

Hepatic gene expression profiling in zebrafish (*Danio rerio*) exposed to the fungicide chlorothalonil

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Abstract

Chlorothalonil (tetrachloroisophthalonitrile) is a fungicide that is widely used on agricultural crops around the world and as such, it is a ubiquitous aquatic contaminant. Despite high usage, the effects of this fungicide on non-target aquatic organisms have not been fully investigated. The aim of the present study was to (1) determine the effects of chlorothalonil toxicity on adult male zebrafish (*Danio rerio*) and (2) characterize the effects of chlorothalonil on gene expression patterns in the liver using two different concentrations of the fungicide, 0.007 mg/L (environmentally-relevant) and 0.035 mg/L (sublethal). These concentrations were selected from range-finding experiments that showed that zebrafish survival was significantly different from control animals at concentrations higher than 0.035 mg/L but not below. Male zebrafish in both treatments of chlorothalonil showed a decrease in liversomatic index. A commercial *Danio rerio* microarray (4x44k) was used to determine gene expression profiles in male zebrafish liver following a 96h toxicological assay. Microarray analysis revealed that males exposed to both 0.007 mg/L or 0.035 mg/L of chlorothalonil showed increased transcriptional sub-networks related to cell division and DNA damage and decreased expression of gene networks associated with reproduction, immunity, and xenobiotic clearance. This study improves knowledge regarding whole animal exposures to chlorothalonil and identifies molecular signaling cascades that are sensitive to this fungicide in the fish liver.

Key words: chlorothalonil, toxicity, microarray, transcriptomics, pesticides

1. Introduction

Chlorothalonil (TICN, 2,4,5,6 tetrachloroisophthalonitrile, CAS 1897-45-6), is a broad spectrum, non-systemic fungicide widely used in several countries. It is primarily used on agricultural and horticultural crops, and acts as a fungicide, bactericide, nematocide, and as a mildewicide in adhesives and paints (U.S. Environmental Protection Agency, EPA, 1999; Wilkinson and Killeen, 1996). It is also used as an alternative to tributyltin (TBT), an additive agent in antifouling paints (Voulvoulis *et al.*, 1999), which is applied to the hulls of ships and boats to prevent the growth of bacteria, macroalgae, mussels, and other invertebrates. Chlorothalonil, over a period of time, is released into the aquatic environment and concentrations ranging from 0.008 to 1.38 ug/l have been detected in marinas and harbors in the Mediterranean region, as well as in the UK coastal environment (Voulvoulis *et al.*, 2000).

Approximately 14 million pounds of chlorothalonil are applied annually for agriculture in the United States (EPA, 1999). In the province of Prince Edward Island, Canada, chlorothalonil was detected in rivers in excess of the Freshwater Aquatic Life

Guidelines (FWALG) (CCME, 1999), with observed values of 1.34 µg/L compared to its FWALG value of 0.18 µg/L (Mutch *et al.*, 2002). In runoff water, Shuman *et al.* (2000) detected chlorothalonil concentrations of ≤ 290 µg/L, and in groundwater at concentrations ≤ 272 µg/L.

In a pesticide study of an agricultural area in Sinaloa, México, the highest average concentration detected in soil corresponded to chlorothalonil levels at 0.15 µg /kg (Leyva, 2014). A unique feature of the degradation of chlorothalonil in soil is that it is suppressed by the repeated application of the fungicide (Katayama *et al.*, 1991; Takagi *et al.*, 1991). This results in a significant contamination problem; due to multiple applications to a crop in a season, with short intervals between applications (DeLorenzo and Fulton, 2012), non- metabolized chlorothalonil seeps into the groundwater and enters bodies of water, posing a risk to aquatic organisms. Chlorothalonil has an estimated average 96-h LC50 value of 0.032 mg/L for estuarine fish species (Mayer, 1986), and it is estimated that it has a bioaccumulation factor ranging from 18 to 260 (US EPA, 1999; Tsuda *et al.*, 1992) for freshwater and estuarine fish, respectively. In natural seawater, Voulvoulis *et al.*, (2000) reported that it degrades after four weeks. A major mechanism for the breakdown of chlorothalonil is the microbial activity, and in simulated marine environments, Caux *et al.*, (1996) found that it takes 8–9 d to degrade during periods of high microbial activity. However the problem remains that it is continuously used throughout the crop seasons.

The precise mechanisms of action for chlorothalonil are not fully established, but studies suggest that chlorothalonil is a multi-site inhibitor affecting various enzymes and other metabolic processes in fungi (MAFFBC, 2004). For example, this fungicide reduces fungal intracellular glutathione molecules to alternate forms that cannot participate in essential enzymatic reactions, and this ultimately leads to cell death (Monadjemi, *et al.*, 2011).

The EPA has registered this fungicide as a probable carcinogen, but relatively little is known about the effect of waterborne chlorothalonil exposure in aquatic species. In non-target organisms, chlorothalonil is toxic to fish, aquatic invertebrates and marine organisms with a LC50 of 0.1 mg/L for rainbow trout (*Oncorhynchus mykiss*), 5.9 mg/L for blue mussel (*Mytilus edulis*), and 35 mg/L for soft-shell clam (Ernst *et al.*, 1991). In vitro, chlorothalonil negatively affects tunicate hemocyte functionality (Cima *et al.*, 2008) and suppresses oyster hemocyte reactive oxygen species production (Baier-Anderson and Anderson, 2000). Previous reports of chlorothalonil toxicity in fish had found that chlorothalonil may inactivate key respiratory chain enzymes in either the liver or gills (Davies., 1987), and studies report that there is increased glutathione GSH biosynthesis in the liver, gills, and posterior kidney of channel catfish (*Ictalurus punctatus*) following exposure (Gallagher *et al.*, 1992). The

importance of GSH mediated detoxification of chlorothalonil in fish is supported by previous studies of glutathione S-transferase (GST)-catalyzed chlorothalonil metabolism in channel catfish, (Gallagher et al., 1991), Galaxiid species, (Davies, 1985a), and rainbow trout (Davies, 1985b). Chlorothalonil can also inhibit glycolysis by binding with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Caux *et al.*, 1996).

The objective of this study was to determine the effects of chlorothalonil on adult survival, and based on these data, to measure the molecular responses following exposure to two sublethal concentrations of chlorothalonil that represent an environmentally relevant concentration and a higher physiological concentration to better characterize its mode of action. We used the zebrafish (*Danio rerio*), a well-established genetic model used to study development and chemical toxicity. As such, we characterized the transcriptomic response in the zebrafish liver using microarray and gene expression analysis (qPCR).

2. Methods

2.1 Fish Husbandry

AB strain adult zebrafish, between six and nine months old, were obtained from AquaBios (México city) in May 2013, and the experiment was conducted in the toxicological laboratory in the Research Center for Food and Development A.C. (Mazatlán, Sin. México). Fish were maintained in a 200 L circular tank for 2 wk for acclimation to the environment and for initial observation. The temperature of the holding tank was maintained at 25 – 27 °C and dissolved O₂ was between 95–100%. Fish were fed until satiation twice a day on a diet of TetraMin tropical flakes.

2.2 Experimental Design and Sample Collection.

Two acute toxicity tests were carried out to determine the maximum tolerated concentration of chlorothalonil in which survival did not significantly differ from control animals over 96 h. The first bioassay contained five nominal concentrations of chlorothalonil that were 0.005, 0.01, 0.02, 0.04, 0.08 mg/L, and a control group. Each replicate (n=3 per dose) contained 5 adult zebrafish randomly selected (male and female) per container, and animals were kept under a light:dark cycle 12:12 h, constant aeration and a temperature of 26°C. Over a 96 h period, survival was recorded every 2 h for the first 24 h, follow by every 4 h until 96 h. Mortality only occurred with concentrations of 0.04 and 0.08 mg/L and were 40 and 100% mortality respectively. A second experiment was conducted with a narrower range of chemical concentrations (0.03, 0.035 and 0.038 mg/L) under the same conditions as the first experiment, and at the concentration of 0.038 mg/L, there was 27% mortality while

there was none in the lower concentrations. Thus, it was reasoned that 0.035 mg/L was the maximum tolerated concentration with 100% of survival after 96 h.

