solute carrier family 16 (monocarboxylic acid transporters), member 8 [Danio rerio]	NP_997797	2.0
solute carrier family 39 (zinc transporter), member 7 [Danio rerio]	CAM56703	3.3
TNFAIP3 interacting protein 1 [Danio rerio]	NP_001073421	1.5
transcobalamin-2 precursor [Danio rerio]	NP_001116703	2.3
transforming protein RhoA [Danio rerio]	NP_001038815	1.9
tubulin beta-1 chain-like [Oreochromis niloticus]	XP_003455078	1.6
tubulin beta-2C [Hippoglossus hippoglossus]	ACZ63699	2.1
uncharacterized protein LOC100216204 [Xenopus (Silurana) tropicalis]	NP_001135645	1.6
uncharacterized protein LOC393524 [Danio rerio]	NP_956846	2.7
unnamed protein product [Tetraodon nigroviridis]	CAF96699	3.3
X-box binding protein 1B [Danio rerio]	AAL05527	1.6

Table S4. Fold-change in abundances of transcripts in livers of fathead minnows exposed to ozone-treated OSPW.

Description	Accession #	Fold Change
acetylserotonin O-methyltransferase-like [Danio rerio]	CAQ14999	-1.7
acyl-CoA dehydrogenase family member 11 [Danio rerio]	NP_956472	-2.1
apolipoprotein B, like precursor [Danio rerio]	NP_001025233	-2.7
arrestin domain-containing protein 3 [Danio rerio]	NP_001073498	-2.2
ATPase, Na+/K+ transporting, alpha 1b polypeptide [Danio rerio]	NP_571765	-1.8
atrial natriuretic peptide-converting enzyme-like [Danio rerio]	XP_003201291	-3
bactin1 protein [Hypophthalmichthys nobilis]	ACO51127	-2.2
beta actin [Paramyxine atami]	BAJ14767	-1.5
carboxypeptidase N, polypeptide 1 [Danio rerio]	AAI54780	-2.3
CDC42 effector protein (Rho GTPase binding) 4 [Danio rerio]	NP_001002183	-3.4
deltaC [Danio rerio]	AAI62095	-2.6
enolase superfamily 1 [Danio rerio]	NP_001070210	-3
far upstream element-binding protein 3 [Danio rerio]	NP_001007777	-2.4
fatty aldehyde dehydrogenase [Salmo salar]	ACN10482	-1.7
fucosidase-alpha-l	NP_001003559	-3.5
glucose-6-phosphate translocase [Danio rerio]	NP_999903	-1.8
glycerol-3-phosphate dehydrogenase 1b [Danio rerio]	NP_956000	-2.6
golgin subfamily A member 7 [Danio rerio]	NP_001099069	-1.8
heparan-alpha-glucosaminide N-acetyltransferase-like [Oreochromis niloticus]	XP_003452198	-2.3
hepatic leukemia factor 1 [Danio rerio]	NP_001070802	-2.1
heterogeneous nuclear ribonucleoprotein U-like protein 1-like [Danio rerio]	XP_003198760	-3
insulin-like growth factor binding protein 2b [Danio rerio]	ABS30427	-3.3
integral membrane protein GPR137C [Danio rerio]	NP_001007434	-2.9
lancl1 protein, partial [Danio rerio]	AAH61705	-2.5
LOC100000238 protein [Danio rerio]	AAI15141	-2.2
mitochondrial glycine cleavage system H protein [Danio rerio]	NP_001002579	-1.5
neural adhesion molecule L1.2 [Danio rerio]	AAI62464	-2.1
novel carboxylesterase domain containing protein [Danio rerio]	CAI12062	-4.2
novel MHCII beta chain protein [Danio rerio]	CAD60678	-18.6
novel protein similar to H.sapiens CORIN, [Danio rerio]	CAQ13859	-3.5
novel protein similar to vertebrate apolipoprotein B (APOB) [Danio rerio]	CAN88170	-3.5
novel protein similar to vertebrate fibulin 5 (FBLN5, zgc:103575) [Danio rerio]	CAX14183	-2.4
novel protein similar to vertebrate galactosidase, beta 1 (GLB1) [Danio rerio]	CAM13012	-4.2
novel protein similar to vertebrate ubiquitin associated protein 2-like (UBAP2L) [Danio rerio]	NP_001076535	-2.1
phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase [Danio rerio]	CAI21320	-2.6
carboxypeptidase D [Danio rerio]	XP_691464	-3.2
CKLF-like MARVEL transmembrane domain-containing protein 6-like [Danio rerio]	XP_003200286	-15.4
dipeptidase 3-like [Danio rerio]	XP_003199021	-12.3

