Toxicogenomic Responses to Inorganic and Organic Mercury in *Caenorhabditis elegans*

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
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ABSTRACT

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# Abstract

Mercury is a toxic metal that can exist in multiple chemical forms, all of which are toxic to humans. Despite years of research, only a fragmented understanding of the molecular mechanisms of toxicity exists. Furthermore, it is not known to what extent different mercury species act similarly or dissimilarly at the molecular level. The objective of this study was to investigate the extent to which inorganic and methylmercury act differently at the molecular level.

The relative toxicity of mercuric chloride ($HgCl_2$) and methylmercury chloride (MeHg) in *C. elegans* was determined by testing the effect of mercurial exposure on growth, reproduction and induction of stress-response genes. MeHg was more toxic than $HgCl_2$, though the difference in toxicity between the two mercurials varied by assay. Using approximately sub-, low- and high toxic exposures to both mercurials, microarrays were performed to determine the effects of the $HgCl_2$ and MeHg on transcription. A total of 473 genes were differentially expressed in the three $HgCl_2$ treatments, while a total of 2865 genes were differentially expressed in the three MeHg treatments. Analysis of the microarray data by hierarchical clustering, principal components analysis and a self-organizing map indicated that the transcriptional effects of the two mercurials were vastly different. Gene Ontology analysis and pathway

mapping indicated that the two mercurials had very different effects on biological processes as well.

The biological function of genes up-regulated by mercurials was tested using RNA interference (RNAi). The effect of RNAi and mercury co-exposure on *C. elegans* growth was tested for 599 genes. Knock-down of 18 of these genes was found to significantly affect growth of *C. elegans* exposed to mercury. Of these 18 genes, only 2 were found to significantly affect growth in response to both mercurials.

ABCG2 (ATP-binding cassette, sub-family G (WHITE), member 2), BACE1 (β-site APP cleaving enzyme 1), BACE2 (β-site APP cleaving enzyme 2), CHKA (choline kinase α), CHKB (choline kinase β), ELOVL3 (elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3), ELOVL6 (ELOVL family member 6, elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)), GCLC (glutamate-cysteine ligase, catalytic subunit) and PARG (poly (ADP-ribose) glycohydrolase) are human homologs of the genes found to significantly affect growth of *C. elegans* in mercury exposure. The effect of sub-, low-, and high-toxic treatments of both mercurials on expression of these genes was tested using three cell lines: SK-N-SH, HepG2 and HEK293. Of these 162 cell-gene-mercury-toxicity combinations, there were 36 in which the gene was differentially expressed. In 24 of these, the gene was significantly differentially expressed by only one of the mercurials. The evolutionary conservation of function of these genes in mercury exposure was tested using RNAi. A total of 11

significant gene-mercury interactions were found between the three cell lines, but there was not a cell type-gene combination in which exposure to both mercurials was found to significantly affect cytotoxicity.

In whole organism and cell culture studies, inorganic and methylmercury were found to have different effects on transcription. In both systems, there was very little overlap in the genes involved in mercury resistance and susceptibility. These data indicate that molecular mechanisms of toxicity differ by mercurial.

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# 1. Introduction

Mercury, the 80th element of the Periodic table, is in Group IIB of the transition metals, along with zinc and cadmium. The toxicity of mercury has been documented since the 16th century, starting with Paracelsus. Mercury toxicity is still of great concern, as it has ranked #3 on the Agency for Toxic Substances & Disease Registry's Superfund priorities list since the list's inception. Despite this, mechanisms of mercury toxicity are poorly understood. In particular, little is known about the extent to which the molecular mechanisms of toxicity differ by mercury species.

## *1.1 Mercury sources and exposure*

Mercury exists as either inorganic or organic species. In an organic mercury species, mercury is covalently bound to at least one carbon (e.g. methylmercury, phenylmercury, dimethylmercury). Inorganic mercury species include elemental mercury (liquid or vapor; $Hg^0$), mercury salts and ionic mercury ($Hg^+$ or $Hg^{2+}$). In regards to human exposures, the most relevant forms of inorganic mercury are mercury vapor and ionic mercury. The organic mercurials to which humans are most exposed are methylmercury and ethylmercury. Mercury, in its various forms, is ubiquitous in the environment, thus mercury exposure is inevitable. Mercury is unique among metals in that it is the only metal to exist as a liquid at ambient temperature, and is easily volatilized during industrial combustion. This, coupled with mercury's ability to switch between different chemical species, results in complex cycling in the environment. Like

all metals, once mercury is released into the environment, it cannot be removed. While the natural degassing of the earth's land and oceans has always been a prominent source of mercury in the environment, anthropogenic emissions are now the dominant source of mercury [1]. It is estimated that approximately 1/3 of mercury emissions are natural, 1/3 are recycled anthropogenic emissions, and 1/3 are new anthropogenic emissions [2]. Coal-fired power plants represent the largest source of mercury emissions into the environment, with 54% of these emissions coming from Asia [3]. Artisanal gold mining is the second largest emitter of mercury into the environment [4]. According to Streets et al., the amount of global mercury emissions in 2050 may be double those of 2006 [5].

Once in the environment, mercury undergoes complex speciation and transport cycles (Figure 1). Mercury vapor, from natural or anthropogenic emissions, can have an atmospheric residence as long as 2 years [1], so local mercury emissions can have a global impact. Coal combustion also results in particulate-bound and oxidized mercury species, which tend to deposit locally [6]. Atmospheric mercury vapor is eventually oxidized, dissolved in water and returned to the earth's surface in rain [7]. In the aquatic environment, microbes can add a methyl group to divalent mercury [8]. This methylated mercury undergoes biomagnification, so that top predators (e.g. swordfish, tuna) have mercury levels that are several orders of magnitude higher than those found in the environment [9]. MeHg exposure through fish consumption represents the mercury exposure of greatest concern to human health.

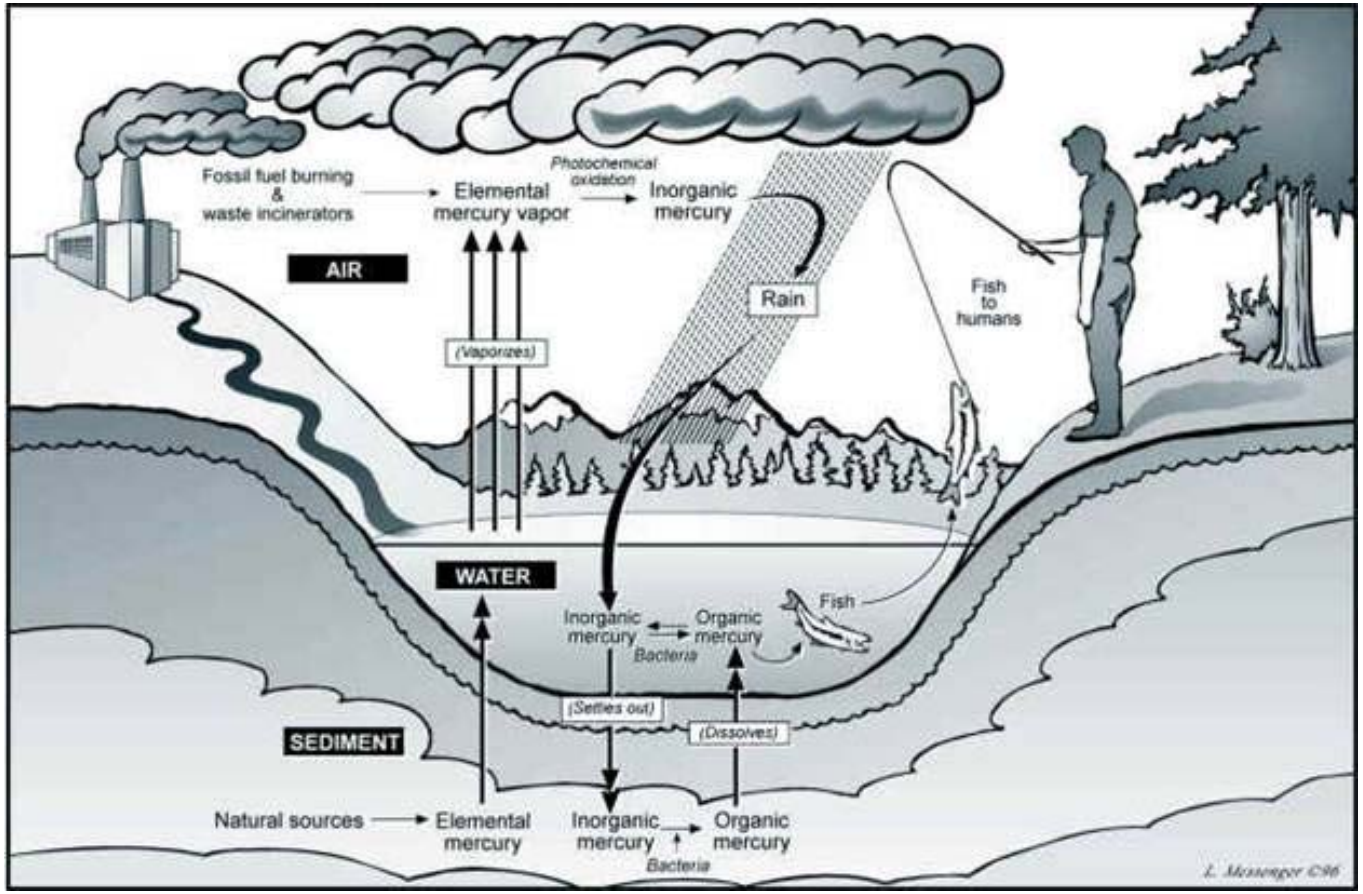



**Figure 1: Environmental cycling of mercury** (Utah Department of Environmental Quality)

Humans are also exposed to various forms of inorganic mercury. Elemental mercury has long been used as the principle component of dental amalgams. This results in exposure to mercury vapor to both those wearing the amalgams, as well as the dental professionals that make and install them. Since the 1960's the "amalgam wars" have raged in which there has been fierce debate as to whether or not these amalgams were of concern to human health [10, 11]. Other occupational exposures to mercury vapor include workers in chlor-alkali plants, fluorescent lamp factories and artisanal gold mining [12, 13]. The ethylmercury-containing compound thimerosal is currently used as a vaccine in multi-use vaccine containers (e.g. influenza). It was previously used in the U.S. as a preservative for childhood vaccines, but its use was discontinued in 2001 over concerns that children may be exposed to excessive levels of mercury.

## *1.2 Mercury exposures and human health*

### 1.2.1 Absorption, Distribution, Metabolism, Excretion

The primary routes of exposure to mercury are through ingestion and inhalation. Different mercurials are absorbed with widely varying efficiency from the gastrointestinal tract. As much as 95% of ingested methylmercury (MeHg) is absorbed from the GI tract [14]. This is in stark contrast to elemental mercury which is not absorbed in the liquid state [15]. Mercuric mercury (the most stable and common ionic form of mercury) is poorly absorbed from the digestive tract, with only about 7% being absorbed [16]. Approximately 75% of inhaled mercury vapor is retained by the body

[17]. Dermal exposure to solutions of mercury salts results in negligible absorption. The dermal absorption of organic mercurials varies. MeHg is absorbed moderately well, while dimethylmercury is absorbed extraordinarily well through the skin [18, 19].

Intestinally-absorbed MeHg first travels to the liver. MeHg that is removed from blood by the liver is bound to glutathione, and transported out of the liver in bile [20]. In the bile, glutathione is broken down to its amino acid constituents, freeing the MeHg to be reabsorbed [21]. Intestinal microbes are able to convert MeHg to divalent inorganic mercury, and this conversion facilitates the excretion of MeHg through feces [22]. MeHg levels in the brain are typically 5 times higher than blood levels [23]. Liver, kidney and muscle are also sites of MeHg accumulation [23, 24]. MeHg has a half-live of ~35 days in the brain [25]. This half-life does not indicate the excretion of MeHg, but rather, the phagocyte-mediated conversion of MeHg to mercuric mercury [26]. Mercuric mercury has a half-life of 1-2 years in the brain [27]. Methylmercury is excreted in hair, so measurement of hair mercury levels is a valuable biomarker for MeHg exposure [28].

Mercuric mercury that is absorbed from the intestine primarily accumulates in the kidney [29]. Virtually no mercury from this exposure is found in the brain [30]. Mercury vapor readily passes through cell membranes, and approximately 10% of inhaled mercury vapor accumulates in the brain [17]. Mercury vapor is not believed to be toxic, but once in the body, it is rapidly oxidized by catalase to the mercuric form [18]. After exposure to mercury vapor, the mercury levels in the liver and kidney are

approximately 10-fold higher than brain mercury levels [30]. Mercury in the kidney has a 58 day half-life, and is excreted in urine by the breakdown of cells, which makes urine a useful biomarker for inorganic mercury body burden [15].

### 1.2.2 Health effects of Methylmercury

The threat posed by the organic mercurial, MeHg, through fish consumption was first brought to the public's attention due to a large scale MeHg poisoning in the 1950's and 1960's in the areas surrounding Minamata Bay in Japan. The nearby Chisso chemical factory used inorganic mercury as a catalyst in the production of acetaldehyde. During this process, MeHg was generated as a waste product, which was discharged into Minamata Bay. This lead to MeHg levels in fish that were ~100 times higher than background levels [31]. In adults, consumption of contaminated fish lead to paresthesia, ataxia, loss of vision, and even death [32]. What was particularly striking, however, was how sensitive individuals were to an *in utero* exposure. Mothers with no overt toxicity gave birth to children with gross cognitive and anatomical defects [33].

Two large epidemiological studies have investigated the effects of prenatal mercury exposure in populations that consume large amounts of seafood. The Faroe Islands lie between Scotland, Norway and Iceland. The primary source of MeHg in this population is the consumption of whale meat. A study of over 1000 singleton births found a significant correlation between mothers' mercury levels during gestation and cognitive deficits at both 7 and 14 years of age [34, 35]. A recent study of Faroese men

suggests that MeHg exposure may also be associated with an increased risk of cardiovascular disease [36]. The Seychelles is an archipelago north of Madagascar in the Indian Ocean. A study similar to the Faroe Islands study was conducted with individuals from close to 700 singleton births. Although the mercury levels in mothers were similar to those found in the Faroe Islands study, there was no correlation between increased mercury levels in mothers and decreased cognitive function in their children [37]. It is not clear what accounts for the difference between the Faroe and Seychelles studies.

One of the hallmarks of MeHg toxicity is a long delay from the time of exposure to the appearance of pathology. As MeHg is de-methylated to mercuric mercury *in vivo*, this has led some to speculate that it is mercuric mercury, and not MeHg that is the proximate agent of toxicity in MeHg exposures [38].

### 1.2.3 Health effects of Inorganic mercury

The toxic effects of exposure to mercury vapor have been known since the time of Paracelsus, and were described in detail by the 18$^{th}$ century physician Joannes Antonius Scopoli [39]. Mercury vapor is a neurotoxicant that produces tremors and psychological disturbances in severe cases, and has been associated with Alzheimer's disease [40]. A study of former fluorescent lamp factory workers found that occupational mercury exposure lead to deficits in information processing speed, psychomotor speed and manual dexterity [41]. The same study also found an increase in symptoms of

depression and anxiety. As this was an assessment of former workers, exposure to mercury can have long-term effects. There is some evidence that, in industrial areas, environmental exposure to inorganic mercury is sufficient to cause an increase in mortality from kidney disease [42].

## *1.3 Molecular mechanisms of mercury toxicity*

There is only a fragmental knowledge of the molecular mechanisms involved in mercury toxicity. The affinity of mercury for sulfhydryl groups is 10-12 orders of magnitude greater than its affinity for hydroxyl or amine groups [43]. This is likely the underlying chemistry behind the toxicity of mercury. However, many metals have very high affinities for sulfhydryl groups, yet the pathology resulting from metal exposure varies greatly by metal [43]. While much remains to be elucidated about mercury toxicity, the following mechanisms are known to be involved.

### 1.3.1 Oxidative Stress

Both inorganic and organic mercury have been shown to cause oxidative stress [44-46]. Neither mercurial engages in Fenton-type chemistry, so it is believed that the oxidative stress is primarily caused by the depletion of glutathione and other antioxidants [43]. In fact, antioxidant supplementation has been shown to mitigate toxicity to both mercurials in multiple systems [47-51]. Mercury exposure can also disrupt the antioxidant system itself. After a 21 day MeHg exposure, adult mice had significantly reduced levels of

glutathione peroxidase activity and increased levels of lipid peroxidation [52]. This susceptibility can continue after cessation of mercury exposure. Mice exposed *in utero* to MeHg were shown to have reduced glutathione, glutathione reductase and glutathione peroxidase levels in the cerebellum even after cerebellar mercury levels returned to baseline levels [53]. Mercury may also cause oxidative stress by directly targeting enzymes involved in antioxidant defense, as both $HgCl_2$ and MeHg inhibit thioredoxin reductase by binding to the selenol-thiol in the active site of the enzyme [54].