For the third bioassay, a lower, environmentally-relevant concentration of chlorothalonil was obtained from the literature by compiling data on fungicide concentrations detected in water (mean value of 0.007 mg/L; Hugh *et al.*, 1994; Davies *et al.*, 1994; EPA, 1999; Gómez *et al.*, 2001). Thus a bioassay was performed to assess differences in morphometric and gene expression patterns in zebrafish exposed to chlorothalonil at 0.007 (environmentally-relevant) and 0.035 mg/L (sub-lethal). A solvent control (acetone) was included at a concentration less than 0.1%. Three 20 L aquaria were used per treatment, using 29 male zebrafish (0.32 ± 0.027 g wet weight) per aquarium under a light:dark cycle 12:12 h, constant aeration, and chlorothalonil was applied only once at the beginning of the experiment (time 0 h) to mimic a pulse exposure from a pesticide application. After 96h of exposure, fish were sampled to measure gene expression profiles in the liver. Following administration of tricaine anesthetic (0.02 mg/ml), adult zebrafish were individually weighed (± 0.001 g) and total body length determined (± 0.1 mm) for the calculation of the condition factor [$k = 100 \times (\text{body weight} / \text{length}^3)$]. Before dissection, zebrafish were sacrificed by severing the spinal cord. Livers were excised, weighed for calculation of the liver somatic index [$LSI = 100 \times (\text{liver weight} / \text{body weight})$] and then preserved in RNAlater®. Sex was carefully determined before the experiment using morphometric landmarks, including the size and shape of the body and fins (Mills, 1994), and verified during dissection by visual inspection of the gonad. All survival data were analyzed in Prism (6.0) using a Log-rank or Mantel-Cox test.

2.3 Analytical Chemistry

Analytical grade chlorothalonil (98% purity) was purchased from Sigma–Aldrich (San Diego, Ca, USA) and acetone (HPLC grade from Fisher Scientific Co.) was used as the carrier. Water from the main experiment was analyzed using mass spectrometry, six samples were run, one sample per treatment (control, concentration low and high). Water samples were collected from each aquarium, three at the start and three at the end of the experiment after 96h. Samples were extracted from 1 L of water by the method 3510C EPA liquid-liquid extraction (EPA, 1996), the percentage of recovery for chlorothalonil with this method is 60-80%. Measured chlorothalonil levels were determined using an Agilent 240 Ion Trap GC/Mass Spectrometer 7890B. The standard was chlorothalonil analytical grade prepared for injection with a concentration of 0.208 ng/ μ l in acetone with a retention time of 14.268 ± 0.200 min. To run the standard and samples, 1 μ l was injected. Chromatographic column was an Agilent CP7841 for pesticides. The main oven temperature program comprised an isothermal period at 70 °C for 2 min, a ramp of 20 °C min⁻¹ to 185 °C for 1.25 min, followed by a ramp of 4 °C min⁻¹ to 275 °C for 7.5 min and a final period of

10 °C min⁻¹ to 300 °C for 2.5 min, for a run time of 45 min. Injector was set to splitless mode, heater 275 °C, total flow 105 mL/min, injection pulse pressure 40 psi until 0.8 min and the purge flow to split vent 100 mL/min at 0.75 min. The carrier gas was helium at a pressure of 28.128 psi, corrected constant flow rate of 2 mL/min, average velocity 40.309 cm/sec, holdup time of 2.0674 min and the flow program was set to 2 mL/min for 8 min, then 0.5 mL/min per min to 1.5 mL/min for 36 min, for a run time of 45 min. Mass spectra were acquired in the range *m/z* 45–500 at the acquisition rate of 100 spectra s⁻¹. The ion source temperature was set at 230 °C and the transfer line temperature was set at 250 °C. The detector voltage was 1500 V and the ionization electron energy was set at 70 eV.

2.4 RNA Extraction.

RNA was obtained from livers for microarray analysis and real time PCR for targeted genes. Total RNA was isolated using TRIzol (Life Technologies Inc., Burlington, ON, Canada) according to the manufacturer's protocol. RNA was column purified using the RNeasy Mini Kit (Qiagen, Mississauga, Ont., CAN.) following the manufacturer's protocol. RNA quantity was determined using a NanoDrop ND2000 (Nanodrop Technologies, Wilmington, DE, USA) and RNA quality was determined using a 2100 BioAnalyzer (Agilent Technologies, Mississauga, ON, Canada) for all samples. Mean RNA integrity number (RIN) for samples used in the microarray analysis and real-time PCR was 9.14 ± 0.54 (mean ± SD).

2.5 Microarray Analysis.

Commercial *Danio rerio* microarrays (V3, 4 x 44K format GPL14664, Agilent Technologies Inc.) were used to identify transcripts differentially regulated by chlorothalonil. There were 15 microarrays performed in total, and sample sizes were as follows; n=6 for controls, n=5 for 0.007 mg/L and n=4 for 0.035 mg/L. RNA labeling, microarray hybridization, and microarray analysis were performed according to Agilent's One-Color Microarray-Gene Based Expression Analysis protocol (Version 6.5, May 2010; Agilent, Mississauga, ON, CAN). Raw expression data were extracted from TIFF images using Feature Extraction Software (Version 10.7.3.1.). Microarray data were deposited into Gene Expression Omnibus (GSE67600) and are MIAME compliant (<http://www.ncbi.nlm.nih.gov/geo/info/MIAME>). Intensity data were imported into JMP® Genomics (version 6.1) and data were normalized using Quantile normalization. DEGs (differentially expressed genes) were identified using one-way ANOVA and a false-discovery rate of 5% (FDR = 0.05) after removing all control spots from the normalized dataset. Probes that showed signal intensity below 1.5 were filtered to a value of 1.5, as this was determined to be the limit of detection of

the microarray based upon the lowest standard curve points and Agilent negative controls.

2.6 Bioinformatics

To broaden our analysis of the transcriptomic responses, any probe that showed a change with respect to the control ($p < 0.05$) prior to FDR adjustment in one or both concentrations of chlorothalonil was used for the PCA and for clustering. This was done to determine if there was any evidence of separation in transcriptomes among groups. Principal Component Analysis on probes was performed in JMP Genomics and rows and columns were scaled prior to PCA. Two-way hierarchical clustering of differentially expressed probes was performed using the Fast Ward algorithm. Rows were centered to a mean of zero prior to clustering and were also scaled to a variance of one. Gene set enrichment on gene ontology (GO) terms was conducted using the Parametric Analysis of Gene Set Enrichment (PAGE) algorithm which is a two-sided z-score for gene ontology categories as described in Kim and Volsky (2005). Pathway Studio 9.0 (Ariadne, Rockville, MD, USA) and ResNet 9.0 were utilized for sub-network enrichment analysis (SNEA). SNEA uses known relationships (i.e. based on expression, binding, common pathways) between genes to build networks focused around gene hubs. This approach has been applied in biomarker discovery in aquatic organisms and additional details can be found in Chishti et al. (2013) and Langlois and Martyniuk (2013). "Name + Alias" was used for mapping zebrafish genes to mammalian homologs in Pathway Studio, successfully mapping 20,694 probes. For all analyses, annotated pathways in Pathway Studio were expanded to include cell processes and functional classes in target gene seeds, as well as diseases related to chlorothalonil (p-value cut-off for Enrichment analysis was set at $p < 0.05$). The analysis used the function "highest fold change, best p-value" for duplicate probes. This bioinformatics method leverages the entire dataset regardless of p-value and builds a distribution based on fold change to statistically test for enrichment of processes.

2.7 Real-time PCR

Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were followed (Taylor et al., 2010). For real-time PCR experiments, RNA was DNase-treated for 30 min at 37 °C with the TURBO DNA-free Kit (Ambion) following manufacturers' protocol prior to cDNA synthesis. DNase treated RNA (100 ng total RNA) was converted to cDNA using the iScript cDNA synthesis kit (BioRad) as per manufacturer's protocol.

Real-time PCR analysis using the CFX Connect™ Real-Time PCR Detection System was prepared with 12 biological replicates for each treatment in duplicate,

including 4 samples that did not receive reverse transcriptase during cDNA synthesis and 4 samples that did not receive cDNA template (negative controls). Normalized gene expression values were extracted using CFX Manager 3.1 software (Bio-Rad laboratories) using a relative $\Delta\Delta C_q$ method as per previous methods (Chishti et al., 2013). Of all the genes assessed for normalization (*bactin*, *rps18*, *gapdh*), the combination of *bactin* and *rps18* gave the most stable baseline (based on the M-value in CFX Manager 3.1) for real-time PCR experiments and were used to normalize all expression data. These transcripts did not significantly change across experimental groups in terms of expression (data not shown).

2.8 Statistics

Shapiro-Wilks test for normality and homogeneity of variance (Levene's test) were conducted on morphometric and real time PCR gene expression data. If data were normal, a one-way ANOVA was used, followed by a post-hoc analysis (Dunn's test) to test for differences between each treatment relative to the control. If data were non-normal, a non-parametric Kruskal-Wallis test was used, followed by a Dunn's test. Significance was set at $\alpha = 0.05$. All statistical analyses for real-time PCR and morphometrics were conducted in GraphPad Prism V6.0.