elongation factor 2 [Danio rerio]	XP_697966	-1.7
fibulin-7-like [Danio rerio]	XP_001918972	-17.1
glycogen phosphorylase, liver form-like isoform 1 [Oreochromis niloticus]	XP_003442910	-1.9
hypothetical protein LOC100149918 [Danio rerio]	XP_001923568	-6.5
hypothetical protein LOC100536798 [Danio rerio]	XP_003199642	-4.7
hypothetical protein LOC100537866 [Danio rerio]	XP_003201142	-2
hypothetical protein LOC555305 [Danio rerio]	XP_682868	-3.7
lysosome membrane protein 2 [Danio rerio]	XP_003200850	-3.9
nuclear receptor subfamily 1 group D member 2 [Danio rerio]	XP_691607	-5
protein Jumonji [Danio rerio]	XP_001345885	-2.3
protein NLRC3-like [Danio rerio]	XP_003197823	-2.8
similar to OSJNBa0059D20.6 [Strongylocentrotus purpuratus]	XP_786277	> 23.2
transmembrane protein 176B-like [Danio rerio]	XP_002665065	-1.5
proliferator-activated receptor gamma coactivator 1 alpha [Schizothorax prenanti]	AEL21373	-2.8
protein NDRG1 isoform 2 [Danio rerio]	NP_998513	-2.5
protein phosphatase 1 regulatory subunit 3C-B [Danio rerio]	NP_957128	-3.9
putative glycerol kinase 5 [Danio rerio]	NP_001071271	-1.6
ras GTPase-activating-like protein IQGAP1 [Danio rerio]	NP_001121812	-3.6
sarcolemmal membrane-associated protein [Danio rerio]	NP_001070722	-1.8
serine/threonine-protein kinase 11-interacting protein [Danio rerio]	NP_956582	-3.5
Si:ch211-219i10.1 protein, partial [Danio rerio]	AAH90504	-1.9
Si:ch211-93f2.1 protein [Danio rerio]	AAI54654	-2.3
solute carrier family 22 member 6 [Danio rerio]	NP_996960	> 23.2
solute carrier family 25, member 32a [Danio rerio]	NP_956550	-1.9
solute carrier family 35 (UDP-glucuronic acid/UDP-N-acetylgalactosamine dual transporter), member D1b [Danio rerio]	NP_956769	-1.8
solute carrier organic anion transporter family member 1c1 isoform 1 [Danio rerio]	AAI63443	-4.7
sorting nexin-7 [Danio rerio]	NP_001002229	-2
splicing factor 3A subunit 1 [Danio rerio]	NP_956388	-9.7
UDP glucuronosyltransferase 1 family polypeptide b7 precursor [Danio rerio]	NP_001166240	-3.7
uncharacterized protein LOC100002878 [Danio rerio]	NP_001107947	-6.5
uncharacterized protein LOC557909 [Danio rerio]	NP_001038309	-2.5
uncharacterized protein LOC790940 [Danio rerio]	NP_001073554	-2.7
unnamed protein product [Tetraodon nigroviridis]	CAG12468	-4.7
upf0498 protein kiaa1191 homolog [Danio rerio]	NP_001035083	-1.6
Zgc:103654 [Danio rerio]	AAI54761	-2
Zgc:172210 [Danio rerio]	AAI71601	-2
zinc finger protein c3h type-like 1 [Danio rerio]	NP_001070621	-2.5