### 1.3.2 Microtubule disruption

Microtubule disruption is another critical mechanism of toxicity, because microtubules are critical in mitosis, cell migration, and neurite growth. Inorganic mercury has been shown to both inhibit microtubule assembly and inhibit myosin ATPase *in vitro* [55, 56]. In cell culture, tyrosinated microtubules are more susceptible to disassembly upon exposure to MeHg, though total tubulin levels are unaffected [57, 58]. Tyrosinated microtubles are associated with neurite growth, and are considered to be less mature [59, 60]. MeHg has also been shown to disrupt microtubules *in vivo*. Neurons isolated from neonatal mice that were treated with MeHg at gestational day 15 exhibited defects in neurite growth that resulted from dissolution of microtubles [61].

### 1.3.3 Mitochondria permeability transition

Both inorganic and methylmercury can inhibit mitochondrial function [62, 63]. In addition to being the primary producer of ATP in the cell, the mitochondria are also

important regulators of intracellular calcium levels [64]. Both mercurials have been shown to increase intracellular levels of calcium both through promoting the release of calcium from intracellular stores and by facilitating the influx of calcium from extracellular stores [65-68] . Mitochondria are calcium buffers that remove excess calcium from the cytosol [69]. However, excessive mitochondrial calcium accumulation, particularly in conjunction with another stress, can be pathogenic to mitochondria [70]. This can lead to formation of the mitochondrial permeability transition pore, a large pore that allows molecules up to 1500 Da to pass freely through the mitochondrial membrane [71]. This results in loss of mitochondrial membrane potential, which leads to the death of the cell [71]. MeHg exposure has been shown to result in mitochondrial permeability transition pore formation in cultured cerebellar granule cells [72]. Calcium channel blockers have been shown to protect against toxicity to both inorganic and organic mercury in cell culture [73] and against MeHg toxicity *in vivo* [74]. Additionally, there may be an age-dependent effect, as Dreiem et al. found that striatal synaptosomes isolated from young rats had higher baseline reactive oxygen species (ROS) levels, lower baseline mitochondrial membrane potentials, and were more susceptible to MeHg [75].

## *1.4 Mercury regulated signaling pathways*

Mercury has been shown to affect cell signaling pathways. The MAPK pathways have been shown to be affected by multiple stressors, and mercury is no exception [76]. In macrophages, inorganic mercury was shown to increase levels of phosphorylated p38,

which was likely mediated by ROS [77, 78]. In kidney cells, inorganic mercury had a dose-dependent effect on MAPK activation [79]. A sub-toxic treatment resulted in a decrease in phosphorylated JNK, while a toxic treatment increased levels of phosphorylated JNK and p38. However, phosphorylation of ERK was not significantly affected by inorganic mercury treatment. In neuronal cells, MeHg has been shown to decrease levels of phosphorylated ERK by itself or with retinoic acid co-exposure [80, 81]. It has also been shown that inorganic mercury can activate the redox-sensitive transcription factors NF-kB and AP-1 [82, 83], while MeHg has been shown to activate Nrf2 [84].

Bland and Rand demonstrated that MeHg can activate Notch signaling, and also proposed a model by which MeHg could specifically target the Notch signaling pathway [85]. In order for Notch signaling to occur, the Notch receptor must first be cleaved by a member of the ADAM/TACE/Kuzbanina family [86]. ADAMs (*a d*isintegrin *a*nd *m*etalloprotease) are inactive proenzymes that have previously been shown to be activated by organomercurials [87]. It is believed that mercury acts by binding to a conserved cysteine which results in the exposure of the metalloprotease active site and subsequent cleavage of the Notch protein, which results in activation of Notch signaling [85, 88] (Figure 2).

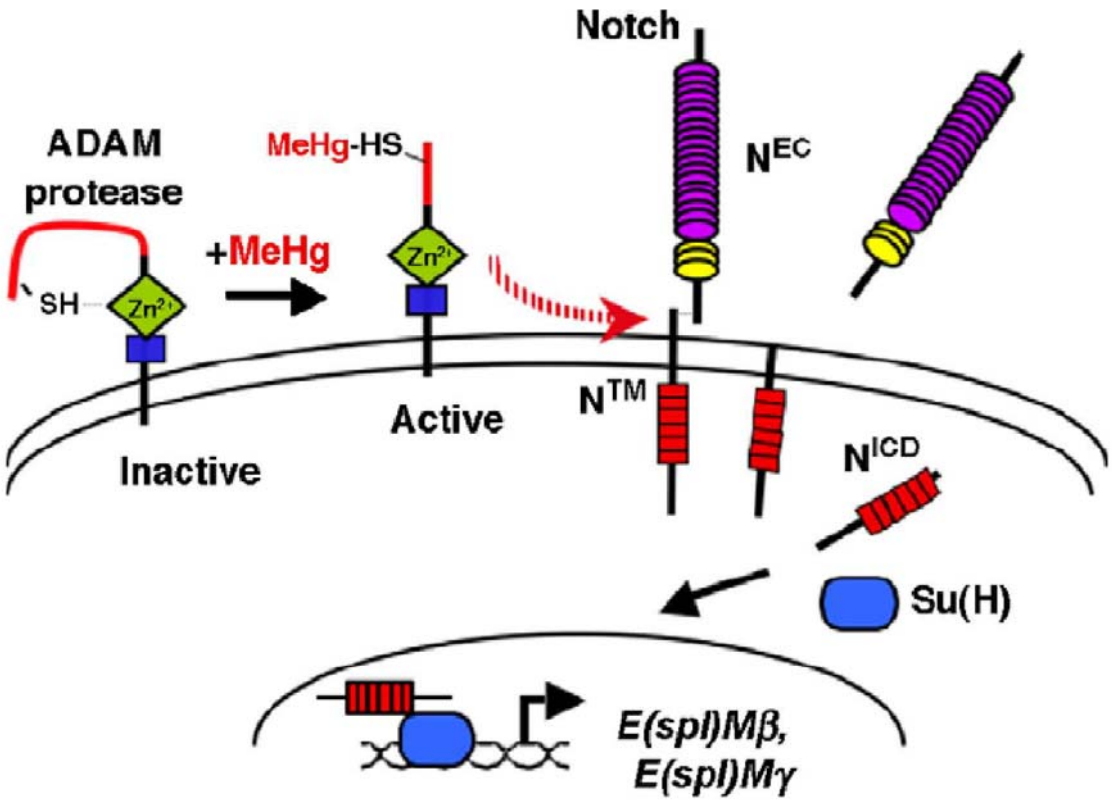



**Figure 2: Proposed mechanism of MeHg activation of Notch signaling** (Bland et al., Neurotoxicology 27(6): 982-991, 2006)

## *1.5 Mercury regulated gene expression*

There have been several studies that used microarrays to investigate the global effects of mercury on transcription. In yeast, Jin et al. found that a 3 h exposure to $HgCl_2$ up-regulated genes involved in the detoxification of reactive oxygen species [89]. In human hepatocellular carcinoma cells exposed for 6 hours to $HgCl_2$, Kawata, et al. also found an up-regulation of genes associated with oxidative stress [90]. Likewise, Ung et

al. found that genes associated with oxidative stress were up-regulated in the liver of zebrafish exposed for various times (8-96 hours) to a sub-lethal concentration of $HgCl_2$ [91]. The authors also found an up-regulation of genes associated with protein degradation, mitochondrial dysfunction and Wnt signaling. Klaper et al. examined the effects of both acute (4 days) and chronic (600 days) MeHg exposure on global transcription in the liver of the fathead minnow [92]. While Ung et al. found an approximately equal number of up- and down-regulated genes in response to $HgCl_2$, Klaper et al. found that the majority of differentially expressed genes were down-regulated in response to MeHg. A 25 day exposure to MeHg up-regulated genes involved in oxidative and endoplasmic reticulum stress [93].

Microarray studies were done to investigate the effects of MeHg on mouse embryo fibroblasts (MEFs) and mouse embryos undergoing neurulation. MEFs were exposed to 2.5 μM MeHg for 24 hours [94]. Here again, there were a greater number of down-regulated genes than up-regulated genes. MeHg exposure resulted in significant up-regulation of antioxidant and phase II detoxifying enzymes. MeHg also up-regulated several genes involved in the ubiquitin-proteasome pathway. In order to assess the effect of MeHg on neurulation, Robinson et al. isolated gestational day 8 mice that had been exposed to MeHg for 8 or 12 hours [95]. In this case, the majority of differentially expressed genes were up-regulated. As in the liver of zebrafish treated with $HgCl_2$, there was an enrichment of up-regulated genes involved in the Wnt signaling pathway. Both

inorganic and organic mercury appear to activate the oxidative and general stress response, as well as Wnt signaling. However, there has not been a study that directly compares the effects of these mercurials on transcription.

## *1.6 C. elegans as a model system*

*C. elegans* are non-parasitic, soil dwelling nematodes that are ~1 mm in length. While males do exist, >99% of *C. elegans* are hermaphrodites. At 20°C, it takes ~72 hours for a fertilized egg to pass through the four larval stages (L1-L4) and become a self-fertilizing adult. The exact lineage of each of the 959 somatic cells in the *C. elegans* hermaphrodite has been established [96]. Additionally, the location and connections for all hermaphrodite neurons has been described. While the total number of cells is small, *C. elegans* do have well developed digestive, muscular, neural and reproductive systems [96].

*C. elegans* have many attributes that make them a good model system in which to investigate the molecular and toxicological responses to mercurials. The genome is fully sequenced and well annotated. RNAi is available for ~90% of the genes, and gene knock-down is accomplished by simply feeding *C. elegans* bacteria that generate dsRNA targeting the gene of interest [97]. A large number of *C. elegans* mutants exist as well. While the evolutionary distance between *C. elegans* and humans is considerable, there is a remarkable conservation of signaling pathways (e.g. MAPK, Wnt, Notch, TGF-β).

There is also a high degree of genetic homology between the two species as there are *C. elegans* homologs for 60-80% of human genes [98]. The small size and transparency of *C. elegans* facilitates their use in medium and high-throughput toxicity assays. As reviewed in Boyd et al., it is possible to assess the effects of toxicants on large numbers of animals using various endpoints [99]. To date, there has been little research investigating the effects of mercury on *C. elegans*. The impressive array of genetic tools coupled with the ability to study whole organism effects makes *C. elegans* a useful model organism in which to study mercury toxicity.

There have been a few studies that have investigated the effect of mercurials in *C. elegans*. The 24 h LC50 of $HgCl_2$ in adult *C. elegans* exposed on agar plates was determined to be 500 μM [100], while the 24 h LC50 of *C. elegans* exposed to $HgCl_2$ in liquid culture, was 90 μM [101]. This difference is likely due to greater availability of $HgCl_2$ in liquid. Exposure to $HgCl_2$ has also been shown to inhibit feeding in a dose-dependent manner [101]. A more detailed study was conducted to examine the effects of MeHg in *C. elegans* [102]. The authors found that the susceptibility of *C. elegans* to MeHg exposure differs between developmental stages. The MeHg LC50 was 4-fold lower in L1 nematodes compared to L4 nematodes. MeHg was found to inhibit feeding and larval development, but did not affect neuronal morphology. Despite these studies, much remains to be elucidated about the molecular effects of mercurials in *C. elegans*.

## *1.7 Dissertation Objectives*

Mercury is a ubiquitous toxicant that exists in multiple forms that are of great concern to public health. It has been well established that different mercury species are toxic, though the absorption, distribution, metabolism and excretion (ADME) in the body varies by mercurial. Various studies have found that different mercurials share some common mechanisms of toxicity. These findings, coupled with the delayed toxicity and conversion of MeHg to mercuric mercury, have led some to suggest that differences in mercury-induced pathology are due to differences in ADME, rather than differences in the molecular mechanisms of different mercurials. There has, however, been little investigation that directly compares the molecular mechanisms of toxicity of the different mercurials. Thus, the overall goal of this dissertation was to ascertain if inorganic and organic mercury behave differently at the molecular level. Throughout this study, $HgCl_2$ was used as the representative inorganic mercurial and $CH_3HgCl$ (MeHg) was used as the representative organic mercurial.

Four specific aims were proposed to address this issue:

### 1.7.1 Specific Aim 1: Assess toxicity of inorganic and organic mercury in *C. elegans*

*C. elegans* was chosen as the model organism for these studies due to the ability to study both whole organism and molecular effects quantitatively and in great detail. It was first necessary to determine the organismal effects of $HgCl_2$ and MeHg so that

approximately equitoxic treatments could be established for use in the molecular studies. To do this, the effects of both $HgCl_2$ and MeHg on growth and reproduction were determined in *C. elegans*. Additionally, the effect of these mercurials on the induction of stress-response genes was also determined. Based on these results, conditions were determined in which to compare the effects of the two mercurials on transcription.

### 1.7.2 Specific Aim 2: Investigate *C. elegans* transcriptional response to inorganic and organic mercury exposure

In order to determine the effects of $HgCl_2$ and MeHg on the *C. elegans* transcriptome, nematodes were exposed for 24 h to sub-, low- and high-toxicity concentrations for both mercurials. Global transcriptional changes were measured using Agilent whole-genome, 1-color microarrays. The transcriptomes resulting from the different treatments were then compared using hierarchical clustering, principal components analysis and a pattern analysis tool. Gene Ontology analysis and pathway mapping were applied to better understand the specific biological effects of mercury exposure.

### 1.7.3 Specific Aim 3: Identify genes related to mercury resistance in *C. elegans*

In order to investigate the biological significance of mercury induced-transcriptional changes, RNA interference (RNAi) -mediated gene inhibition was employed to study the importance of genes in the response to inorganic and

methylmercury. The effect of RNAi on genes up-regulated in the microarray in the presence of either mercurial was tested in both mercurials. The effect of RNAi alone, mercury alone, and RNAi plus mercury on inhibition of nematode growth was assessed. Genes in which the combined effect of RNAi and mercury was significantly different than the predicted additive effects of RNAi and mercury were deemed to be relevant genes in the nematodes' response to mercury.

### 1.7.4 Specific Aim 4: Determine evolutionary conservation of function of genes relevant to mercury resistance

In order to determine if the function of genes involved in the response to mercury were evolutionarily conserved, human homologs of the *C. elegans* genes found in the RNAi screen were identified. The expression of these genes was knocked down in three human cell lines using siRNA. To look for significant gene-mercury interactions, the combined effect of RNAi and mercury on cell viability was assessed.

# 2. Methods

## 2.1 Maintenance of C. elegans

The wild-type N2 Bristol and the NL2099 (pk1426) strains were used in this study. The N2 Bristol strain was used in the toxicity assays and the microarray experiments. The NL2099 strain harbors a homozygous deletion of *rrf-3* and will be referred to as rrf-3 hereafter [103]. This is an RNAi sensitive strain, and was used in the RNAi screen. *C. elegans* were maintained at 20°C on K-agar (2% bacto-agar, 0.25% bacto-peptone, 51 mM sodium chloride, 32 mM potassium chloride, 13 μM cholesterol) plates with the *Escherichia coli* OP50 strain as food.

## 2.2 Reproduction assay

A previously described (Boyd, 2010) reproduction assay that uses the COPAS Biosort (Union Biometrica, Holliston, MA) to directly count the number of offspring was used to test the effect of mercurials on reproduction. In this assay, five age-synchronized L4 larvae are dispensed into a 96-well plate containing varying concentrations of either $HgCl_2$ (VWR, Bridgeport, NJ) or MeHg (VWR) in complete K medium (51 mM sodium chloride, 32 mM potassium chloride, 3 mM calcium chloride, 3 mM magnesium sulfate, 13 μM cholesterol) with OP50. 6 wells were used to test each concentration (total of 30 L4s for each concentration), 12 conditions (11 test concentrations plus untreated control) were tested for $HgCl_2$, while 17 conditions (16 test concentrations plus untreated control) were tested for MeHg. The concentrations tested for $HgCl_2$ were: 0.3 μM, 0.48 μM, 0.75

μM, 1.2 μM, 1.9 μM, 3 μM, 4.8 μM, 7.5 μM, 12 μM, 19 μM and 30 μM. The concentrations tested for MeHg were: 0.03 μM, 0.048 μM, 0.075 μM, 0.12 μM, 0.19 μM, 0.3 μM, 0.48 μM, 0.75 μM, 1.2 μM, 1.9 μM, 3 μM, 4.8 μM, 7.5 μM, 12 μM, 19 μM and 30 μM. After 48 h incubation at 20°C, the number of *C. elegans* was measured. 3-8 replicates were performed for each test concentration. The EC50s were estimated by fitting the Hill function.