3. Results

3.1 Water analysis and experimental observation

Chlorothalonil was not detected in the control aquaria. The concentration measured in the water at the beginning of the experiment was below detection limits of the bioassay. However both the low and high concentrations were detectable in the water at the beginning of the experiment (Table 1). There were no mortalities during the exposure to chlorothalonil but some fish exhibited abnormal behavior in the 0.035 mg/L treatment, including loss of equilibrium, reduced feeding, and erratic swimming, which was suggestive of toxic effects.

3.2 Concentration response.

The percentage of zebrafish survival following chlorothalonil exposure was the following; 0% for 0.08 mg/L, 60% for 0.04 mg/L, and 100% survival for 0.02, 0.01, and 0.005 mg/L after 96h of exposure (Fig. 1, left pane). There was a significant difference among survival curves (d.f. = 5; Chi square = 363.3, $P < 0.0001$). In the second experiment 73.3% of the individuals survived following 0.038 mg/L treatment, and there was 100% survival at 0.035 mg/L and 0.030 mg/L respectively (Fig. 1, right pane). There was a significant difference between the three survival curves (d.f. = 3; Chi square = 33.7, $P < 0.0001$). We point out that in the figure, treatments with 100% survival overlap.

3.3 Morphometrics.

There were differences in mean condition factor and liversomatic index (LSI) between the control group and the two concentrations of chlorothalonil (Fig. 2). Significant differences were detected in condition factor among treatments (Kruskal-Wallis, $H=9.7$, $p=0.008$) and there was an increase in individual condition factor with both treatments. Conversely, mean LSI decreased in individuals from the treatment groups compared to the control group (ANOVA, $F=12.06$, $p<0.001$). No differences were detected between the two concentrations of chlorothalonil for condition factor and LSI.

3.4 Gene expression analysis.

All gene expression data from the microarray analysis are provided in Appendix 1. Microarray analysis revealed that for individuals exposed to the low concentration (0.007 mg/L), 2390 probes were significantly increased and 3197 probes were significantly decreased with $p \leq 0.05$ (prior to FDR correction). In this group, the highest changing probes for 0.007 mg/L, based on fold change, were vitellogenin (*vtg*) 1 to 7 (*vtg1*, *vtg2*, *vtg3*, *vtg4*, *vtg5*, *vtg6* and *vtg7*), which showed a 71 to 178-fold increase following exposure. Zona pellucida (*zp*) glycoprotein 2 and 3 (*zp2* and *zp3*) and immunity-related GTPase family f4 (*irgf4*) were also affected in this group. Gene expression changes at 0.035 mg/L included 2252 probes that were significantly increased and 2290 probes that were significantly decreased following chlorothalonil treatment. The probes with the highest fold change were *zp* glycoprotein 2 and 3 (*zp2* and *zp3*), claudin d (*clndd*), (>50-fold) and spermatogenesis associated 6-like (*spata6l*) (34.3-fold). Hierarchical clustering revealed a clear separation between treatments based upon global gene expression (Fig.3.). We point out that these probes were those that were significant at an $\alpha=0.05$ prior to a post-hoc test. Following a stringent FDR at 5%, only myosin binding protein (*mybph*) Ha and keratin 97 (*zgc:92061*) in the low dose and *mybph* in the high dose passed the FDR.

Principal Component Analysis (PCA) supported the cluster analysis. Each stage grouped separately from each other along three PCAs (Suppl. Fig. 1). PCA1 explained 93.9% of the variation in expression; PCA2 explained 1.9%, while PCA3 explained 0.6%. PAGE analysis with FDR corrected p-value ($p < 0.05$), categorized by molecular function (Gene Ontology term), revealed that 27 molecular functions were affected at the gene level in individuals in the low treatment, including cell division, glycolysis, cell redox homeostasis, response to estrogen stimulus. In the high concentration, there were 20 molecular functions affected at the gene level in individuals and these included cell redox homeostasis, protein disulfide oxidoreductase activity, innate immune response, RNA processing, negative

regulation of transcription, nucleosomal DNA binding and DNA-dependent (Appendix 2).

Genes were categorized by function to identify cellular and biological process differentially affected by chlorothalonil. Data from the SNEA analysis are presented in Appendix 3. SNEA analysis for the 0.007 mg/L group indicated that 165 biological processes were affected (FDR corrected p-value $p < 0.05$). Cellular processes affected by chlorothalonil at the transcriptome level in the liver included reproduction, immune response, detoxification, regulation of cell, and DNA replication (positive and negative). Molecular networks affected in the liver of zebrafish exposed to 0.007 mg/L chlorothalonil were related to VEGFR signaling, histone and DNA methylation, Toll like receptors signaling, transcription factor, and pyruvate metabolism. SNEA analysis for animals exposed in the 0.035 mg/L treatment revealed that 155 processes were affected at the gene level (FDR corrected p-value, $p < 0.05$). Biological processes affected in this group included immune system, stress response, detoxification, and glucose biosynthesis. Common biological processes that contained differentially expressed genes were DNA and cell replication, detoxification, DNA damage, and metabolic processes, for example gluconeogenesis (Table 3).

Pathway Studio was used to identify gene sub-networks that were significantly affected by chlorothalonil. Appendices 3 and 4 contains all the significantly affected pathways identified using SNEA and GSEA (enrichment $P < 0.05$) respectively. A Venn diagram revealed that there were 84 molecular pathways (based on SNEA) unique for the low concentration treatment, 57 unique for high concentration, and 87 in common between both concentrations (Fig.4.) Pathways identified in the 0.007 mg/L group totaled 164, while those in the 0.035 mg/L totaled 154. Gene networks related to reproduction and immune system were down-regulated in zebrafish liver exposed to the low concentration (0.007 mg/L), and gene networks related to the stress response and immune system were down-regulated following exposure to 0.035 mg/L chlorothalonil. Vascular Endothelial Growth Factor (VEGF) signaling pathway was down-regulated by 0.007 mg/L chlorothalonil (Fig.5). Glycogen pathway at the mRNA level was also affected in the liver by 0.035 mg/L chlorothalonil (Fig. 6), the glycogen metabolism was upregulated following exposure.

3.5 Real-time PCR

The steady state mRNA levels of *Cyp1a* were significantly decreased by the high concentration of chlorothalonil (Fig. 7A). *Vtg1* showed congruence between real-time PCR and microarrays, and was expressed in individuals from each treatment group but not in individuals from the control group. However, there appeared to be differences in individual sensitivity to the chemical in terms of *vtg* induction and not all fish expressed the transcript in the liver. Real-time PCR assays for *gsr*, *hif1a*,

esr1 and *vtg* were not significantly different between groups (Fig.7B-E); however, the microarray and qPCR data showed a significant and positive relationship for relative fold change from control (Fig7F).

4. Discussion

In this study, we evaluated the effects of an environmentally relevant and sub-lethal concentration of chlorothalonil in the water on adult male zebrafish. This is the first study exposing *Danio rerio* to the fungicide chlorothalonil, and the first report of molecular responses in fish following exposure. Our data suggest that there are prevalent biological themes affected by exposure, which we describe below. Moreover, gene expression patterns show a concentration-dependent response, and there were differences between responses at a relevant environmental concentration compared to a sub-lethal concentration, however we do point out that there were overlapping molecular processes that were affected by both concentrations of the fungicide.

The main mode of action of chlorothalonil has been attributed to its ability to bind and inhibit cellular glutathione (GSH) and glyceraldehyde 3-phosphate dehydrogenase GAPDH, as well as other enzymes necessary for glycolysis (Long and Siegel, 1975, Caux *et al.*, 1996). This mode of action was supported in this study based on the observation that genes differentially expressed between control and treated animals were related to metabolism of glutathione, amino acid and lipoprotein metabolism, pyruvate and lactate metabolism, and carbohydrate metabolism. Further findings indicated that chlorothalonil also elicits responses for transcripts that participate in glycogen, gluconeogenesis, noradrenaline metabolism, methionine, and homocysteine metabolism.

The toxicity observed in rodent kidneys is thought to be related to the metabolism of chlorothalonil by the action of γ -glutamyl transpeptidase and cysteine-conjugate β -lyase, resulting in the production of di- and tri-thiols (IARC, 1999). These compounds are bound to macromolecules and affect mitochondrial respiration in the kidney, leading to ATP depletion, cytotoxicity, necrosis, and compensatory hyperplasia, which may eventually progress to tumor formation (Wilkinson and Killeen, 1996). Both concentrations of chlorothalonil used here down-regulated the levels of transcripts coding for the gene cystathionine-beta-synthase b (CBS), which catalyzes the pyridoxal 5'-phosphate (PLP)-dependent β -replacement reaction, in which the thiolate of L-homocysteine replaces the hydroxyl group of L-serine. Mutations in the CBS gene can alter mRNA or enzyme stability, activity, binding of PLP, or impair allosteric regulation (Wilson and Kraus, 2004). Also, in the 0.035 mg/L chlorothalonil treatment, the protein-coding thiol-disulfide exchange pathway was

significantly affected. These results suggest that chlorothalonil may be interfering with enzymes due to its affinity for thiol groups, which is one of the most recognized modes of action of this fungicide. However, these data are at the transcript level and additional studies are required to verify impaired enzyme function in the zebrafish liver following exposure.

Pescador *et al.* (2010) propose that the hypoxia-inducible factor (HIF) mediated increase in glycogen stores is a hypoxia-triggered adaptation that prepares cells to cope with further oxygen restrictions by ensuring adequate substrate supply for anaerobic glycolysis. Based on microarray data, there was an overexpression of HIF1a in both concentrations and an upregulation of the glycogen metabolism pathway in fish exposed to the sublethal concentration. Pelletier *et al.* (2012), demonstrated that the mRNA and protein levels of the first enzyme of glycogenesis, phosphoglucomutase 1, were increased in hypoxia and established that hypoxia-induced glycogen stores are rapidly mobilized in cells that are starved of glucose. In our study, the mRNA for phosphoglucomutase was upregulated. Based on this, we hypothesize that chlorothalonil interrupts cellular respiration, and induces oxidative stress and hypoxia. This results in a higher demand for energy, which manifests as glucose imbalance due to general stress, leading to a transcriptome response that upregulates genes involved in the glycogen metabolism pathway in the fish. To verify this mode of action, future studies should measure glycogen and triglycerides stores in the liver.

Morphometric measurements showed a decrease in the size of the zebrafish liver exposed to chlorothalonil compared to control fish after only 96 h. Although it is not clear how chlorothalonil can have such a rapid effect on the liver size, expression analysis indicates that this fungicide inhibits the pyruvate oxidation route and the tricarboxylic acid cycle, suppresses gluconeogenesis, disrupts oxidative phosphorylation and consequently reduces the production of ATP. In support of this, there was a decrease in genes related to gluconeogenesis in both treatments. Therefore it is plausible that the energy reserves in the liver are rapidly consumed in response to the deficiency of energy production. Further studies are necessary to confirm the energetic and functional liver responses, as well as tissue-level responses following exposure of chlorothalonil. However, it is worth mentioning that in the 0.007 mg/L exposure, transcripts involved in the vascular endothelial growth factor receptor (VEGFR) pathway was significantly down-regulated, and included *vegfc*, *vegfa*, *stat1*, *stat3*, *stat6* and *foxo3*. The *VEGF* pathway is related to angiogenesis and neovascularization, and its suppression has been suggested as a possible cancer therapy (Wood, 2000). Shah *et al.* (2007) hypothesized that the *VEGF* signal transduction pathways, when activated or inhibited, play a key role in drug-induced liver injury due to oxidative stress (Shah *et al.*, 2013 & Han *et al.*,

2010). It has also been suggested that *VEGF* induces the release of the hepatic mitogens, IL-6 and hepatocyte growth factor from endothelial cells (Le Couter *et al.*, and Davidson *et al.*, 2003). In this regard, if chlorothalonil inhibits *VEGF* and is also able to elicit liver damage, a possible explanation of liver reduction could be related to the impairment of the angiogenesis processes. However, this hypothesis must be further tested.

The USEPA categorizes chlorothalonil as a probable carcinogen in humans due to lack of evidence (USEPA, 1999). Under the conditions of our assay, we found transcriptional enrichment of functions that are known to be active in carcinogenic responses. In both chlorothalonil treatments, induction of genes involved in cellular functions related to the development of cancer were observed (oncogenesis, histone and DNA methylation, oxidative stress, lipid peroxidation, reactive oxygen species, DNA damage). Lodovici *et al.* (1997) reported that exposure to chlorothalonil resulted in the generation of reactive oxygen species capable of inducing cell genetic damage in liver DNA of rats. Our data indicate that genes related to liver DNA oxidative damage are induced by chlorothalonil, which is consistent with the report of Yamano and Morita (1995), who demonstrated *in vitro* chlorothalonil-induced lipid peroxidation in rat hepatocytes. Rosenkranz *et al.* (1997) reported that chlorothalonil was able to inhibit gap junctional intercellular communication, which is related to tumor promotion. We therefore hypothesize that oxidative damage may contribute to the mutagenicity and carcinogenicity suspected of this fungicide.

Lastly, the bioinformatics analysis suggested that chlorothalonil may affect transcriptional networks associated with reproduction, as networks that included fertilization, male reproduction, and testis function among others were altered. Chlorothalonil has been associated with acute and chronic toxicity in a number of species (Caux *et al.*, 1996) and impaired reproductive processes may be a secondary effect of the chemical. For example, chlorothalonil was observed to suppress aromatase activity in human placental microsomes, but the authors proposed that this was more likely due to cytotoxicity of the compound than a specific mode of action (Andersen *et al.*, 2002). Many fungicides are known to impact the reproductive system, and can interfere with the androgen signaling pathway (e.g., dicarboximide (vinclozolin) and conazole fungicides (prochloraz) (Hotchkiss *et al.*, 2008, Martinović *et al.* (2011). Of interest, we point out that *zona pellucida (zp)* mRNA was identified by the microarray analysis as a potential target of chlorothalonil. In both the low and high treatment, this transcript showed a 45 and 54-fold change in expression prior to an FDR correction. Moreover, *vtg* appeared to be induced in some males exposed to the low concentration 0.007 mg/L. The wide variability in response may be due to individual differences related to susceptibility and uptake as previously reported for fish (Marlatt *et al.* 2010).

Nevertheless, based on the network analysis, we suggest that additional studies investigate reproductive impacts of chlorothalonil in fish as current literature is scarce on this topic.

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632

633 Legends

634 **Fig.1. Survival curves from experimental concentration of chlorothalonil in the**
635 **water.** Range finding experiments identified the maximum tolerated nominal
636 concentration for zebrafish to obtain a concentration that showed 100% survival. A)
637 Percent survival from experiment 1 and B) Percent survival for experiment 2. In both

graphs, there was 100% survival (A) for control, 0.005, 0.01, and 0.02 mg/L and (B) for control, 0.03 and 0.035 mg/L therefore the data overlap in the figure.

Fig.2. Condition factor and liversomatic index of zebrafish exposed to two concentrations of chlorothalonil, 0.007mg/L and 0.035 mg/L. Shown are each individual measurements, and the horizontal bar is mean \pm SEM.

Fig.3. Hierarchical clustering of gene expression data. Gene probes used to cluster were those that were differentially expressed ($p < 0.05$) in one or both treatments. Blue indicates low relative mRNA levels of the gene and red indicates high relative mRNA levels of the gene after centering rows.

Fig.4. Venn diagram for the sub-network enrichment analysis (using Venny, Oliveros 2007-2015). Cell L SNEA corresponds to 0.007 mg/L and Cell H SNEA corresponds to high 0.035 mg/L concentrations ($P < 0.05$).

Fig.5. Gene network showing a down regulation of genes involved in VEGFR signaling in the low concentration (0.007 mg/L). The color blue indicates that the gene is decreased in the network and grey indicates that the gene was not present on the zebrafish array or was below detection limit of the analysis. Abbreviations are provided in the abbreviation list.

Fig.6. Gene network showing an upregulation of genes involved in glycogen metabolism signaling in the high concentration (0.035mg/L treatment). Red indicates that the gene is increased and blue indicates that the gene is decreased in the network. Abbreviations are provided in the abbreviation list.

Fig.7. Normalized expression data from real-time PCR (A) *cyp1a* (B) *gr* (C) *hif1a* (D) *esr1* and (E) *Vtg1*. Graph (F) shows the comparison of fold changes between real-time PCR data on the X-axis and microarray data on the Y-axis for the same genes.

Table 1. Chlorothalonil water concentrations at time 0 and time 96 hours. BDL = below detection limit.

Table 2. Oligonucleotide primers used for real-time polymerase chain reaction assays.

Table 3. Sub-network enrichment analysis (SNEA) for cellular processes in *Danio rerio* ($p < 0.05$). Pathways were manually grouped into major biological themes.

Legends to supplementary files

670 **Supplemental Figure 1.** Principle component analysis of transcriptomic responses
671 in the liver of zebrafish. The control and treatment groups shows separation, with a
672 few overlapping points.

673 **Supplemental Figure 2.** Venn diagram for common genes between concentrations
674 (uncorrected p 0.05).

675 **Supplemental Figure 3.** Venn diagram for sub-networks. Common gene ontologies
676 after PAGE analysis uncorrected p 0.05.

677 **Supplemental Figure 4.** Pathway for Reproduction affected in the low concentration
678 0.007 mg/L.

679 **Supplemental Figure 5.** Gene network for Double Strand Homologous DNA repair
680 at the high concentration 0.035 mg/L.