## 2.3 Growth assay

A growth assay that uses the COPAS Biosort was described previously by Smith et al. [104]. In this assay, the size, (determined by the optical density (EXT)), of 50 synchronized L1 larvae is measured as they are dispensed into a 96-well plate containing a range of concentrations of either $HgCl_2$ or MeHg in complete K medium plus OP50. The nematodes are incubated at 20°C for 48 h. Under these incubation conditions, untreated L1 larval stage nematodes will develop to the L4 larval stage. At the end of 48 h, the EXT of each nematode was measured to determine its size. This assay does not measure lethality, as nematodes with zero growth are alive, but have not developed past the L1 larval stage. Twelve conditions (11 test concentrations plus untreated control) were tested for both $HgCl_2$ and MeHg. The concentrations tested for $HgCl_2$ were: 1.5 μM, 2.1 μM, 3 μM, 4.3 μM, 6.1 μM, 8.7 μM, 12 μM, 18 μM, 25 μM, 35 μM, and 50 μM. The concentrations tested for MeHg were: 1 μM, 1.3 μM, 1.8 μM, 2.5 μM, 3.3 μM, 4.5 μM, 6 μM, 8.1 μM, 11 μM, 15 μM and 20 μM. Each condition was tested

in 6 wells per 96-well plate (~300 nematodes/condition/plate), and 3 replicates were performed. Growth was calculated by subtracting the mean EXT of the loaded L1 larvae from the EXT measurement of each nematode at the end of the 48 hour exposure. The effective concentrations were estimated by fitting the Hill function.

## *2.4 Treatment and RNA isolation of mixed-stage C. elegans*

40 L4 stage N2 Bristol nematodes were placed on K-agar plates and allowed to grow for 4 days at 20°C. All nematodes were then transferred into a flask containing Complete S-medium (100 mM sodium chloride, 50 mM potassium phosphate, 10 mM potassium citrate pH 6.0, 3 mM calcium chloride, 3 mM magnesium sulfate, 50 μM EDTA, 25 μM iron sulfate, 10 μM manganese chloride, 10 μM zinc sulfate, 1 μM copper sulfate, 13 μM cholesterol) with OP50 and were grown in a 200 rpm shaker for 4 days at 20°C. Aliquots from this flask were put into separate flasks, and the appropriate amount of mercury was added to each test flask. Nematodes were exposed to mercury for 24 h in a 200 rpm shaker at 20°C. Nematodes were then rinsed and living nematodes were isolated by sucrose floatation, frozen in liquid nitrogen and then stored at -80°C [105]. Nematodes were ground with a liquid nitrogen-cooled mortar and pestle, and total RNA was isolated using the RNeasy Midi Kit (Qiagen, Valencia, CA) according to manufacturer's instructions.

## *2.5 Quantitative reverse transcription-real time-PCR*

Quantitative reverse transcription-real time-PCR (qRT-PCR) was used to measure the effect of mercury on the steady-state levels of the following *C. elegans* genes: *gcs-1* (γ-glutamylcysteine synthetase), *gst-38* (glutathione S-transferase), *hsp-16.2, hsp-70* (heat shock protein) *mtl-1, mtl-2* (metallothionein) and the following human genes: ABCG2 (ATP-binding cassette, sub-family G (WHITE), member 2), BACE1 (β-site APP cleaving enzyme 1), BACE2 (β-site APP cleaving enzyme 2), CHKA (choline kinase $\alpha$), CHKB (choline kinase β), ELOVL3 (elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3), ELOVL6 (ELOVL family member 6, elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)), GCLC (glutamate-cysteine ligase, catalytic subunit) and PARG (poly (ADP-ribose) glycohydrolase). cDNAs were generated from 500 ng total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. cDNAs were subsequently used in qRT-PCR using Power SYBR Green according to manufacturer's instructions (Applied Biosystems, Foster City, CA). qRT-PCR was performed in an ABI 7900 HT Fast Real-Time System (Applied Biosystems), and fold changes in mRNA levels was calculated using the ΔΔCT method [106]. Myosin light chain-2 was used as reference mRNA for *C. elegans*, and β-actin was used as reference mRNA for human cells. Results are presented as mean ± standard error (n = 3-4). Data

were analyzed using a 1-way ANOVA with a Dunnett's post-hoc test. The criterion for statistical significance was set at $p < 0.05$.

Primers were designed using the open source Primer3 program and were purchased from Integrated DNA Technologies (Coralville, IA) (Table 1).

**Table 1: Primers used in qRT-PCR experiments**

| Gene | Forward (5′→3′) | Reverse (5′→3′) |
|---|---|---|
| *mlc-2* | TTGACAGGAACTGACCCAGAGG | ATAGCCTTGACCTCATCCTCG |
| *gcs-1* | CCACCAGATGCTCCAGAAAT | TGCATTTTCAAAGTCGGTCA |
| *gst-38* | AAGATAACAGACTTACCGATGAGGA | GAAGCTGGTTGTATGGGGTTT |
| *hsp-16.2* | TGCAGAATCTCTCCATCTGAGT | TGGTTTAAACTGTGAGACGTTGA |
| *hsp-70* | CGGTATTTATCAAAATGGAAAGGTT | TACGAGCGGCTTGATCTTTT |
| *mtl-1* | CGCCAAATCTCATCACAAA | CGTGAATGTTGCAAACACCT |
| *mtl-2* | CCGAACAATTGAACGGTCAC | CCTGCACAAAGACTTCCTGG |
| β-actin | GATATCGCTGCGCTGGTCGTC | ACGCAGCTCATTGTAGAAGGTGTGG |
| ABCG2 | AGCTCGAAGGAAAGATCCAA | TCGCCATCACAACATCATCT |
| BACE1 | CCAGAGGCAGCTGTCCAGCAC | GTGACGTTGGGGCCATGGGG |
| BACE2 | CAGCTGGCGTGCTGGACGAA | GGGCTGAATGTAAAGCTGAGGCAGG |
| CHKA | TGAATTTCAAGGGGCTGAGGCCA | CGCCGGCTCGGGATGAACTG |
| CHKB | CAGCCCGAGGAGCTGAGGGT | AGGGAGTCCACGCCCTGCAA |
| ELOVL3 | TTCGAGGAGTATTGGGCAAC | AAGATTGCAAGGCAGAAGGA |
| ELOVL6 | GTGCTCTTCGAACTGGTGCT | CCCAGAATTTGCTGACAGGT |
| GCLC | GTGGATGTGGACACCAGATG | GCGATAAACTCCCTCATCCA |
| PARG | TTTTGCGAGCAGGAGAAGTT | CAGTTCGCTCACCATTCTCA |

## *2.6 Microarray experiments*

Mixed-stage *C. elegans* were treated to sub-(2 μM $HgCl_2$, 0.75 μM MeHg) low-(7.5 μM $HgCl_2$, 2 μM MeHg) and high-(20 μM $HgCl_2$, 7.5 μM MeHg) toxic concentrations of mercury for 24 hours. Total RNA was isolated from treated cultures as well as untreated control. Independent *C. elegans* cultures were used to generate 3 biological replicates for each treatment condition. The quality of the isolated RNA was assessed using the Agilent 2100 Bioanalyzer (Palo Alto, CA). The RNA was then submitted to the NIEHS Microarray Group for reverse transcription labeling, probe hybridization and microarray scanning. The Agilent *C. elegans* Gene Expression Microarray (015061) was used in a single channel (1-color) design. This array contains 43,803 probes and includes target probes, in duplicate, for all *C. elegans* open reading frames. Data was obtained using the Agilent Feature Extraction Software (v9.5), using 1-color defaults for all parameters. The Agilent Feature Extraction Software performed error modeling, adjusting for additive and multiplicative noise. To determine differentially expressed genes, Rosetta Resolver software was used to compare untreated samples to treated samples using an error-weighted, 1-way ANOVA with a Bonferroni correction. A p-value $< 0.01$ was considered to be significant. Additionally, a 2-fold change in expression in treated samples was required in order for a gene to qualify as differentially expressed.

## 2.7 Microarray data analysis

The EPIG (Extracting microarray gene expression Patterns and Identifying co-expressed Genes) pattern analysis tool was used to compare the transcriptional profiles of genes across different treatments [107]. In EPIG, the expression of a gene in each replicate is compared to the average expression of that gene in all untreated replicates. Genes with similar expression patterns are grouped together using the following parameters: correlation value, signal to noise ratio and magnitude of change. The graphed expression patterns are based on the 6 most highly correlated genes for each pattern.

EPIG analysis resulted in a greater number of differentially expressed genes (DEGs) than the 1-way ANOVA with a Bonferroni correction described above. As inclusion of a greater number of genes gives a better assessment of the global effects of transcription, genes identified as differentially expressed by EPIG were used in hierarchical clustering and principal components analysis (PCA). Hierarchical clustering was performed using an agglomerative clustering method with Euclidean dissimilarity. PCA was performed with a correlation dispersion matrix and normalized eigenvector scaling. Both hierarchical clustering and PCA were performed using Partek Genomic Suites version 6.5 software (Partek Incorporated, St. Louis, MO).

Gene Ontology (GO) analysis was performed using Gene Ontology Enrichment Analysis Software Toolkit (GOEAST). Unlike other GO analysis software, the GOEAST

program accounts for the hierarchical structure of GO. This greatly reduces the number of related GO terms, leaving only the most specific significantly enriched GO term. GO terms listed in tables met the following criteria: included 4 or more differentially expressed genes and p-value < 0.05. P-values are the results of Fisher's Exact Test and False Discovery Rate multi-testing adjustment.

KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathways enriched with differentially expressed genes were found using the DAVID bioinformatics resource [108]. P-values are the results of Fisher's Exact Test.

Cytoscape [109, 110] was used to identify sub-networks of interacting genes enriched by genes found to be differentially expressed in the microarrays. A network comprised of known *C. elegans* protein-protein interactions and predicted interactions was used for this analysis [111, 112]. The JActive Modules Cytoscape plug-in was used to find the sub-networks enriched with genes differentially expressed in the microarrays. These sub-networks are identified by their central nodes. Central nodes were defined as those genes with the most edges (interactions) in a sub-network.

## *2.8 Assessing knock-down of C. elegans genes on mercury exposure*

RNAi was performed using two libraries of bacteria able to generate dsRNA to specific genes (MRC Gene Service, University of Cambridge, UK; Open Biosystems, Huntsville, AL). Each bacterial clone contains a lactose-regulated pL4440-Dest-RNAi

vector that drives the expression of dsRNA to a specific gene. Control bacteria contain the same vector, but without an insert that results in dsRNA. This control is referred to as "open vector" hereafter. The RNAi-sensitive rrf-3 strain was used to increase the sensitivity of the assay [103].

A two generation approach was used in order to ensure that the gene of interest was knocked down throughout all life stages. On day 1, bacterial cultures were started and grown at 37°C and 200 rpm for 14 h overnight. On day 2, 2 mM (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to cultures, which were incubated at 37°C and 200 rpm for 1 h. Cultures were then spun down and resuspended in K+ media. Open vector or test bacteria, along with K+ media were added to appropriate wells in a 96-well plate. 9 L4 nematodes were then added to each well and the plate was place in a 20°C incubator. On day 3, bacterial cultures were started and grown at 37°C and 200 rpm for 14 h overnight. On day 4, 2 mM (IPTG) was added to cultures, which were incubated at 37°C and 200 rpm for 1 h. Cultures were then spun down and resuspended in complete K medium. Open vector or test bacteria, along with complete K medium and either $HgCl_2$ or MeHg were added to appropriate wells in a 96-well plate (Figure 3). Using the COPAS Biosort, 50 L1 larval stage offspring from the nematodes plated on day 2 were removed from each well of the original plate and added to the corresponding well on the new plate. The growth of these nematodes was assessed 48 h later. This protocol ensures gene knock-down throughout the

development of the nematode, but allows for the same analysis of growth that is used in the standard growth assay. Each experimental and control condition was replicated in 4 wells on each plate.

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *gcs-1* | *gcs-1* | *gcs-1* + MeHg | *gcs-1* + MeHg | Rinse | Rinse | open vector | open vector | open vector | open vector | Rinse | Rinse |
| $HgCl_2$ | $HgCl_2$ | $HgCl_2$ | $HgCl_2$ | Rinse | Rinse | MeHg | MeHg | MeHg | MeHg | Rinse | Rinse |
| RNAi | RNAi | RNAi | RNAi | Rinse | Rinse | RNAi + $HgCl_2$ | RNAi + $HgCl_2$ | RNAi + $HgCl_2$ | RNAi + $HgCl_2$ | Rinse | Rinse |
| RNAi + MeHg | RNAi + MeHg | RNAi + MeHg | RNAi + MeHg | Rinse | Rinse | RNAi | RNAi | RNAi | RNAi | Rinse | Rinse |
| RNAi + $HgCl_2$ | RNAi + $HgCl_2$ | RNAi + $HgCl_2$ | RNAi + $HgCl_2$ | Rinse | Rinse | RNAi + MeHg | RNAi + MeHg | RNAi + MeHg | RNAi + MeHg | Rinse | Rinse |
| RNAi | RNAi | RNAi | RNAi | Rinse | Rinse | RNAi + $HgCl_2$ | RNAi + $HgCl_2$ | RNAi + $HgCl_2$ | RNAi + $HgCl_2$ | Rinse | Rinse |
| RNAi + MeHg | RNAi + MeHg | RNAi + MeHg | RNAi + MeHg | Rinse | Rinse | RNAi | RNAi | RNAi | RNAi | Rinse | Rinse |
| RNAi + $HgCl_2$ | RNAi + $HgCl_2$ | RNAi + $HgCl_2$ | RNAi + $HgCl_2$ | Rinse | Rinse | RNAi + MeHg | RNAi + MeHg | RNAi + MeHg | RNAi + MeHg | Rinse | Rinse |

**Figure 3: 96-well plate layout for *C. elegans* RNAi experiments.** The effect of knocking a gene in mercury exposure was tested in 4 genes on each plate. Different genes are represented by different colors in the figure. Rinse wells contained water and were used to ensure nematodes from different treatments were not mixed during growth measurements. Knock-down of *gcs-1* in the presence of MeHg was used as a positive control.

This experiment was done in 2 phases. In the first phase, a liberal assessment of gene-mercury interaction was done by visual observation. In the first phase, bacterial cultures were started directly from one of the two libraries. As not all of these bacterial clones have been verified, there were some cases in which an unintended gene was knocked down. All clones that passed the first phase were sequenced using the NIEHS

sequencing facility to verify the identity of each clone. In the second phase, the growth of the worms was measured using the COPAS Biosort as described in the growth assay. In the second phase, each plate was replicated 3-4 times. As a result, 500-800 individual nematodes were measured for each gene-mercury combination. A 2-way ANOVA was used to test for significant gene-mercury interactions. The criterion for a statistically significant interaction was set at $p < 0.01$.

## *2.9 Maintenance of mammalian cell lines*

Human neuroblastoma (SK-N-SH), hepatocellular carcinoma (HepG2) and embryonic kidney (HEK293) cells were cultured in Minimum Essential Medium supplemented with 10% Fetal Bovine Serum and 2 mM L-glutamine. Cells were grown in a humidified incubator at 37°C under 5% $CO_2$ atmosphere.

## *2.10 Transfection of siRNA into cells*

Cells were transfected 48 hours prior to collection of RNA or neutral red assay. Prior to adding cells, Opti-MEM (20% final concentration) and lipofectamine RNAiMAX (Invitrogen, 0.2% final concentration) were added to wells. siRNA or non-homologous siRNA (25 nM final concentration) (Table 2) were added to appropriate wells and allowed to incubate at ambient temperature for 20 min. SK-N-SH and HepG2 cells were plated at ~30% confluency and HEK293 cells were plated at ~20% confluency. Mercury was added to cells 24 h after transfection.

**Table 2: Sequences of siRNA used in mammalian cell culture**

| Gene | Target Sequence |
|---|---|
| ABCG2 | CTGGTCTAATTTATTAATCTA |
| BACE1 | CACAGTGGCACTAGCATTATA |
| BACE2 | TACATGTGCCACCAACATAAA |
| CHKA | AGCCGGCGATTAGATACTGAA |
| CHKB | CACGAAGATGGCGCAATTTCA |
| ELOVL3 | ACGGTTCATCATCCTGCGTAA |
| ELOVL6 | TAGGTTGATTTAACCCAGTAA |
| GCLC | TAGGATCAGTAAATCCCGATA |
| PARG | CTGGATCACAATGAATGTCTA |

## *2.11 Neutral Red assay*

At the end of the exposure time, media containing 40 μg/ml neutral red (Sigma, St. Louis, MO) and cells were incubated at 37°C for 2 h. The media was then removed and a 1% $CaCl_2$/0.5% Formaldehyde solution was added to the cells and quickly removed. Then a 1% acetic acid/50% ethanol solution was added to the cells. The cells were placed on a shaker for 5 min and the OD540 was measured using the FLUOStar Optima (BMG LabTech, Offenburg, Germany). In the dose-response experiments, data are presented as mean percent control ± standard error (n = 3-6). In the gene-mercury

interaction experiments, OD540 data were analyzed using a 2-way ANOVA with a Bonferroni post-hoc test. Data are presented as percent difference from control.

## *2.12 Isolation of RNA from cells*

RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA concentration was determined using the ND-1000 spectrophotometer (Nanodrop, Wilmington, DE), and RNA was stored at -80°C until use.

# 3. Results

## *3.1 Toxicity of mercurials in C. elegans*

In order to compare the molecular effects of inorganic and organic mercury, it was first necessary to determine the toxicity of the representative mercurials, $HgCl_2$ and MeHg, to *C. elegans*. To do this, the toxicity of the two mercurials was assessed by determining their effects on growth, reproduction, and induction of stress-response genes.