681 **Supplemental Figure 6.** Gene network for histone and DNA methylation at the low
682 concentration 0.007 mg/L.

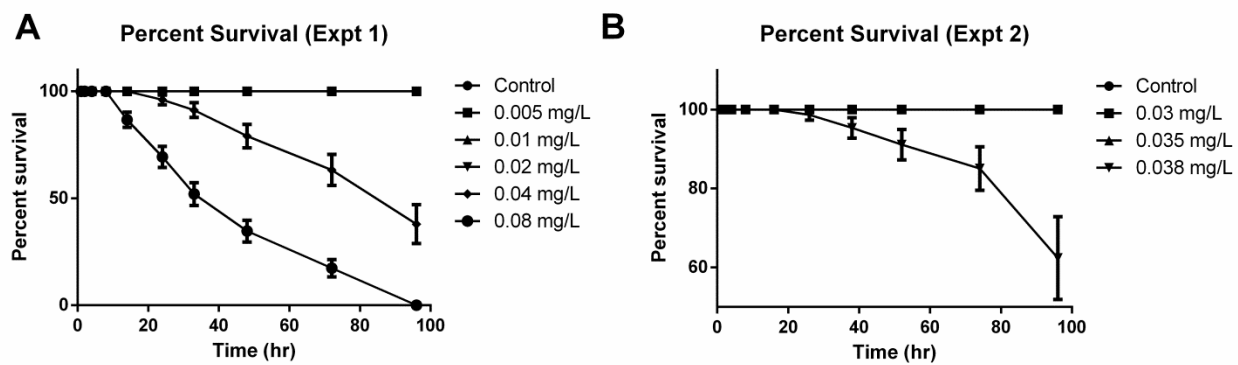


Figure 1.

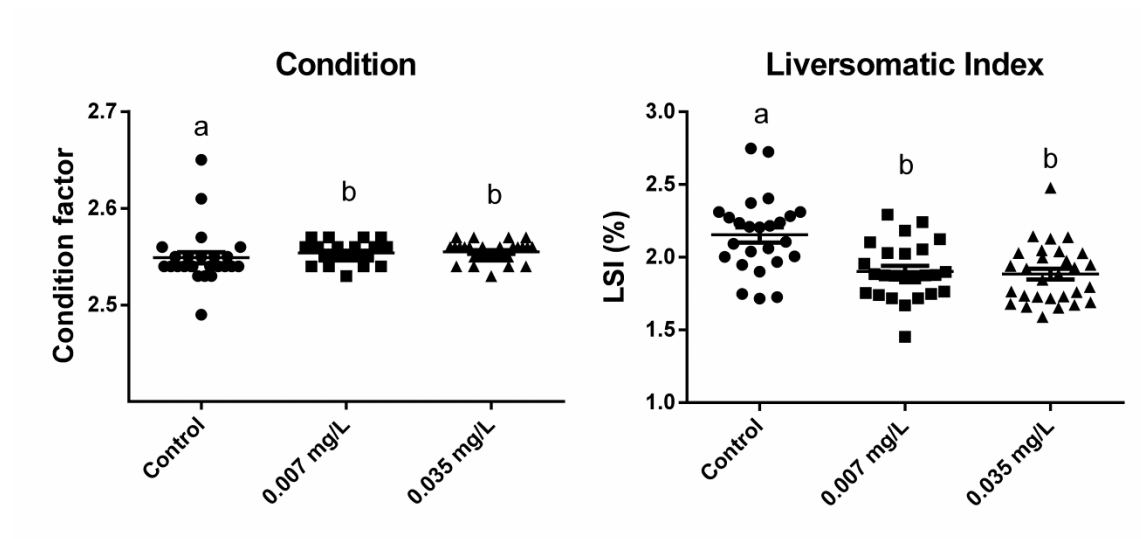


Figure 2.

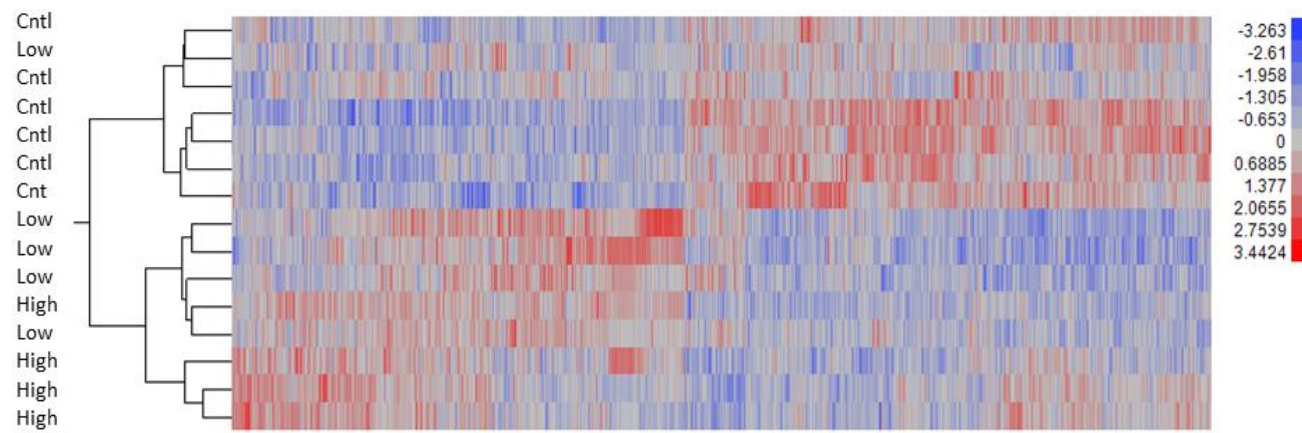


Figure 3.

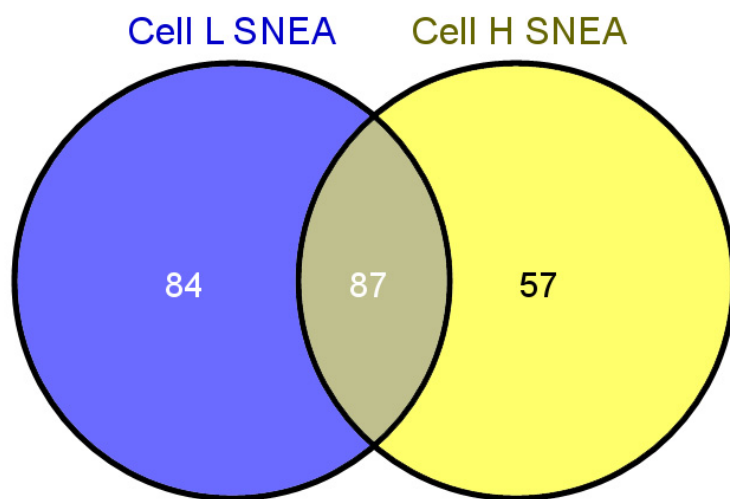


Figure 4.

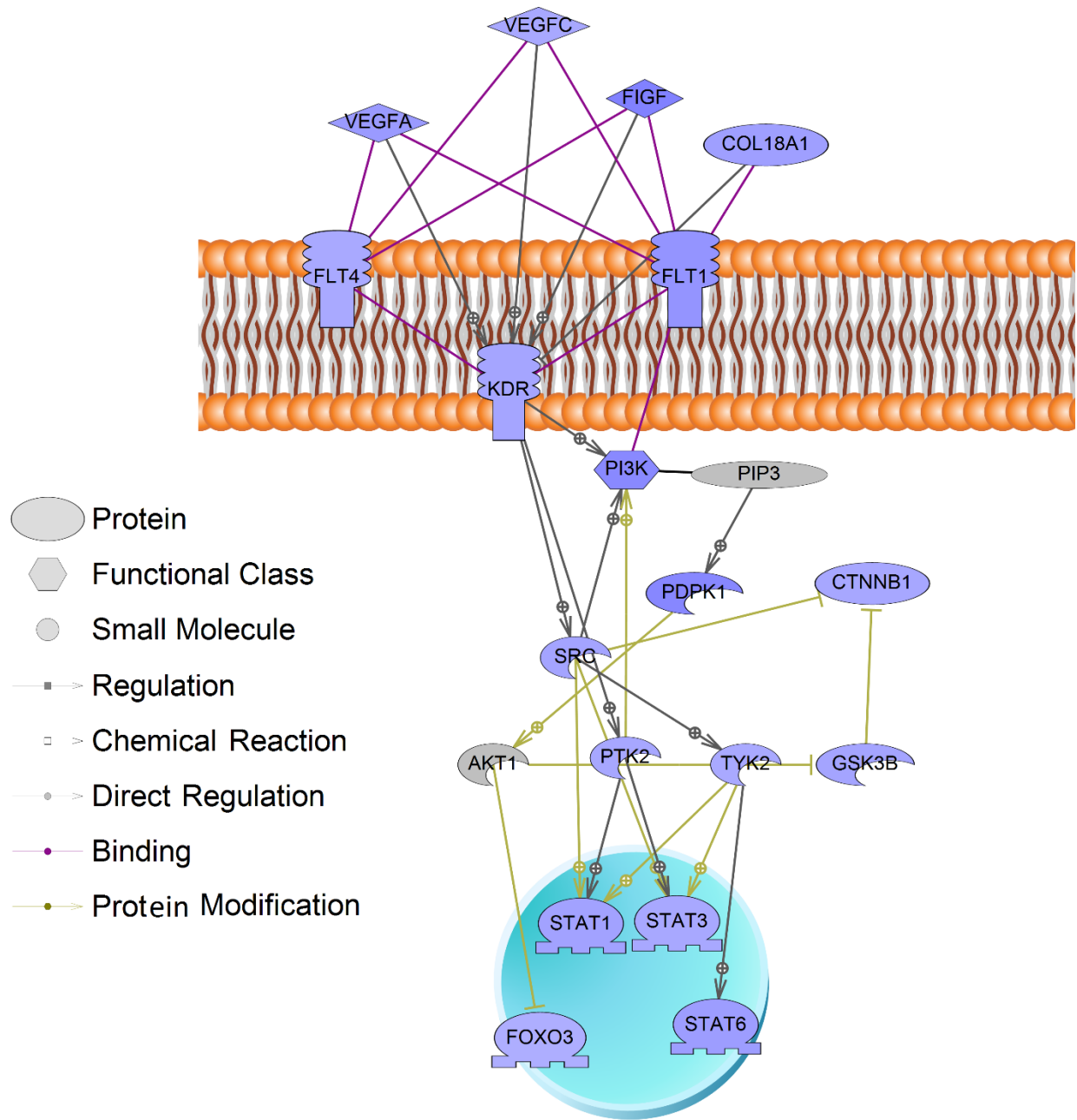


Figure 5.

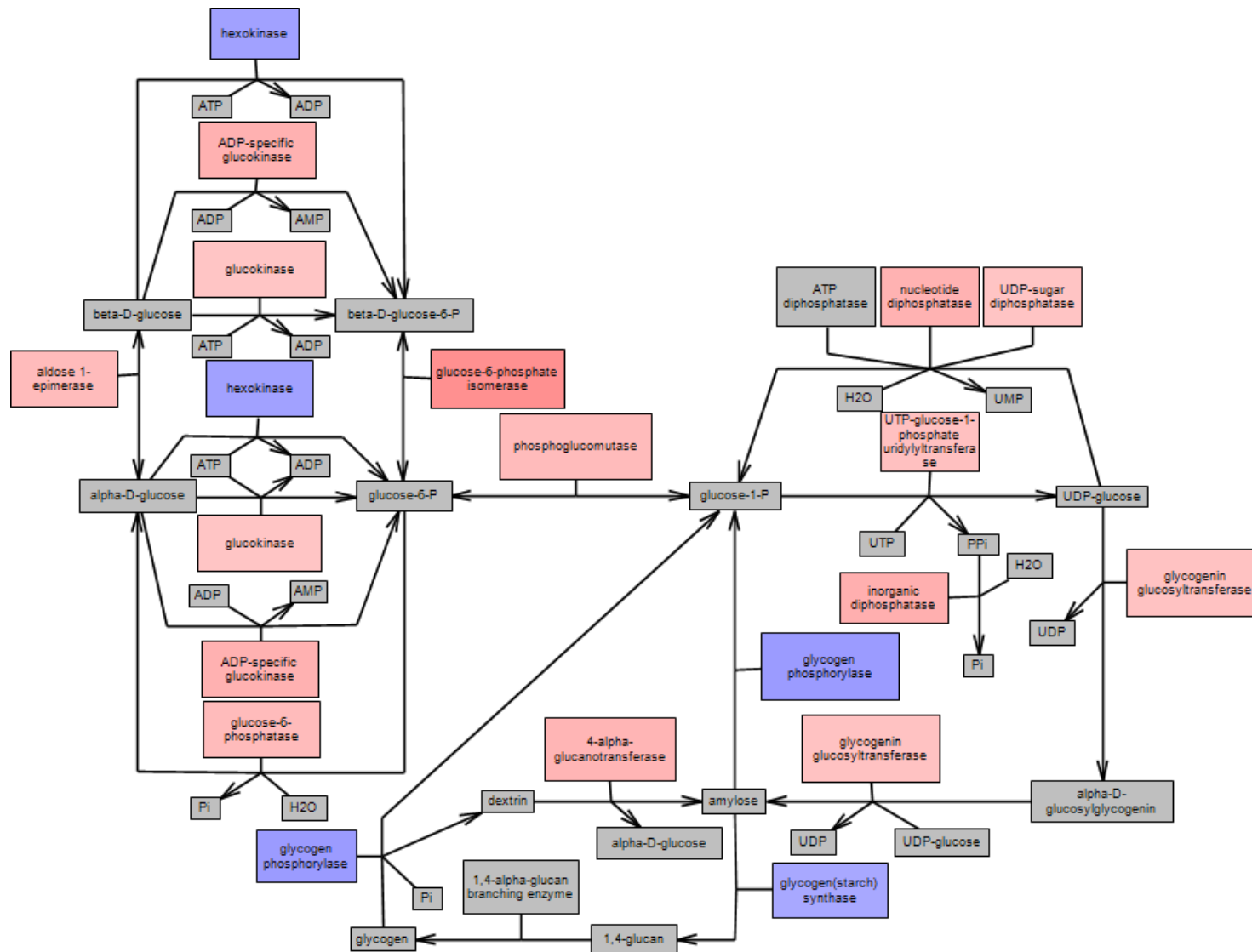


Figure 6.

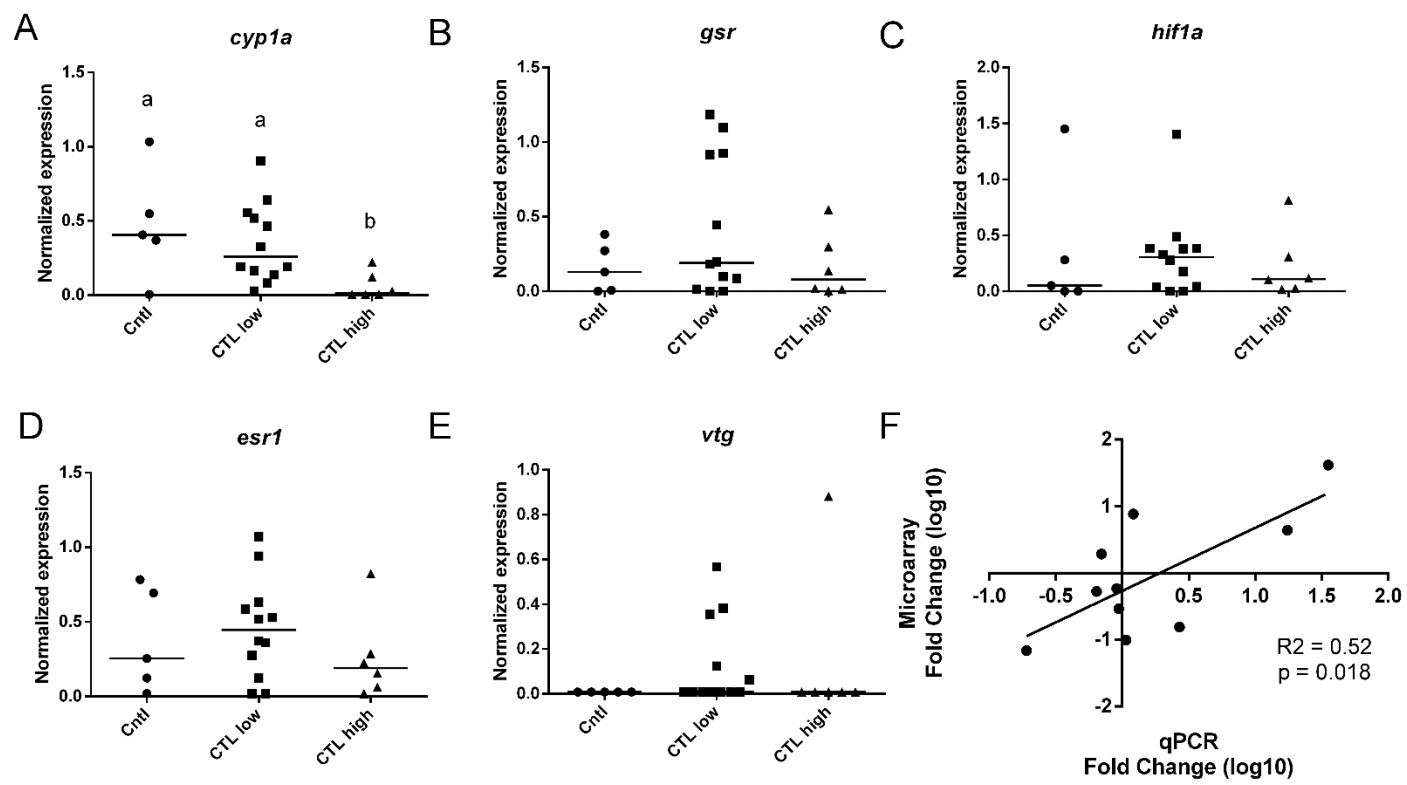


Figure 7.