### 3.1.1 Effect of mercurials on growth

The effect of $HgCl_2$ and MeHg on the growth of *C. elegans* was determined by measuring the 48 h dose-response to 11 concentrations of each mercurial relative to untreated worms. The EC50, which represents a 50% inhibition of growth, was very similar between the two mercurials. The EC50 for $HgCl_2$ was 12 μM (11.9-12.2, 95% CI), while the EC50 for MeHg was 11.3 μM (11.2 -11.5, 95% CI) (Figure 4).

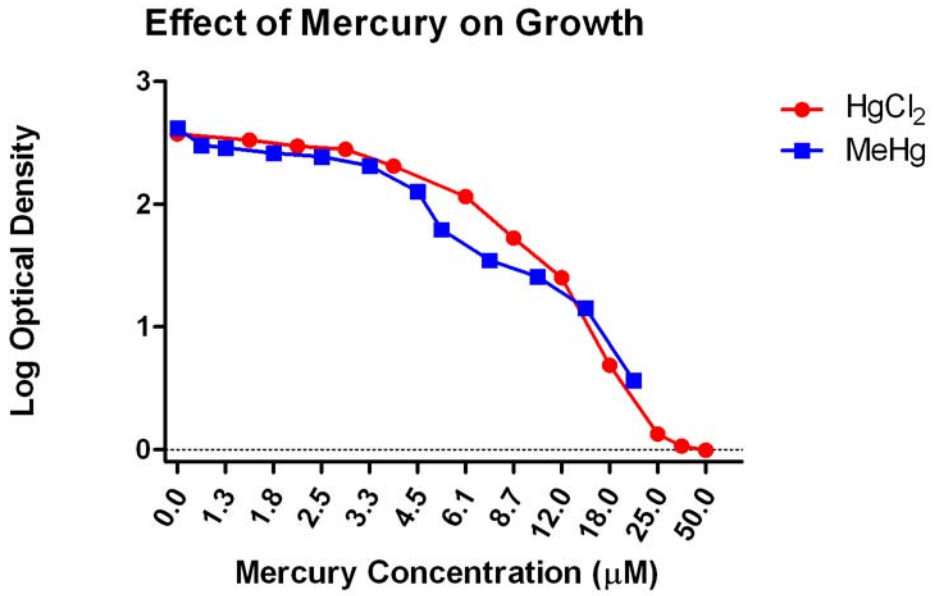



**Figure 4: Results of 48 h dose-response growth assay.** Results are displayed as mean Log optical density ± SEM.

### 3.1.2 Effect of mercury on reproduction

Reproduction in *C. elegans* involves the integration of several processes (e.g. gamete formation, embryonic development and egg lying, which is an integrated neuromuscular function), which makes it a sensitive endpoint in which to measure the effects of toxicants. There was a very large difference between the EC50 of $HgCl_2$ and the EC50 of MeHg. The EC50 for $HgCl_2$ was 14 μM (9.9-18, 95% CI), while the EC50 for MeHg was only 0.81 μM (0.68-0.95, 95% CI) (Figure 5). By this measure MeHg was greater than 15 times more toxic to *C. elegans* than $HgCl_2$.

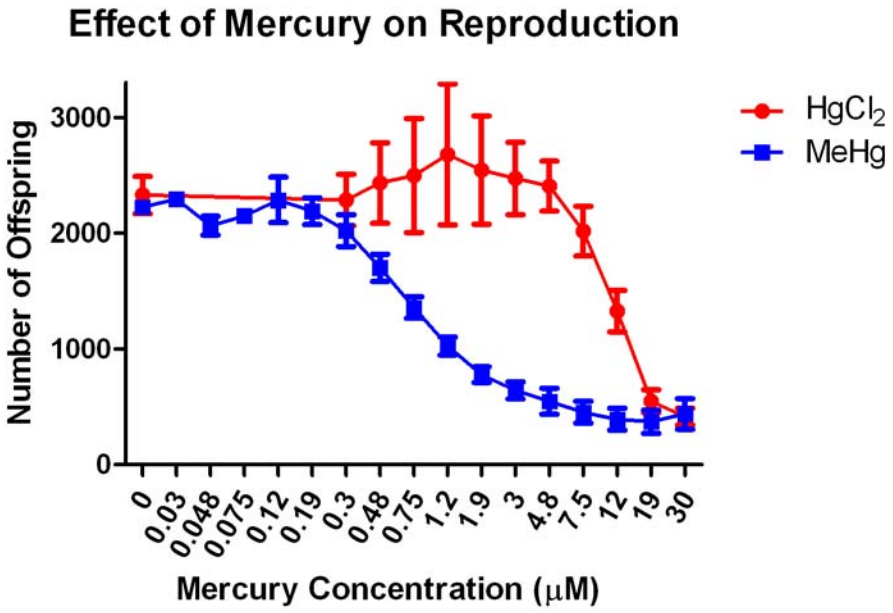



**Figure 5: Effect of mercury on reproduction.** Results are displayed as mean ± SEM.

### 3.1.3 Induction of stress-response genes by mercury

In order to determine the effect of exposure to mercurials at a molecular level, we measured the dose-response of $HgCl_2$ and MeHg on steady-state mRNA levels of 4 stress-responsive genes: *gcs-1*, *gst-38*, *hsp-16.2*, and *hsp-70*. *gcs-1* catalyzes the rate-limiting first step in the synthesis of glutathione and is involved in the oxidative stress pathway [113]. The glutathione S-transferase *gst-38* has been previously shown to be up-regulated in response to multiple stressors [114, 115]. *hsp-16.2* and *hsp-70* are heat shock proteins that are up-regulated by heat shock and other environmental stressors [116, 117]. Steady-state mRNA levels of these genes were measured following a 24 h exposure of a mixed-stage *C. elegans* culture to a range of $HgCl_2$ or MeHg concentrations (Figure

6). For all four genes, there was a dose-dependent increase in mRNA levels after exposure to either mercurial. The heat shock proteins required higher concentrations of mercurials to be induced than did *gcs-1* or *gst-38*. Thus, induction of glutathione-response genes may be a more sensitive indicator of mercury stress than induction of heat shock protein genes. As the rate-limiting enzyme in the production of glutathione, *gcs-1* would be expected to play a critical role in the glutathione-response [113]. In all genes tested, a lower concentration of MeHg was required to induce a significant increase in expression relative to $HgCl_2$.

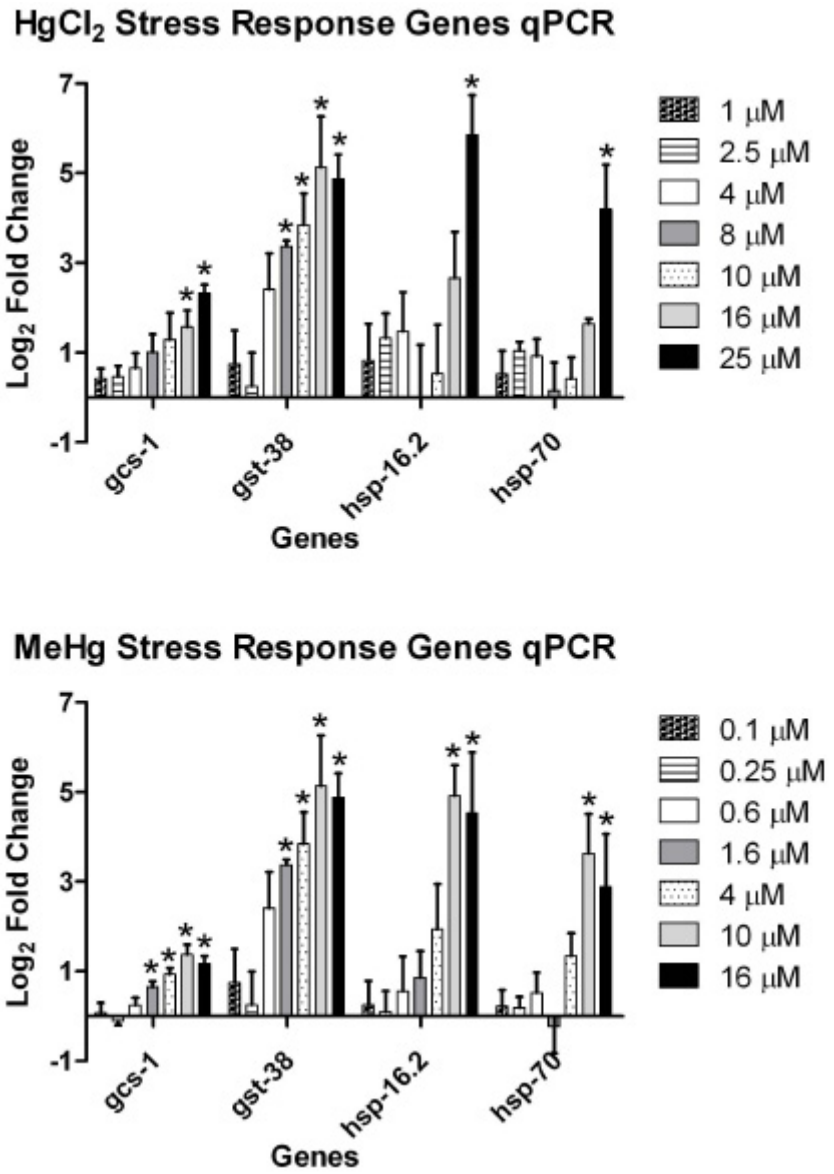



**Figure 6: Dose-response of *C. elegans* stress response gene qRT-PCR.** Results are displayed as mean $Log_2$ ± SEM. Significant differences ($p < 0.05$) relative to untreated *C. elegans* are designated with an asterisk.

## *3.2 Analysis of mercury-induced changes in gene expression*

In order to determine if inorganic and organic mercury act differently at the molecular level, microarrays were performed to determine the global effects of exposure to either $HgCl_2$ or MeHg on transcription.

### 3.2.1 Determination of conditions for microarrays

In order to effectively compare the transcriptional effects of the two mercurials, it was necessary to use approximately equitoxic concentrations of each mercurial. After taking into consideration, the growth, reproduction and stress-response gene qRT-PCR assay results, sub-, low- and high-toxic concentrations were established for each mercurial (table 3).

**Table 3: Mercury concentrations used in the microarray**

| Toxicity | $HgCl_2$ | MeHg |
|---|---|---|
| Sub-Toxic | 2 μM | 0.75 μM |
| Low-Toxic | 7.5 μM | 2 μM |
| High-Toxic | 20 μM | 7.5 μM |

Cui et al. used microarrays to examine the transcriptional effects of a single concentration of cadmium at 4 and 24 h in *C. elegans* [114]. They found a greater number of differentially expressed genes at 24 h compared to 4 h. Furthermore, all genes that

were differentially expressed at 4 h were also differentially expressed at 24 h. For this reason, a 24 h exposure with varying concentrations of mercury was used in this study.

Both mercurials were found to inhibit growth (Figure 4) and reproduction (Figure 5). Therefore, it was necessary to ensure that differences in gene expression were due primarily to the direct effects of mercury, and not differences in the population distribution of developmental stages in the treatment groups. To do this, nematodes were exposed to conditions identical to those used in the microarray. At the end of the exposure, nematodes were measured using the COPAS. The length (time of flight (TOF)) of the first 7000 nematodes was measured and the population distribution of treated nematodes was compared to untreated nematodes (Figure 7).

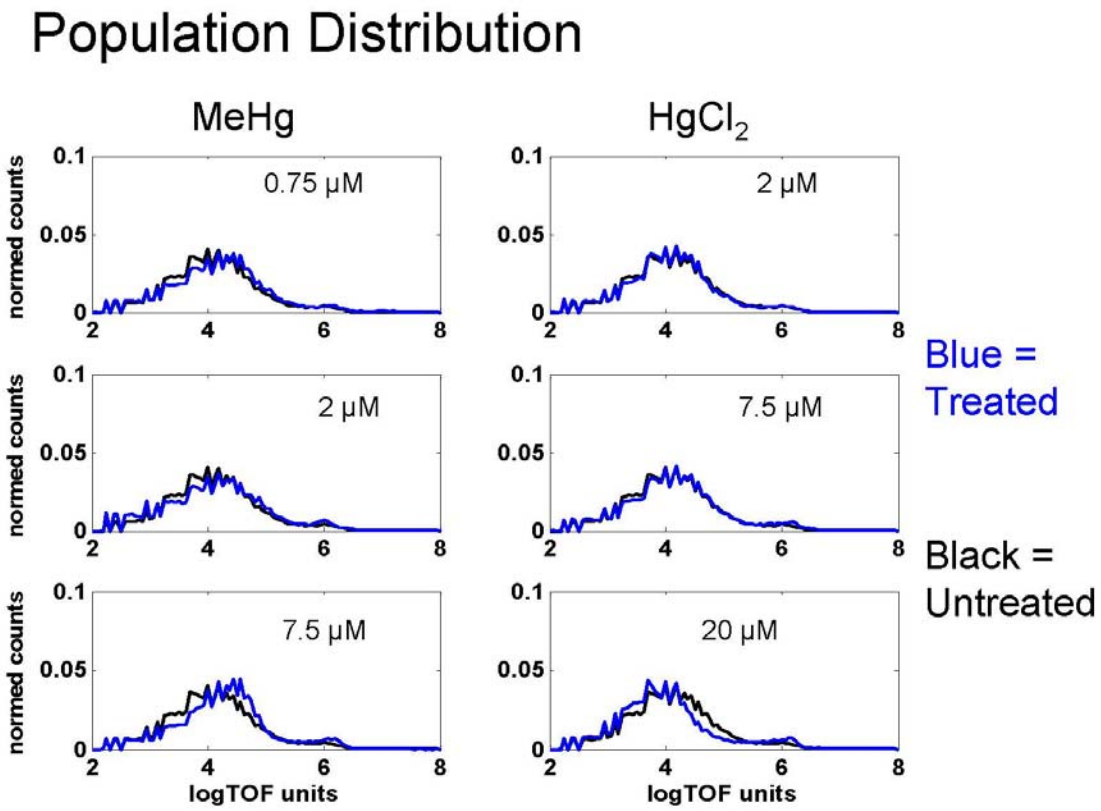



**Figure 7: Effect of mercury exposure on population distribution.** The length (TOF) of nematodes was measured to determine size. Normed counts at each size represent the fraction of the total population.

There were not large differences in the population distribution between treated nematodes and untreated nematodes. Therefore, differences in transcriptional profiles are likely due primarily to the effects of the mercurials.

### 3.2.2 Genes differentially expressed in response to mercury exposure

Differentially expressed genes for each treatment were defined as those for which the difference in expression was both statistically significant and 2-fold or greater, relative to the expression in untreated nematodes. For both mercurials, increasing concentrations of mercury resulted in increasing numbers of differentially expressed genes (DEGs) (Table 4).

**Table 4: Differentially expressed genes**

| $HgCl_2$ | | MeHg | |
|---|---|---|---|
| Concentration | Differentially Expressed Genes | Concentration | Differentially Expressed Genes |
| 2 μM | 8 (8 up, 0 down) | 0.75 μM | 44 (35 up, 9 down) |
| 7.5 μM | 74 (68 up, 6 down) | 2 μM | 419 (247 up, 172 down) |
| 20 μM | 403 (316 up, 87 down) | 7.5 μM | 2791 (1604 up, 1187 down) |

At each level of toxicity, a higher percentage of DEGs were down-regulated by MeHg than $HgCl_2$: sub-toxic (0% $HgCl_2$, 20% MeHg), low-toxic (8% $HgCl_2$, 41% MeHg), high-toxic (22% $HgCl_2$, 43% MeHg). Tables 5-8 list the top 10 up- and down-regulated genes for each treatment condition.