Table 1. Chlorothalonil water analysis concentrations.

Sample	Target concentration	Measured concentration 0h.	Measured concentration 96h.
	mg/L	mg/L	mg/L
Control	BDL	BDL	BDL
Low concentration	0.007	0.00095	BDL
High concentration	0.035	0.0114	5x10 ⁻⁸

Table 2. Oligonucleotide primers used for real-time polymerase chain reaction assays.

Gene name	Simbol	Forward	Reverse	Reference
Beta-actin	ACTB	5'-CGAGCAGGAGATGGGAACC-3'	5'-CAACGGAAACGCTCATTGC-3'	McCurley & Callard <i>et al.</i> , 2008
Ribosomal subunit 18	18s	5'-TCGCTAGTTGGCATCGTTTATG-3'	5'-CGGAGGTTCTGAAGACGATCA-3'	McCurley & Callard <i>et al.</i> , 2008
vitellogenin 1	vtg1	5'-GCTGCTGCATCTGTCAATGT-3'	5'-CTGCTGCTGCTTTCAGAAGA-3'	Wang <i>et al.</i> , 2005
hypoxia induced gene 1	hif1a	5'-TCTCACCTGGACAAAGCCTCCATT-3'	5'-AAGCCATTTCAGCTGACTTTCCAGC-3'	Liu <i>et al.</i> , 2014
glutathione reductase	GR	5'-ATTGGCAGAGAACCAACAC-3'	5'-ACATCCCCGACTGCATAGAC-3'	Song <i>et al.</i> , 2009
Estrogen receptor 1	esr1	5'-CAGGACCAGCCCGATTCC-3'	5'-TTAGGGTACATGGGTGAGAGTTTG-3'	Chandrasekar <i>et al.</i> , 2010
cytochrome P450, family 24, subfamily A, polypeptide 1	CYP1A	5'-TCCACTCGATCGCTCCGGGTT-3'	5'-GCGGTTTAGGCGCATGAGCAGAT-3'	Sander <i>et al.</i> , 2012

Table 3. Sub-network enrichment analysis (SNEA) for cellular processes in *Danio rerio* ($p < 0.05$). Pathways were manually grouped into major biological themes.

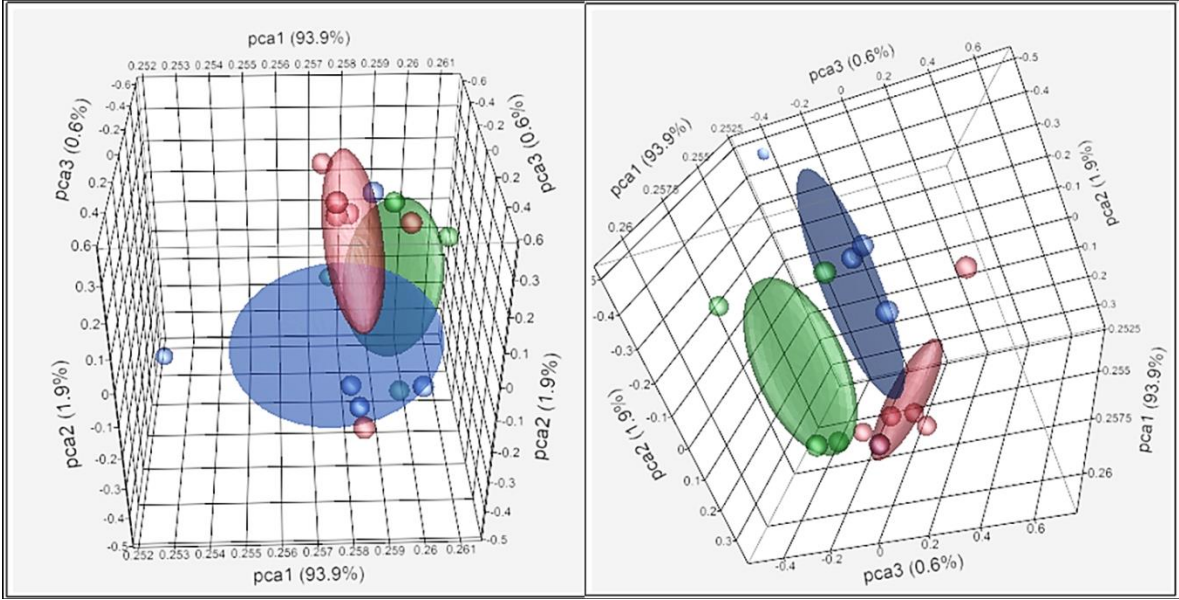
Concentration	Biological process	Gene set seed	Total # in Pathway	# of Measured entities	Median change
Low 0.007 mg/l	Reproduction	male reproduction	36	22	-1.43
		pregnancy	1008	715	-1.24
		testis function	83	63	-1.13
		fertilization	360	258	-1.13
		lactation	249	186	-1.27
		mammary gland growth	39	31	-1.15
		reproductive process	51	34	-1.14
	Immune System	killing of inflammatory cells	29	23	-1.24
		xenobiotic metabolism	54	32	-1.56
High 0.035 mg/l	Stress response	response to stress	251	182	-1.14
		hyperosmotic response	14	12	-1.08
		osmotic pressure	18	12	-1.62
	Immune System	TLR signaling pathway	19	15	1.18
		mitochondrial membrane permeability	188	153	-1.22

		mitochondrial membrane potential	276	224	-1.18	
		encapsidation	26	19	-1.30	
		disease resistance	43	29	-1.02	
		T-cell tolerance	75	47	-1.27	
		necrotic cell death	159	129	-1.26	
					High	Low
	DNA and cell Replication	DNA replication	1410	1073	-1.03	1.01
		DNA replication checkpoint	51	41	1.78	2.11
		DNA replication during S phase	14	12	2.18	3.93
		DNA replication initiation	83	63	1.61	2.01
		DNA replication licensing	13	12	2.67	6.26
		DNA strand elongation	11	8	2.93	6.26
		DNA unwinding	152	124	1.35	1.31
		Okazaki fragment processing	11	10	1.99	2.91
		centromere binding	11	6	2.86	4.61
		chromosome condensation	261	205	1.16	1.18
		chromosome org. and biogenesis	27	24	1.47	1.61
		chromosome segregation	240	189	1.25	1.31
		G1 phase	597	438	-1.07	-1.07
		G1/S transition	659	506	-1.03	1.02

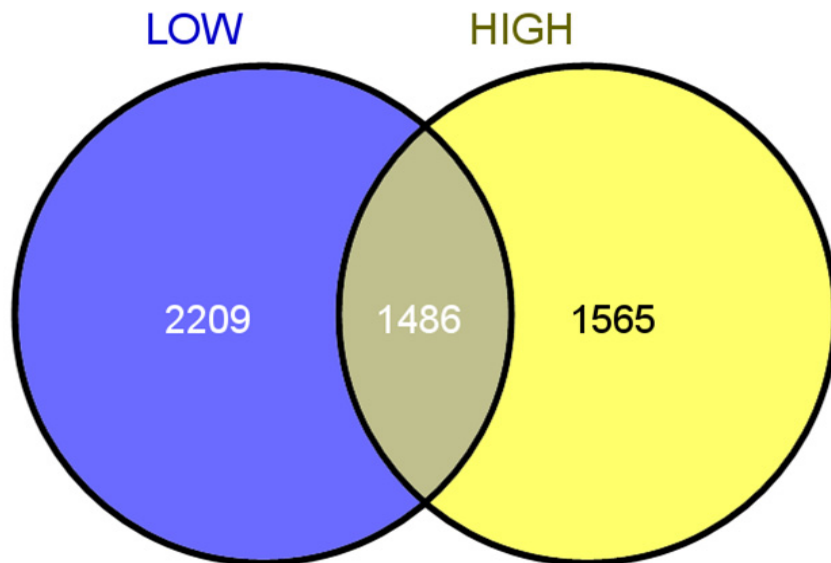
In Common Low and High		G2/M checkpoint	143	117	1.39	1.30
		G2/M transition	704	552	1.01	1.05
		M phase	253	205	1.13	1.18
		M/G1 transition	17	17	1.70	1.48
		meiotic spindle assembly	11	8	3.17	5.54
		mitotic checkpoint	86	70	1.29	1.32
		mitotic spindle assembly	62	55	1.65	1.65
		nucleoside transport	27	19	-1.01	-1.40
		nucleotide biosynthesis	27	22	1.19	1.45
		Ser/Gly metabolism	15	11	-1.51	-1.83
		S-G2 transition	198	159	1.16	1.18
		sister chromatid exchange	91	69	1.35	1.39
		premeiotic DNA synthesis	25	19	2.18	4.55
		kinetochore assembly	176	126	1.49	1.47
	Detoxification	xenobiotic clearance	284	203	-1.16	-1.34
		response to drug	450	362	-1.11	-1.10
		glutathione metabolism	25	19	-1.31	-1.69
	DNA Damage	DNA Damage	861	668	1.08	1.11
		DNA damage checkpoint	127	100	1.38	1.40
		DNA modification	15	13	1.68	1.55
		DNA repair	654	519	1.11	1.16
		mitophagy	37	31	-1.49	-1.51