**Table 5: Top up-regulated genes in $HgCl_2$ exposures**

| 2 µM $HgCl_2$ | | | 7.5 µM $HgCl_2$ | | | 20 µM $HgCl_2$ | | |
|---|---|---|---|---|---|---|---|---|
| Sequence Name | Gene Name | Fold Change | Sequence Name | Gene Name | Fold Change | Sequence Name | Gene Name | Fold Change |
| T08G5.10 | *mtl-2* | 3.1 | T08G5.10 | *mtl-2* | 42 | F22E10.4 | *pgp-15* | 84 |
| K01D12.1 | | 2.1 | T08G5.1 | | 29 | C15B12.8 | | 43 |
| M02D8.4 | | 2.1 | K11G9.6 | *mtl-1* | 12 | C45B2.3 | | 25 |
| F07C4.10 | | 2.1 | F56A4.2 | | 11 | C17H1.8 | | 21 |
| F15H10.8 | | 2.1 | Y39B6A.1 | | 7.9 | Y70C5C.2 | *clec-9* | 21 |
| K01D12.3 | | 2.0 | F21F8.4 | | 7.8 | F26F2.2 | | 19 |
| C08F11.3 | | 2.0 | C15B12.8 | | 6.6 | F15H10.5 | | 17 |
| ZK675.4 | | 2.0 | Y46C8AL.3 | *clec-70* | 5.9 | T10B9.4 | *cyp-13A8* | 16 |
| | | | C32H11.12 | *dod-24* | 4.6 | T08G5.10 | *mtl-2* | 16 |
| | | | C32A3.1 | *sel-8* | 4.5 | F21C10.9 | | 14 |

**Table 6: Top down-regulated genes in $HgCl_2$ exposures**

| 2 µM $HgCl_2$ | | | 7.5 µM $HgCl_2$ | | | 20 µM $HgCl_2$ | | |
|---|---|---|---|---|---|---|---|---|
| Sequence Name | Gene Name | Fold Change | Sequence Name | Gene Name | Fold Change | Sequence Name | Gene Name | Fold Change |
| | | | T25D10.2 | | -3.8 | K03B8.11 | | -6.0 |
| | | | F49H6.12 | | -3.7 | T15B7.3 | *col-143* | -5.2 |
| | | | F29A7.7 | *clec-20* | -3.7 | Y57A10C.1 | | -5.2 |
| | | | F14F8.4 | *srz-103* | -2.9 | K05F6.4 | | -5.2 |
| | | | C43G2.2 | *bicd-1* | -2.8 | F54E7.5 | *sdz-21* | -5.1 |
| | | | T04C9.2 | | -2.6 | B0462.3 | *fbxb-119* | -5.0 |
| | | | | | | F34D6.1 | | -4.8 |
| | | | | | | M151.5 | *fbxb-31* | -4.6 |
| | | | | | | Y82E9BL.3 | | -4.5 |
| | | | | | | Y63D3A.11 | | -4.2 |

**Table 7: Top up-regulated genes in MeHg exposures**

| 0.75 µM MeHg | | | 2 µM MeHg | | | 7.5 µM MeHg | | |
|---|---|---|---|---|---|---|---|---|
| Sequence Name | Gene Name | Fold Change | Sequence Name | Gene Name | Fold Change | Sequence Name | Gene Name | Fold Change |
| Y32G9A.1 | *gst-37* | 9.4 | Y32G9A.1 | *gst-37* | 77 | Y32G9A.1 | *gst-37* | 100 |
| Y1H11.2 | *gst-35* | 8.5 | F11A5.12 | *stdh-2* | 24 | F11A5.12 | *stdh-2* | 98 |
| C29F3.1 | *ech-1* | 4.2 | Y1H11.2 | *gst-35* | 22 | W08E12.2 | | 69 |
| F35E12.5 | | 4.0 | H23L24.5 | *pme-4* | 22 | F22E5.6 | | 68 |
| M199.7 | | 4.0 | Y43F8C.1 | *nlp-25* | 19 | Y53F4B.35 | *gst-31* | 67 |
| F11A5.12 | *stdh-2* | 3.8 | F48C1.9 | | 17 | C14C6.4 | *nhr-155* | 63 |
| C04F5.7 | *ugt-63* | 3.8 | F35E12.5 | | 17 | ZC239.12 | *sdz-35* | 60 |
| ZK546.11 | *gst-30* | 3.7 | F35E12.2 | | 16 | Y46H3A.3 | *hsp-16.2* | 57 |
| C32H11.9 | | 3.5 | C15B12.8 | | 14 | F29F11.2 | *ugt-34* | 52 |
| Y43F8C.1 | *nlp-25* | 3.5 | ZK20.2 | *kin-6* | 11 | F33H12.4 | *sri-74* | 52 |

**Table 8: Top down-regulated genes in MeHg exposures**

| 0.75 µM MeHg | | | 2 µM MeHg | | | 7.5 µM MeHg | | |
|---|---|---|---|---|---|---|---|---|
| Sequence Name | Gene Name | Fold Change | Sequence Name | Gene Name | Fold Change | Sequence Name | Gene Name | Fold Change |
| F37B1.8 | *gst-19* | -4.2 | T26H2.5 | | -11 | C13A2.4 | | -99 |
| T08G5.10 | *mtl-2* | -3.2 | Y32B12A.1 | | -10 | F46B3.17 | *col-163* | -97 |
| T26H2.5 | | -3.1 | R05D8.11 | | -7.8 | R05D8.11 | | -93 |
| C05E4.14 | *srh-2* | -2.2 | F37B1.8 | *gst-19* | -5.9 | F53F4.7 | | -91 |
| ZK666.6 | *clec-160* | -2.1 | E03H12.3 | *clec-176* | -5.4 | T26H2.5 | | -88 |
| C15A11.7 | | -2.1 | F28G4.5 | | -4.8 | C13A2.9 | | -85 |
| F11A5.9 | | -2.1 | T24B8.5 | | -4.6 | C13A2.6 | | -82 |
| C11E4.7 | | -2.1 | Y41D4B.6 | | -4.5 | C13A2.11 | | -59 |
| Y4C6B.6 | | -2.0 | F26D2.14 | | -4.4 | F07G11.3 | | -58 |
| | | | Y38E10A.5 | *clec-4* | -4.3 | Y32B12A.1 | | -58 |

There was surprisingly little overlap among genes whose expression was affected by exposure to inorganic mercury (Figure 8). In fact the only gene that was differentially expressed in all three exposures was the metallothionein *mtl-2*.

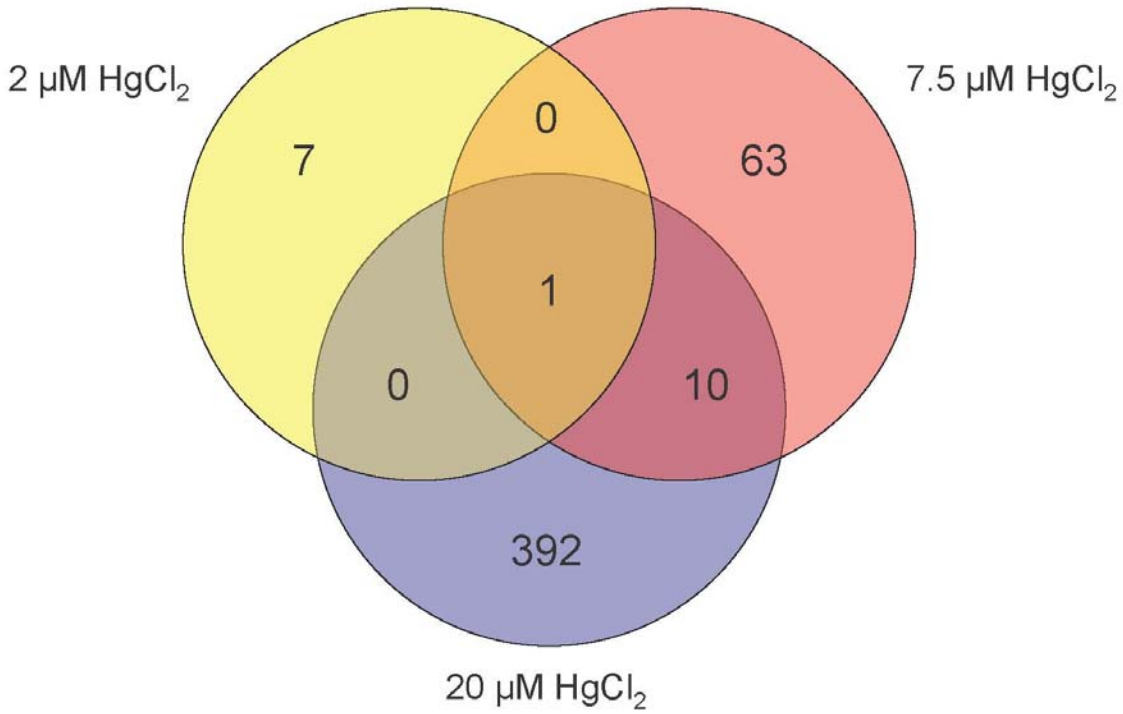



**Figure 8: Overlap of genes differentially expressed in $HgCl_2$ exposures.**

In contrast, there was high degree of overlap in genes differentially expressed after exposure to different concentrations of MeHg (Figure 9). Genes differentially expressed in response to all MeHg exposures are listed in Table 9.

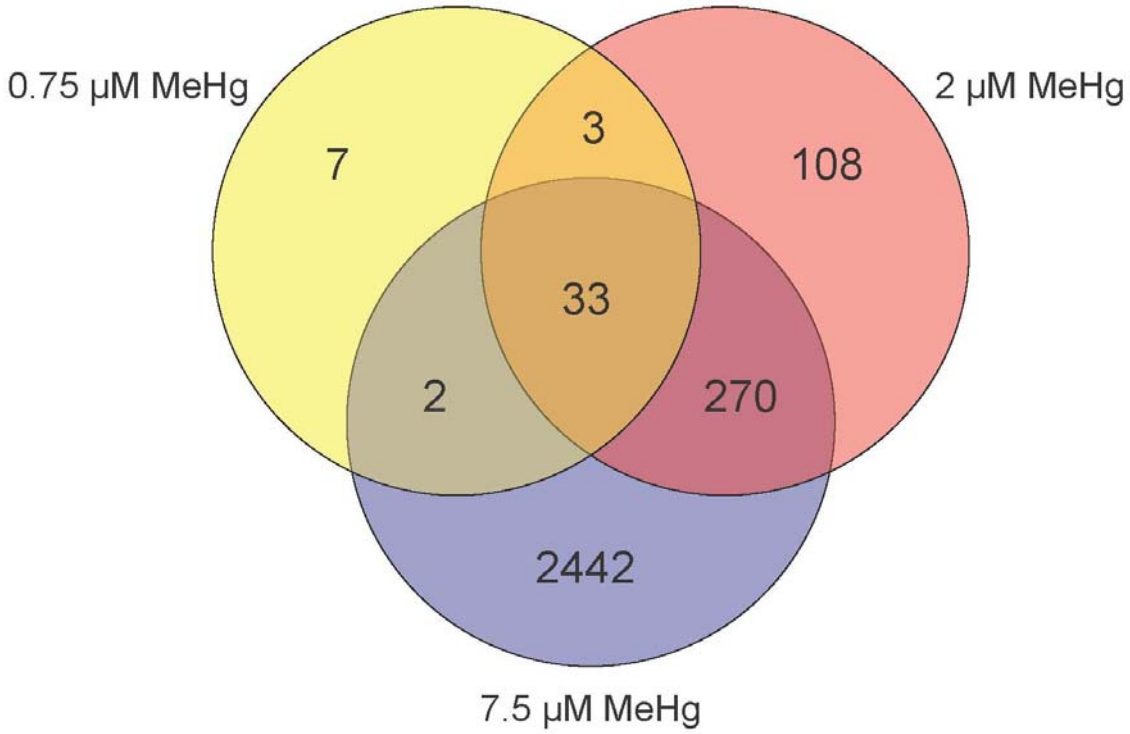



**Figure 9: Overlap of genes differentially expressed in MeHg exposures.**

**Table 9: Genes differentially expressed in all MeHg treatments.** Number under each concentration indicates the fold-change in expression of that gene in that treatment.

| Genes up-regulated in all MeHg exposures | | | | |
|---|---|---|---|---|
| Sequence Name | Gene Name | 0.75 µM | 2 µM | 7.5 µM |
| B0507.8 | | 2.3 | 2.2 | 2.0 |
| B0554.6 | *dod-20* | 2.3 | 3.3 | 8.5 |
| C15B12.8 | | 2.7 | 14 | 14 |
| C33A12.6 | *ugt-21* | 2.1 | 3.7 | 3.5 |
| C34H4.1 | | 2.6 | 6.6 | 13 |
| F07E5.9 | | 2.3 | 4.3 | 4.7 |
| F11A5.12 | *stdh-2* | 3.8 | 24 | 98 |
| F11D11.3 | | 2.5 | 6.5 | 33 |
| F15B9.1 | *far-3* | 2.2 | 3.8 | 6.3 |
| F35E12.5 | | 4.0 | 17 | 32 |
| F37B1.2 | *gst-12* | 2.1 | 7.0 | 39 |
| F53B2.2 | *tsp-4* | 2.2 | 5.3 | 15 |
| F56D5.3 | | 2.2 | 5.0 | 7.0 |
| K08F4.7 | *gst-4* | 2.4 | 4.4 | 10 |
| M199.7 | | 4.0 | 4.9 | 4.1 |
| T04H1.9 | *tbb-6* | 2.4 | 9.6 | 36 |
| W06H8.2 | | 2.9 | 9.9 | 29 |
| Y1H11.2 | *gst-35* | 2.9 | 9.9 | 29 |
| Y32G9A.1 | *gst-37* | 9.4 | 77 | 100 |
| Y39G10AR.6 | *ugt-31* | 2.1 | 4.4 | 5.7 |
| Y43F8C.1 | *nlp-25* | 3.5 | 19 | 17 |
| Y45F10B.1 | *tsp-5* | 2.1 | 5.4 | 17 |
| ZC239.14 | | 2.5 | 4.4 | 11 |
| ZK697.6 | *gst-21* | 2.9 | 4.2 | 2.6 |

| Genes down-regulated in all MeHg exposures | | | | |
|---|---|---|---|---|
| Sequence Name | Gene Name | 0.75 µM | 2 µM | 7.5 µM |
| C05E4.14 | *srh-2* | -2.2 | -3.4 | -4.5 |
| C11E4.7 | | -2.1 | -3.4 | -6.9 |
| C15A11.7 | | -2.1 | -3.9 | -6.7 |
| F11A5.9 | | -2.1 | -2.8 | -8.3 |
| F37B1.8 | *gst-19* | -4.2 | -5.9 | -5.4 |
| T08G5.10 | *mtl-2* | -3.2 | -3.5 | -3.5 |
| T26H2.5 | | -3.1 | -11 | -88 |
| ZK666.6 | *clec-60* | -2.1 | -3.2 | -3.0 |

The overlap between up-regulated genes of equitoxic treatments was compared to gauge the similarity of the transcriptional responses of nematodes in response to $HgCl_2$ and MeHg. When comparing low-toxicity treatments, only two genes, a UDP-glucuronosyl transferase (*ugt-21*) and C15B12.8 (a gene of unknown function) were up-regulated by both mercurials (Figure 10).

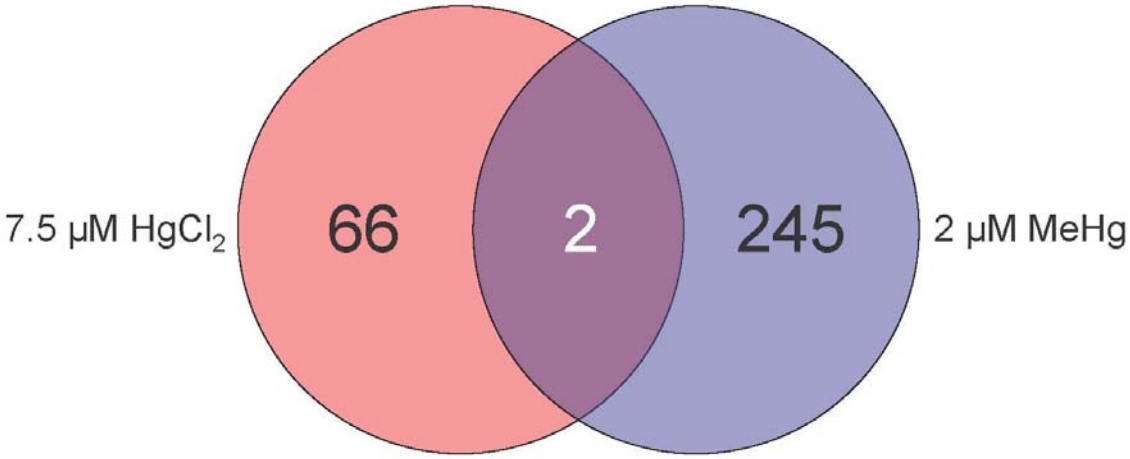



**Figure 10: Overlap of genes up-regulated in low-toxic mercury exposures.**

There was greater overlap in genes up-regulated by high-toxicity mercury treatments, but the majority of genes were only up-regulated after exposure to one mercurial (Figure 11).