		oncogenesis	523	402	-1.06	-1.12
		genetic instability	113	88	1.15	1.25
		genome instability	292	234	1.01	1.18
		genome stability	258	196	1.23	1.28
		response to DNA damage	460	364	1.15	1.19
	Metabolic	gluconeogenesis	254	208	-1.05	-1.26
		pyruvate metabolism	10	10	-1.53	-2.55
		pyruvate oxidation	15	15	-1.53	-1.85
		Pentose-phosphate shunt	44	39	-1.18	-1.38

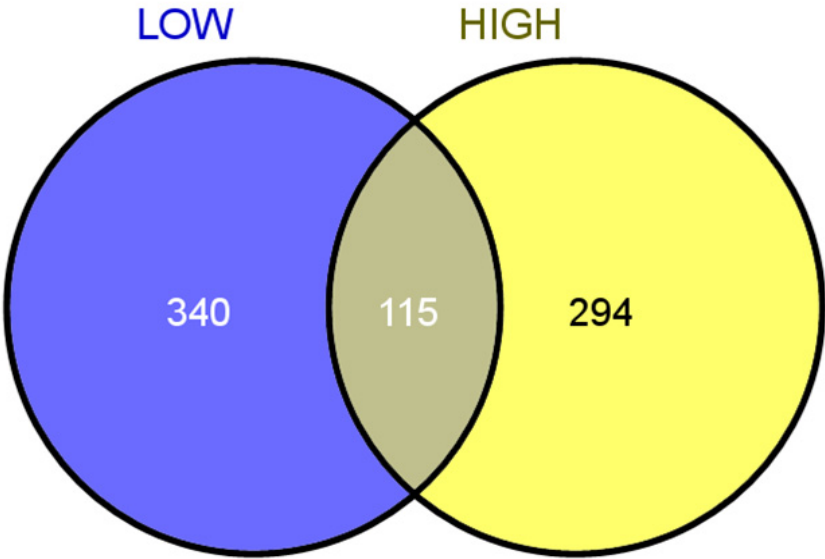
Suppl. 1



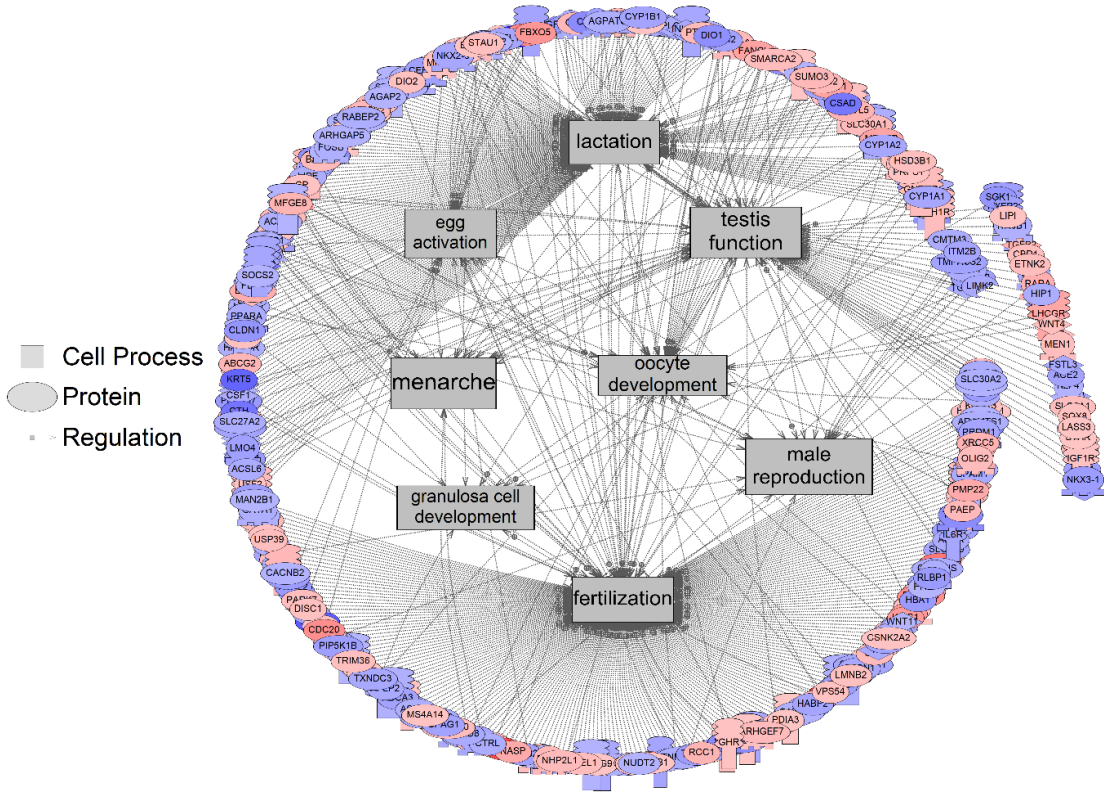
Suppl. 2



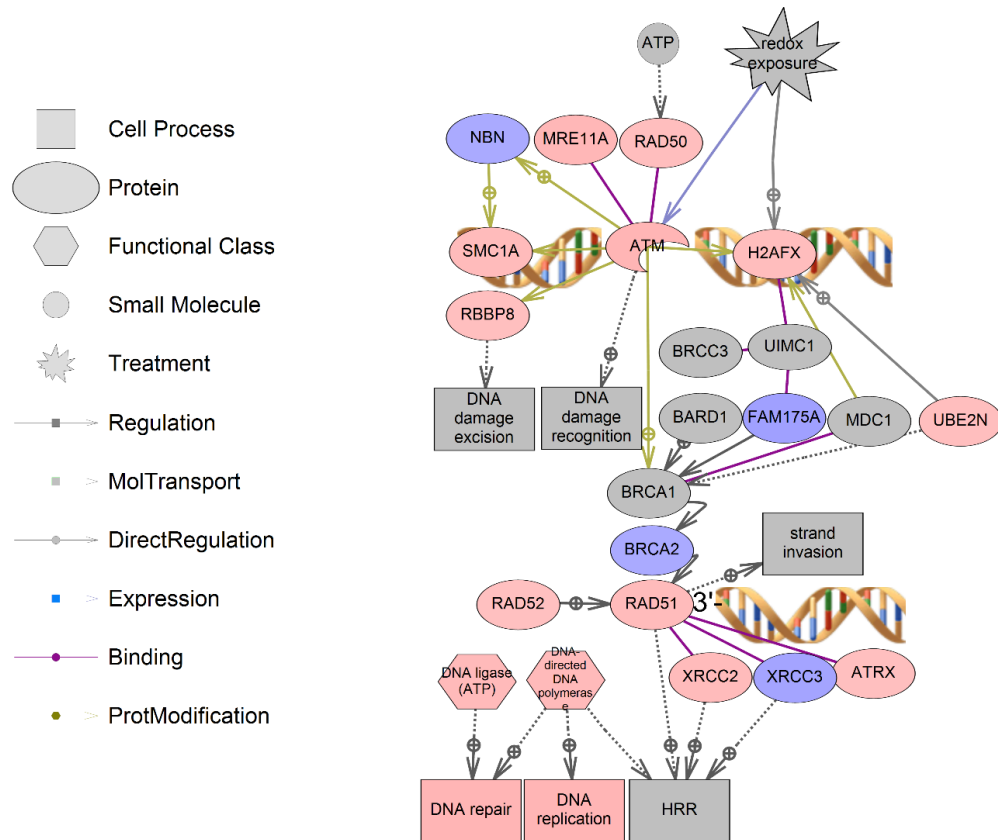
Suppl. 3



Suppl. 4



Suppl. 5



Suppl. 6