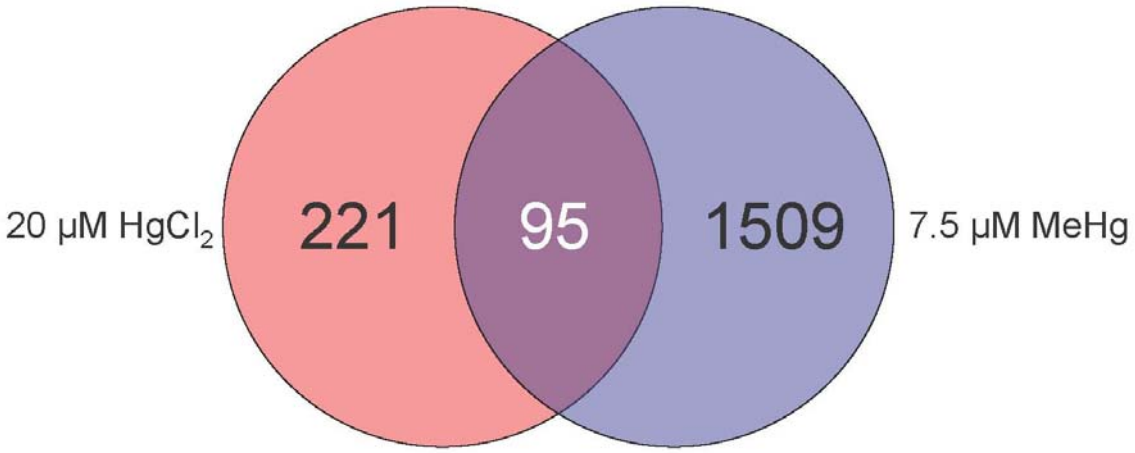



**Figure 11: Overlap of genes up-regulated in high-toxic mercury exposures.**

Metallothioneins are small cysteine-rich, metal-binding proteins whose transcription is up-regulated in response to metal exposure. *C. elegans* have two metallothioneins, *mtl-1* and *mtl-2* [118]. *mtl-1* has greater levels of basal transcription than *mtl-2*, but transcription of both genes can be dynamically regulated [118]. As expected, *mtl-2* was up-regulated by all three $HgCl_2$ treatments. What was not expected was that *mtl*-2 was down-regulated in all three MeHg exposures. To confirm these results, qRT-PCR for both metallothioneins was performed using the RNA used for the microarrays. *mtl-2* was up-regulated in all $HgCl_2$ treatments and *mtl-1* was up-regulated in sub- and low-toxic $HgCl_2$ exposures. *mtl-2* was down-regulated in all MeHg treatments (Figures 12 and 13).

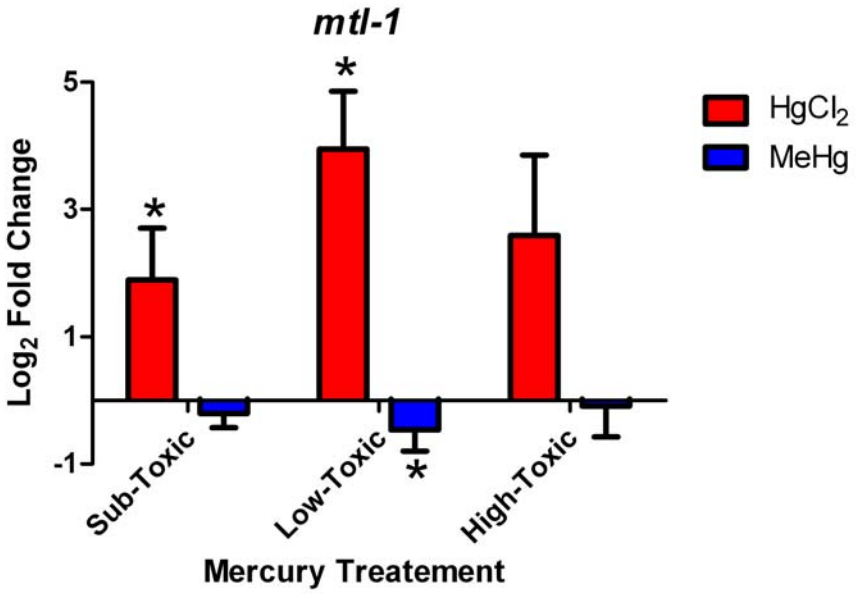



**Figure 12:** ***mtl-1* qRT-PCR.** Results are displayed as mean $Log_2$ ± SEM. Significant differences ($p < 0.05$) relative to untreated *C. elegans* are designated with an asterisk.

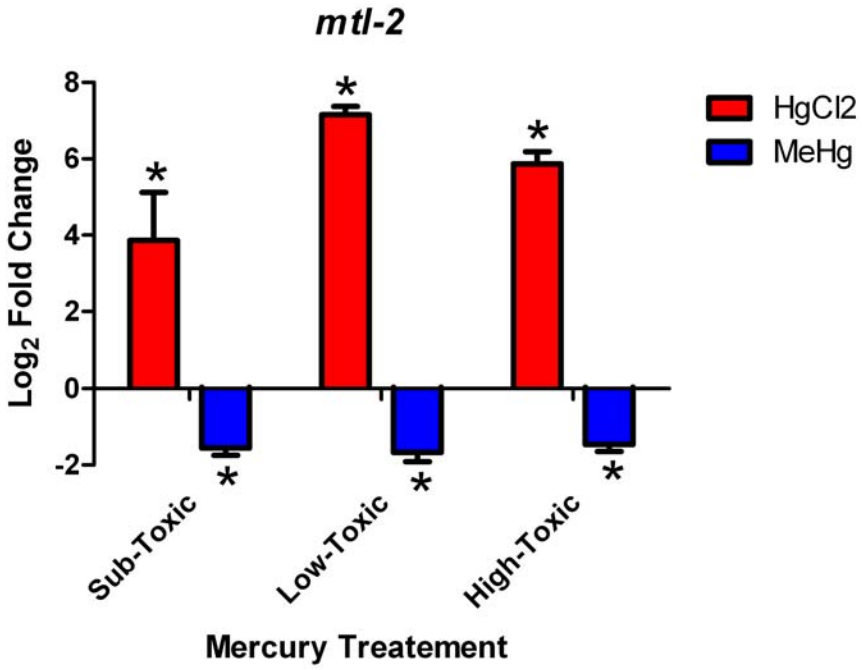



**Figure 13:** ***mtl*-2 qRT-PCR.** Results are displayed as mean $Log_2$ ± SEM. Significant differences ($p < 0.05$) relative to untreated *C. elegans* are designated with an asterisk.

### 3.2.3 Tissue distribution of differentially expressed genes

It is well known that there are differences in the tissue distribution of $HgCl_2$ and MeHg in animal models, and this explains at least some of the differences in toxicity between mercurials. In order to determine if different mercurials targeted different tissues in *C. elegans*, the tissue-specific expression of DEGs was examined. The tissue specific expression for hundreds of *C. elegans* genes is known based on promoter::GFP studies. The expression patterns for 186 DEGs from this study have been previously examined using this technique. Of these 186 genes, which represent 6% of the total

number of DEGs, 11 were differentially expressed in response to both mercurials, 24 were differentially expressed only in response to $HgCl_2$, and 151 were differentially expressed only in response to MeHg. The expression patterns of these genes were grouped into the following tissues: intestine, pharynx, neuron, muscle, hypodermis, reproductive and "other". Many genes were expressed in multiple tissues. In these cases all tissues in which the gene is expressed were included in the analysis. Based on this limited sample, there does not appear to be a substantial difference in the tissues affected by $HgCl_2$ and MeHg (Figure 14).

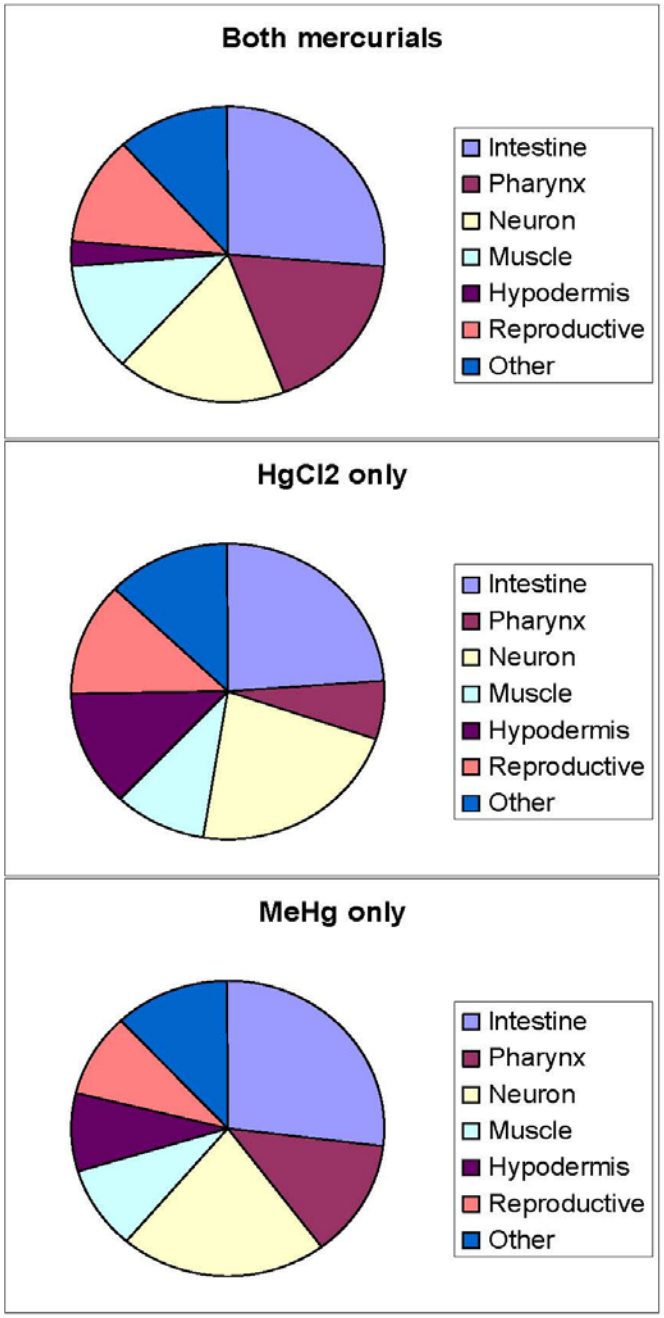



**Figure 14: Relative proportion of differentially expressed genes by treatment and tissue**

### 3.2.4 Bioinformatics analysis of differentially expressed genes

Principal components analysis (PCA) and hierarchical clustering were performed to visualize the effects of inorganic and methylmercury on the *C. elegans* transcriptome. Principal components analysis using all probes (all *C. elegans* genes in duplicate) on the microarray found good spatial positioning of experimental replicates, which indicates good reproducibility between experiments (Figure 15). The first 2 principal components accounted for 55% of the variation. The first principal component, which accounts for 33% of the variation in the data, segregated treatments by mercurial. The second principal component, which accounts for 22% of the variation, segregated treatments by toxicity. The sub- and low-toxic replicates were scattered together in $HgCl_2$ treated nematodes as well as MeHg treated nematodes. The high-toxic replicates were divergent in both $HgCl_2$ and MeHg treated nematodes. In order to limit experimental noise, a principal components analysis was performed using only differentially expressed genes (Figure 16). The overall pattern was similar, though the first two principal components accounted for 85% of the variation in the data. These analyses indicate that the inorganic and methylmercury have different effects on transcription. They also indicate that, for both mercurials, there is a large difference in the transcriptional response to a low-toxic mercury exposure vs. a high-toxic mercury exposure.

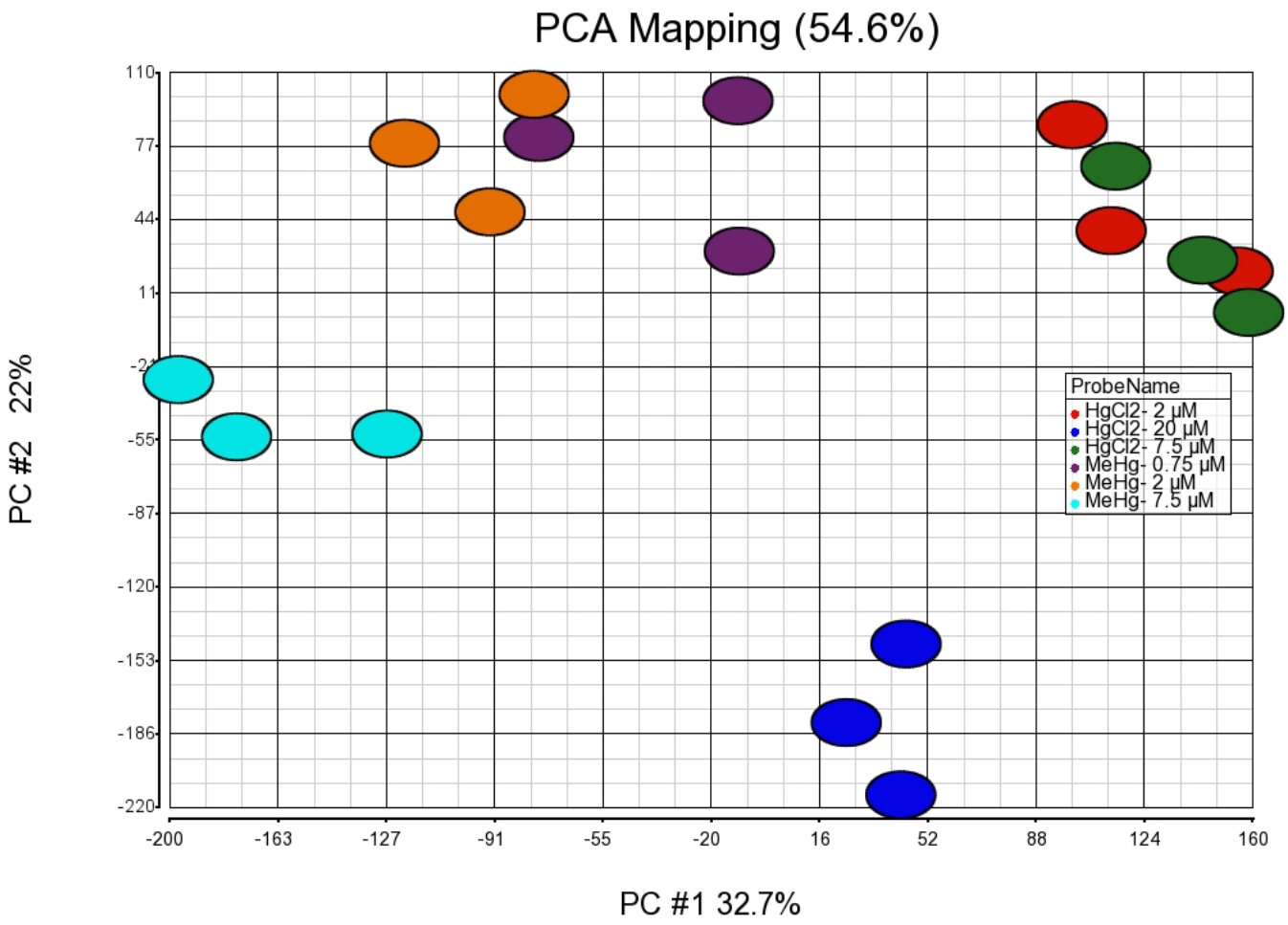



**Figure 15: Principal components analysis using all genes on microarray**

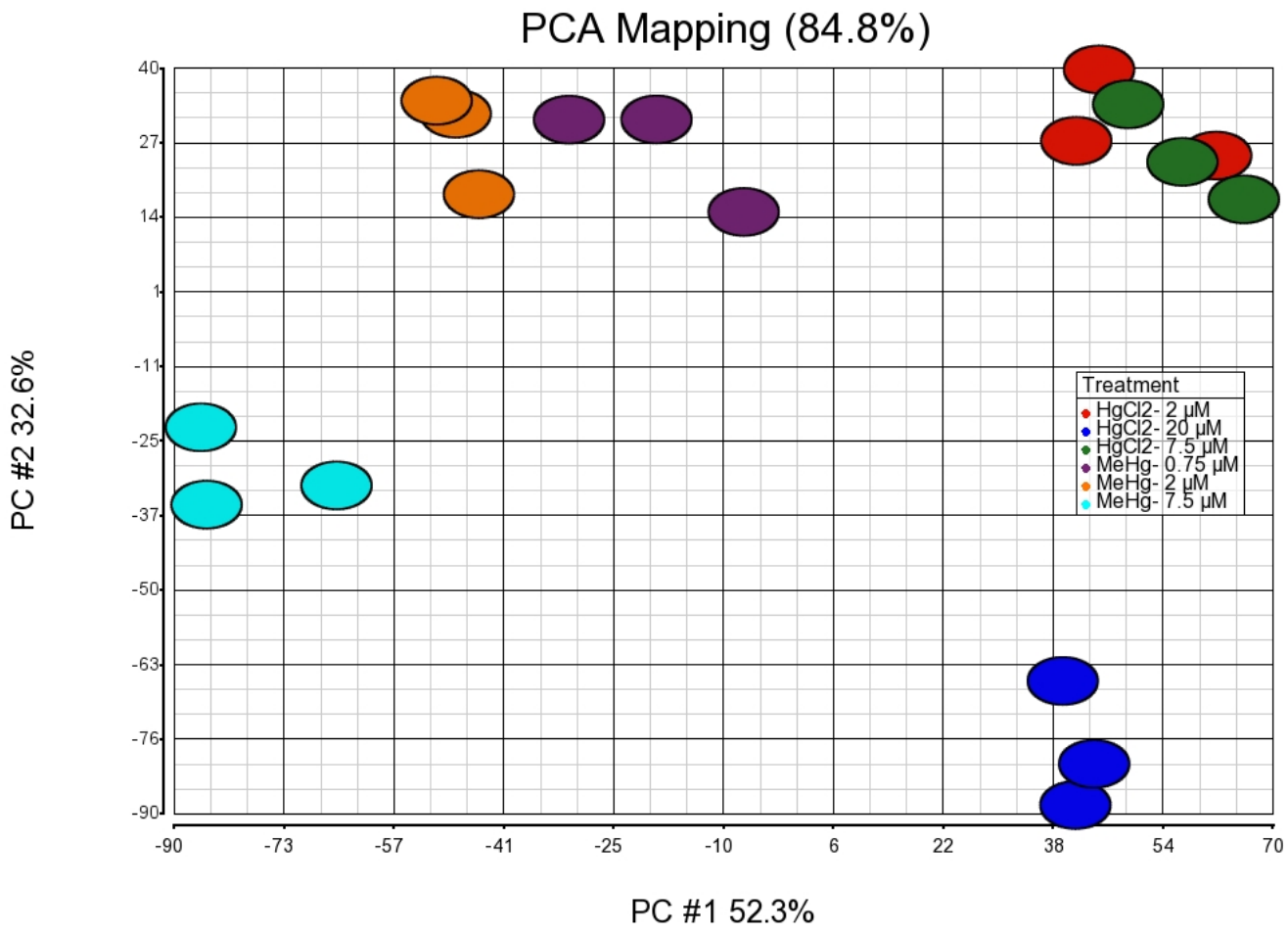



**Figure 16: Principal components analysis using differentially expressed genes**

In the hierarchical clustering analysis of differentially expressed genes, individual replicates also clustered fairly tightly by treatment (Figure 17). This indicates a high degree of reproducibility between experimental replicates. The gene expression profiles for the sub- and low-toxic $HgCl_2$ treated nematodes were very similar. The gene expression profiles for the sub- and low-toxicity MeHg treated nematodes were also very similar. However, the gene expression profiles for the two mercurials at sub- and low-toxic treatments were almost the opposite of one another. The gene expression profiles for the high-toxic exposures in $HgCl_2$ and MeHg treated *C. elegans* were both dissimilar from any other treatment. This is in agreement with the results of the PCA, and strongly suggests that the two mercurials act differently at the molecular level.

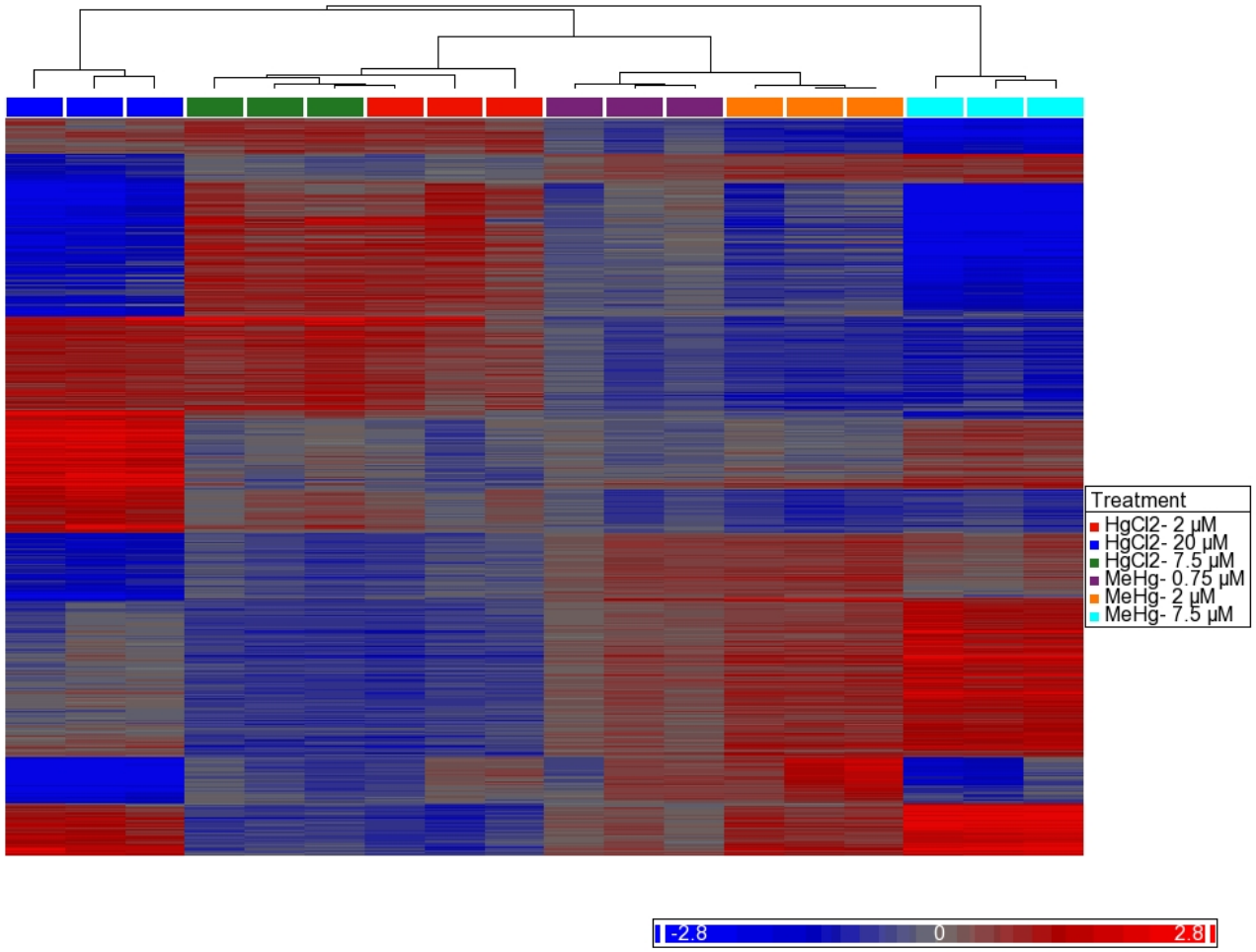



**Figure 17: Hierarchical clustering of microarray experimental replicates**

The microarray results were further analyzed to determine which biological processes were affected by the different mercurials. Genes differentially expressed by the different mercury treatments were examined for enrichment in Gene Ontologies (GO) and KEGG pathways. Gene Ontology provides a controlled vocabulary for describing the characteristics of gene products. A GO-biological process term describes a series of biological events, but it is not equivalent to a pathway. A GO-molecular function term describes a specific biological activity. A KEGG pathway is a manually drawn pathway, curated by the Kyoto Encyclopedia of Genes and Genomes, that represents the current knowledge of molecular interaction networks for a variety of biological pathways. The glutathione metabolism pathway, which was enriched with genes up-regulated by high-toxic $HgCl_2$ and low- and high-toxic MeHg exposures, is shown in Figure 18. Tables 10-16 compare the significantly enriched Gene Ontology Biological Processes, Molecular Function and KEGG pathways between equitoxic mercurial exposures. GO terms or KEGG pathways that are significantly enriched in genes differentially expressed by both mercurials are highlighted in yellow. There was not a significant enrichment of genes differentially expressed in sub-toxic $HgCl_2$ or down-regulated in low-toxic $HgCl_2$ exposures.

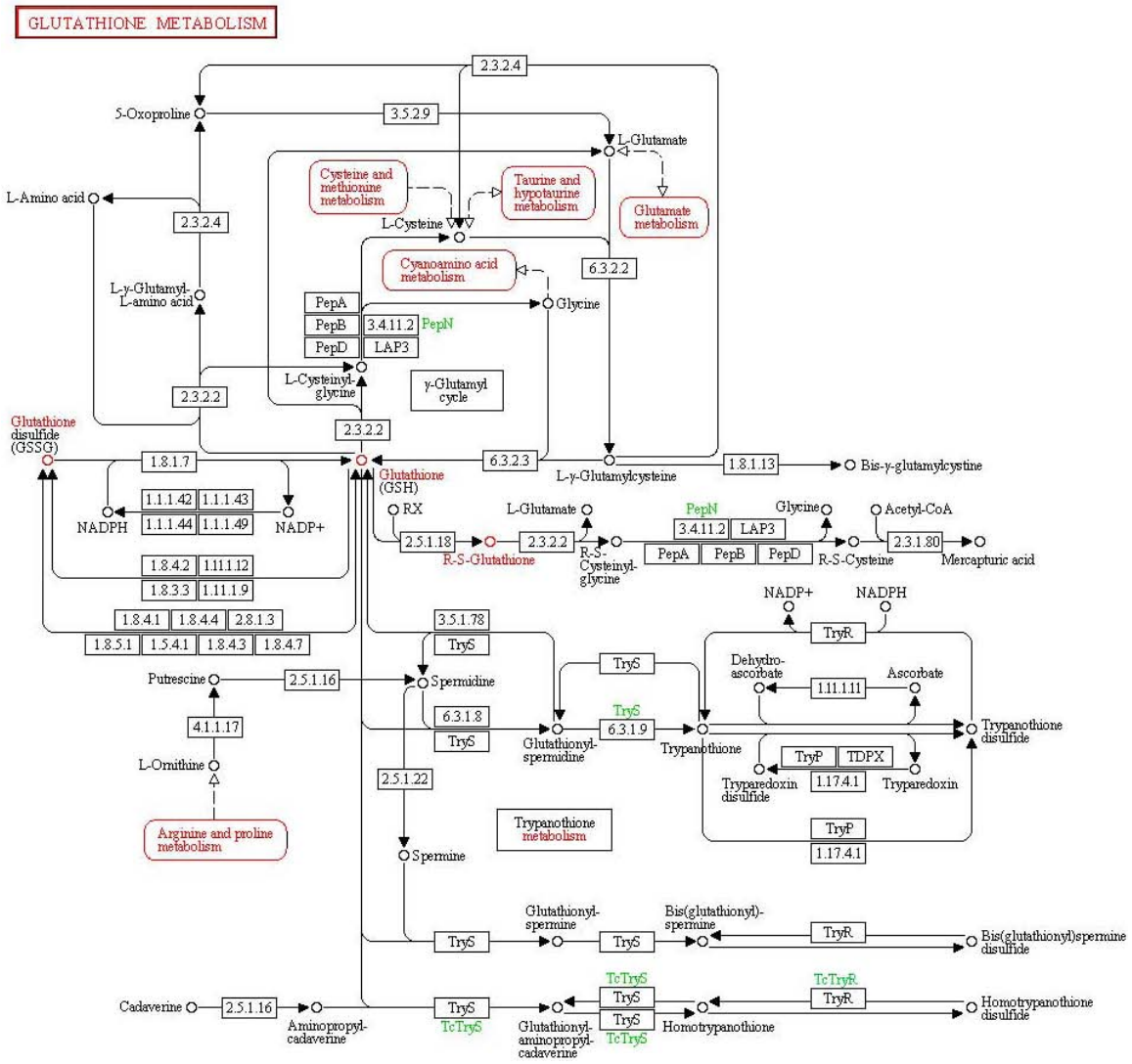



**Figure 18: KEGG glutathione metabolism pathway**

**Table 10: Enriched with genes up-regulated in sub-toxic exposures.** There were no Gene Ontologies enriched with genes down-regulated by exposure to sub-toxic $HgCl_2$.

| GO-Biological Processes | |
|---|---|
| MeHg | |
| Term | p-value |
| metabolic process | 0.00097 |
| | |
| GO-Molecular Function | |
| MeHg | |
| Term | p-value |
| oxidoreductase activity | 9.60E-05 |
| coenzyme binding | 0.00011 |
| | |

**Table 11: Enriched with genes up-regulated in low-toxic exposures.**

| GO-Biological Processes | | | |
|---|---|---|---|
| $HgCl_2$ | | MeHg | |
| Term | p-value | Term | p-value |
| proteolysis | 7.40E-06 | lipid glycosylation | 4.60E-05 |
| | | response to heat | 0.00084 |
| | | carbohydrate metabolic process | 0.0084 |
| | | oxidation reduction | 0.04 |
| | | | |
| GO-Molecular Function | | | |
| $HgCl_2$ | | MeHg | |
| Term | p-value | Term | p-value |
| serine-type peptidase activity | 0.032 | transferase activity, transferring hexosyl groups | 3.80E-06 |
| | | carbohydrate binding | 0.0014 |
| | | oxidoreductase activity | 0.0019 |
| | | transferase activity, transferring acyl groups other than amino-acyl groups | 0.033 |
| | | electron carrier activity | 0.042 |
| | | | |
| KEGG | | | |
| $HgCl_2$ | | MeHg | |
| Pathway | p-value | Pathway | p-value |
| Lysosome | 0.0026 | Metabolism of xenobiotics by cytochrome P450 | 0.00034 |
| | | Drug metabolism | 0.00047 |
| | | Glutathione metabolism | 0.00083 |

**Table 12: Enriched with genes down-regulated in low-toxic exposures.** There were no Gene Ontologies enriched with genes down-regulated by exposure to low-toxic $HgCl_2$.

| GO-Biological Processes | |
|---|---|
| MeHg | |
| Term | p-value |
| regulation of transcription | 0.032 |
| | |
| GO-Molecular Function | |
| MeHg | |
| Term | p-value |
| transcription factor activity | 0.044 |

**Table 13: Gene Ontologies enriched with genes up-regulated in high-toxic exposures.**

| GO-Biological Processes | | | |
|---|---|---|---|
| $HgCl_2$ | | MeHg | |
| Term | p-value | Term | p-value |
| lipid glycosylation | 2.53E-14 | post-translational protein modification | 3.40E-67 |
| transmembrane transport | 1.10E-06 | vitelline membrane formation | 8.50E-07 |
| carbohydrate metabolic process | 2.10E-06 | lipid glycosylation | 1.10E-05 |
| extracellular matrix organization | 1.30E-05 | response to heat | 0.0022 |
| oxidation reduction | 0.0084 | enterobactin biosynthetic process | 0.044 |
| proteolysis | 0.044 | | |
| | | | |
| GO-Molecular Function | | | |
| $HgCl_2$ | | MeHg | |
| Term | p-value | Term | p-value |
| carbohydrate binding | 1.90E-10 | protein tyrosine phosphatase activity | 1.70E-36 |
| transferase activity, transferring hexosyl groups | 4.40E-10 | protein serine/threonine kinase activity | 7.40E-32 |
| ATPase activity, coupled to transmembrane movement of substances | 0.00022 | ATP binding | 4.40E-18 |
| structural constituent of vitelline membrane | 0.00023 | protein tyrosine kinase activity | 6.90E-17 |
| oxidoreductase activity | 0.0018 | structural constituent of vitelline membrane | 5.10E-06 |
| endopeptidase inhibitor activity | 0.0067 | protein tyrosine/serine/threonine phosphatase activity | 0.00072 |
| iron ion binding | 0.0081 | structural molecule activity | 0.0022 |
| electron carrier activity | 0.0096 | carbohydrate binding | 0.019 |
| | | FMN binding | 0.021 |
| | | neurotransmitter:sodium symporter activity | 0.021 |
| | | phospholipase A2 activity | 0.021 |
| | | transferase activity, transferring hexosyl groups | 0.046 |
| | | carboxy-lyase activity | 0.047 |

**Table 14: KEGG pathways enriched with genes up-regulated in high-toxic exposures.**

| KEGG | | | |
|---|---|---|---|
| $HgCl_2$ | | MeHg | |
| Pathway | p-value | Pathway | p-value |
| Metabolism of xenobiotics by cytochrome P450 | 0.00048 | Metabolism of xenobiotics by cytochrome P450 | 0.000023 |
| Drug metabolism | 0.00067 | Drug metabolism | 0.000049 |
| Glutathione metabolism | 0.0012 | Glutathione metabolism | 0.00018 |
| | | Linoleic acid metabolism | 0.032 |

**Table 15: Gene Ontologies enriched in genes down-regulated in high-toxic exposures.**

| GO-Biological Processes | | | |
|---|---|---|---|
| $HgCl_2$ | | MeHg | |
| term | p-value | term | p-value |
| body morphogenesis | 6.75E-10 | cell adhesion | 9.52E-06 |
| locomotion | 0.018 | body morphogenesis | 1.77E-05 |
| morphogenesis of an epithelium | 0.024 | cell-matrix adhesion | 4.68E-05 |
| regulation of multicellular organism growth | 0.036 | chitin catabolic process | 0.00021 |
| | | regulation of transcription | 0.0012 |
| | | cell wall macromolecule catabolic process | 0.0025 |
| | | defense response | 0.0035 |
| | | tail morphogenesis | 0.0044 |
| | | proteolysis | 0.0046 |
| | | cilium morphogenesis | 0.012 |
| | | neuron recognition | 0.021 |
| | | lipid transport | 0.021 |
| | | ubiquitin-dependent protein catabolic process | 0.032 |
| | | response to oxidative stress | 0.035 |
| | | protein-DNA complex assembly | 0.042 |
| | | regulation of cell migration | 0.046 |

| GO-Molecular Function | | | |
|---|---|---|---|
| $HgCl_2$ | | MeHg | |
| term | p-value | term | p-value |
| structural constituent of cuticle | 5.70E-25 | structural constituent of cuticle | 1.46E-15 |
| | | hydrolase activity, hydrolyzing O-glycosyl compounds | 2.64E-06 |
| | | triglyceride lipase activity | 0.004 |
| | | transcription regulator activity | 0.01 |
| | | peroxidase activity | 0.014 |
| | | lipid transporter activity | 0.016 |
| | | serine-type endopeptidase inhibitor activity | 0.036 |
| | | peptidase inhibitor activity | 0.036 |
| | | receptor binding | 0.04 |
| | | sequence-specific DNA binding | 0.043 |

**Table 16: KEGG pathways enriched with genes down-regulated in high-toxic exposures.** There were no enriched KEGG pathways enriched with genes down-regulated in exposure to high-toxic $HgCl_2$.

| KEGG | |
|---|---|
| MeHg | |
| Pathway | p-value |
| TGF-beta signaling pathway | 0.00017 |
| Wnt signaling pathway | 0.00086 |
| Lysosome | 0.002 |
| Ubiquitin mediated proteolysis | 0.0088 |
| Other glycan degradation | 0.03 |

The JActive modules program in Cytoscape was used to identify sub-networks of interacting genes enriched by genes found to be differentially expressed by the different exposure conditions in the microarrays. The interaction network in which sub-networks were found consists of 23, 847 interactions. It was estimated that there may be as many as 200 million potential interactions in *C. elegans*, so this network represents a small fraction of the total *C. elegans* interactome [112]. The central gene in the top 5 sub-networks is listed for each exposure condition (Table 17). In cases, where there were fewer than 5 enriched sub-networks, the central gene from the significant sub-networks is listed. Each condition has unique central genes. This is further evidence that the different mercurials affect different biological processes. A sub-network enriched with genes up-regulated in high-toxic $HgCl_2$ exposure is shown in Figure 19.

**Table 17: Cytoscape central genes in enriched sub-networks**

| Sub-Toxic | |
|---|---|
| $HgCl_2$ | MeHg |
| nhr-46 | Y73C8C.10 |
| | F17A9.5 |
| | F17A9.4 |
| | ZK742.3 |
| | C01B10.3 |
| Low-Toxic | |
| $HgCl_2$ | MeHg |
| hsd-1 | C27A2.5 |
| High-Toxic | |
| $HgCl_2$ | MeHg |
| F56D5.3 | mrt-2 |
| F17A9.4 | eya-1 |
| Y39B6A.1 | gei-3 |
| pqn-5 | F17A9.5 |
| cpb-3 | rps-10 |

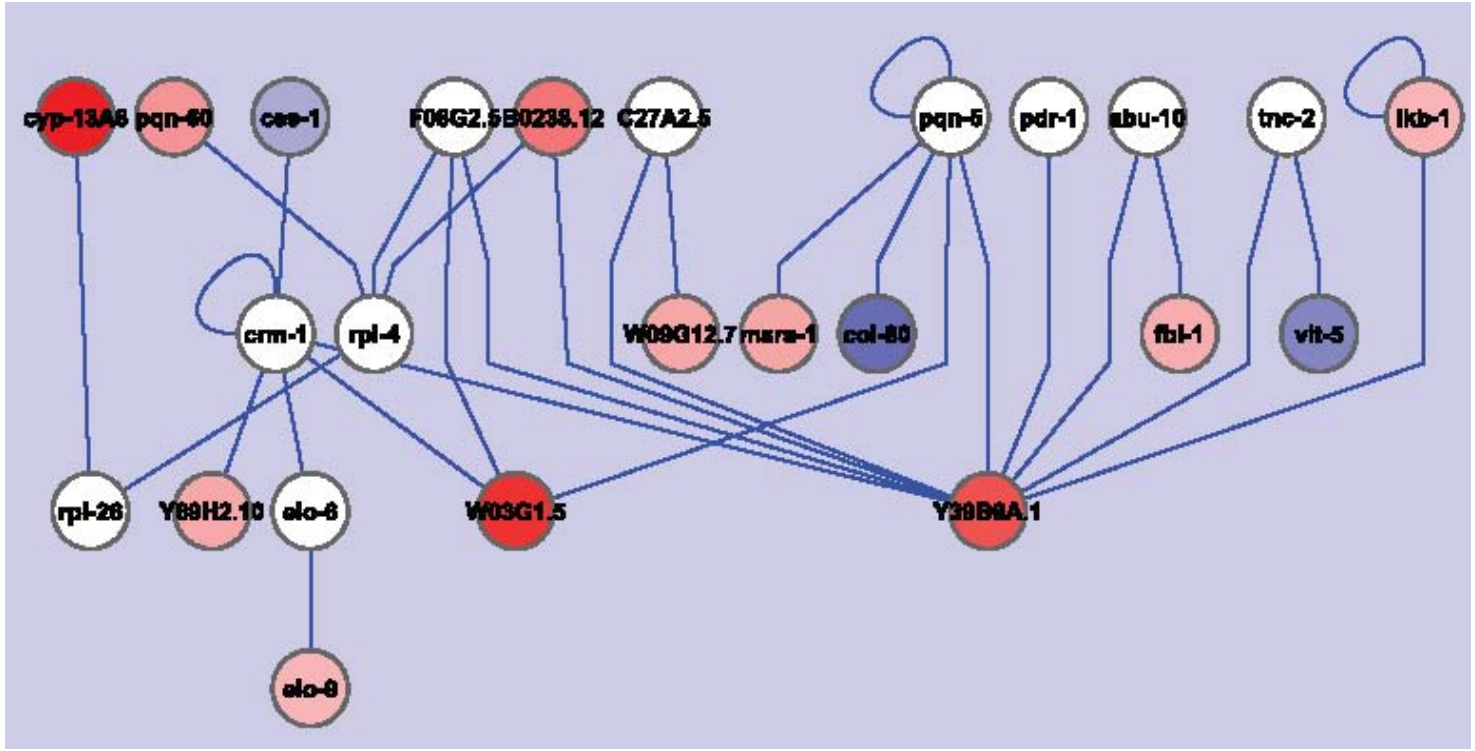



**Figure 19: The Y39B9A.1 sub-network.** Genes that were up-regulated in the high-toxic HgCl2 exposure are in red nodes, while those down regulated are in blue nodes. The darkness of the color of the node is correlated to the magnitude fold-change.

Chou et al. developed a pattern analysis tool called EPIG [107]. This program identifies and groups together genes that have similar patterns of transcription across treatments using correlation values, signal to noise ratios and magnitudes of change. Analysis of the microarray data using EPIG identified 12 different gene expression patterns (Figure 20). What was most striking was how differently $HgCl_2$ and MeHg affected transcription of these sets of genes. There was only one gene set (pattern 10) in which both mercurials had a similar effect on gene expression. In pattern 10, which was comprised of 602 genes, genes were highly down-regulated in high toxicity treatments of both mercurials. Even in this case, the transcription pattern was not identical, as these genes were slightly up-regulated in response to $HgCl_2$ and slightly down-regulated in response to MeHg. In the remaining 11 EPIG transcriptional patterns, there was a marked difference between the effects of $HgCl_2$ and MeHg.

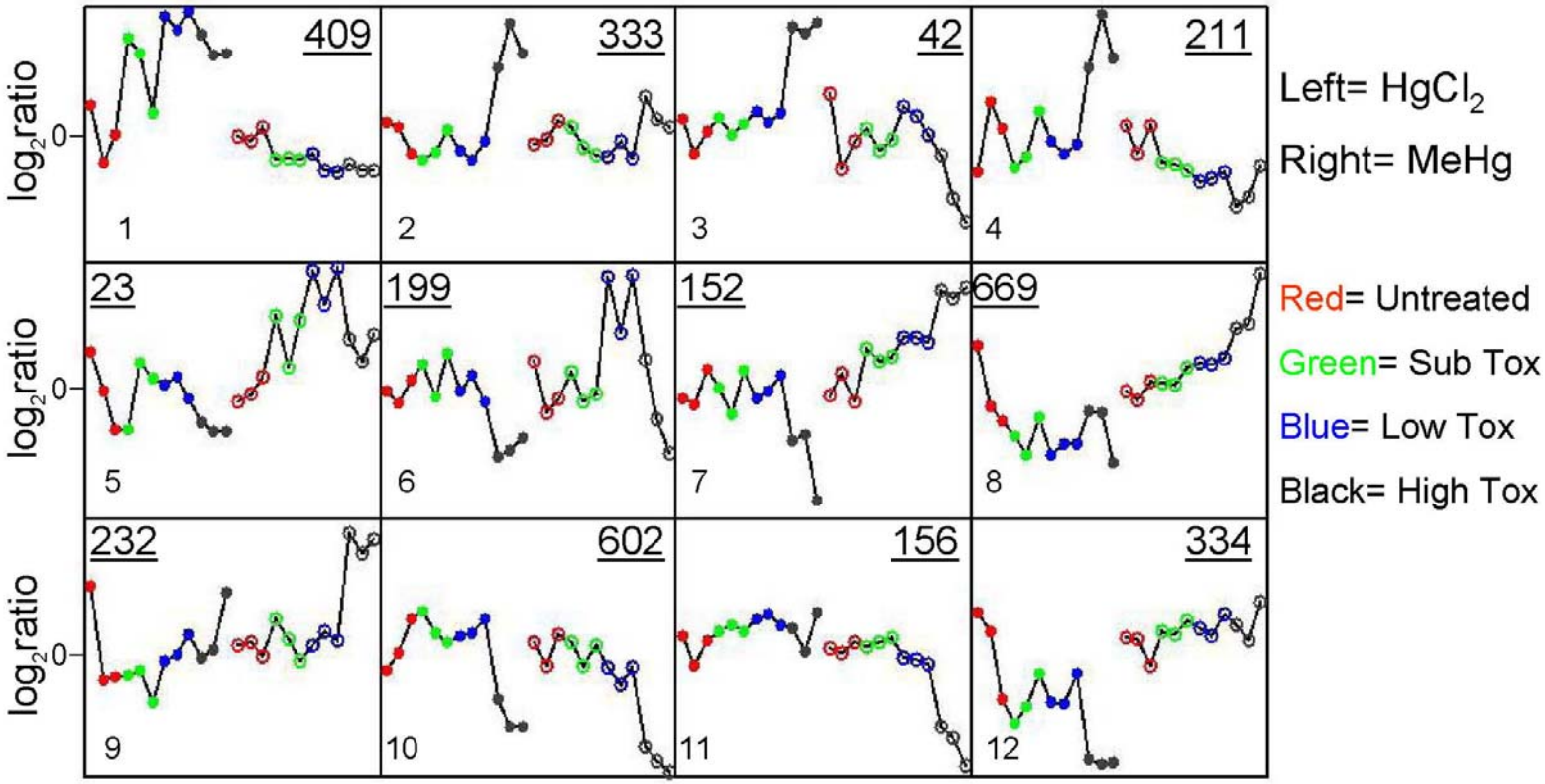



**Figure 20: EPIG clustering of co-expressed genes.** The average $\log_2$ fold-change of genes in the pattern for each experimental replicate and treatment is represented by the individual dots in each box. The number in the lower left hand corner indicates the number of the EPIG pattern. The number in the upper left hand corner indicates the number of genes in the pattern.

Genes that have similar expression patterns across different treatment conditions may be co-regulated and involved in related biological processes. Therefore an analysis of genes that have similar expression across treatments is more likely to discover an enrichment in GO categories and KEGG pathways than an analysis of genes differentially expressed under one condition. A bioinformatics analysis of genes grouped into the different EPIG patterns found enrichment in many more GO categories and KEGG pathways than the bioinformatics analysis of the DEGs for each treatment. Tables 18-30 list the significantly enriched: GO-Biological Processes, GO-Molecular Function, KEGG pathways and central genes in enriched Cytoscape sub-networks for each EPIG pattern.

**Table 18: EPIG Pattern 1 Bioinformatics analysis.**

| GO-Biological Processes | |
|---|---|
| Term | p-value |
| oxidative phosphorylation | 0.00013 |
| ion transport | 0.00020 |
| response to drug | 0.00024 |
| monovalent inorganic cation transport | 0.00031 |
| metal ion transport | 0.00032 |
| transmembrane transport | 0.00056 |
| oxygen transport | 0.0043 |
| response to heat | 0.021 |
| oxidation reduction | 0.045 |
| | |

| KEGG | |
|---|---|
| Term | p-value |
| Oxidative Phosphorylation | 0.00035 |
| Citrate Cycle (TCA cycle) | 0.0030 |

| GO-Molecular Function | |
|---|---|
| Term | p-value |
| ion channel activity | 0.00029 |
| hydrogen ion transmembrane transporter activity | 0.00030 |
| acetylcholine receptor activity | 0.0031 |
| NAD or NADH binding | 0.0054 |
| extracellular ligand-gated ion channel activity | 0.0078 |
| oxygen binding | 0.0088 |
| heme binding | 0.015 |
| calcium ion binding | 0.025 |
| oxidoreductase activity, acting on NADH or NADPH | 0.025 |

| Cytoscape |
|---|
| Gene |
| unc-97 |
| Y43F4B.7 |
| cah-4 |
| F52A8.5 |
| F27C8.2 |

**Table 19: EPIG pattern 2 bioinformatics analysis.**

| GO-Biological Processes | |
|---|---|
| Term | p-value |
| lipid glycosylation | 0.0011 |
| monovalent inorganic cation transport | 0.0043 |
| metal ion transport | 0.0045 |
| transport | 0.026 |

| GO-Molecular Function | |
|---|---|
| Term | p-value |
| ion channel activity | 0.0010 |
| carbohydrate binding | 0.0075 |
| ATP-dependent helicase activity | 0.043 |
| iron ion binding | 0.049 |
| cysteine-type peptidase activity | 0.049 |

| KEGG | |
|---|---|
| Term | p-value |
| Aminoacyl tRNA biosynthesis | 0.022 |

| Cytoscape |
|---|
| Gene |
| lpd-7 |
| Y61A9LA.1 |
| toe-1 |
| rpl-10 |
| atn-1 |

**Table 20: EPIG pattern 3 bioinformatics analysis.**

| GO-Biological Processes | |
|---|---|
| Term | p-value |
| lipid storage | 0.033 |
| | |
| Cytoscape | |
| Gene | |
| Y60A3A.9 | |
| sek-1 | |
| lec-1 | |
| air-1 | |
| ubxn-1 | |

**Table 21: EPIG pattern 4 bioinformatics analysis.**

| GO-Biological Processes | |
|---|---|
| Term | p-value |
| defecation | 3.40E-05 |
| oxygen transport | 0.00029 |
| regulation of pharyngeal pumping | 0.00037 |
| transmembrane transport | 0.0084 |
| neurotransmitter transport | 0.021 |
| transmission of nerve impulse | 0.040 |
| | |
| GO Molecular Function | |
| Term | p-value |
| oxygen binding | 0.00068 |
| calcium ion binding | 0.00069 |
| motor activity | 0.0042 |
| iron ion binding | 0.029 |

| KEGG | |
|---|---|
| Term | p-value |
| MAPK signaling | 0.044 |

| Cytoscape |
|---|
| Gene |
| gei-16 |

**Table 22: EPIG pattern 5 bioinformatics analysis.**

| GO-Biological Processes | |
|---|---|
| Term | p-value |
| cytokinesis | 1.48E-06 |
| embryonic development ending in birth or egg hatching | 0.045 |
| | |
| KEGG | |
| Term | p-value |
| Mismatch repair | 0.012 |
| DNA replication | 0.022 |
| Nucleotide excision repair | 0.023 |
| | |
| Cytoscape | |
| Gene | |
| F44B9.8 | |
| gly-5 | |
| C56G2.7 | |
| C47D12.2 | |
| C54G10.2 | |

**Table 23: EPIG pattern 6 bioinformatics analysis.**

| GO-Biological Processes | |
|---|---|
| Term | p-value |
| meiosis | 0.018 |
| | |
| GO-Molecular Function | |
| Term | p-value |
| nucleic acid binding | 0.00015 |
| zinc ion binding | 0.0089 |
| ATP binding | 0.032 |

| KEGG | |
|---|---|
| Term | p-value |
| DNA replication | 0.012 |
| | |
| Cytoscape | |
| Gene | |
| pnk-1 | |
| pcn-1 | |
| cpb-3 | |
| Y71G12B.27 | |
| pal-1 | |

**Table 24: EPIG pattern 7 bioinformatics analysis.**

| GO-Biological Processes | | | KEGG | |
|---|---|---|---|---|
| Term | p-value | | Term | p-value |
| embryonic development ending in birth or egg hatching | 6.14E-08 | | Spliceosome | 0.026 |
| mitotic spindle organization | 1.19E-05 | | | |
| reproduction | 1.76E-05 | | | |
| cytokinesis | 0.00027 | | | |
| germ cell development | 0.0051 | | | |
| negative regulation of vulval development | 0.0062 | | | |
| growth | 0.0068 | | | |
| receptor-mediated endocytosis | 0.012 | | | |
| nematode larval development | 0.022 | | | |
| positive regulation of multicellular organism growth | 0.026 | | | |
| body morphogenesis | 0.029 | | | |
| hermaphrodite genitalia development | 0.037 | | | |
| oogenesis | 0.042 | | | |
| meiotic chromosome segregation | 0.049 | | | |
| | | | | |
| GO-Molecular Function | | | Cytoscape | |
| Term | p-value | | Gene | |
| GTPase activator activity | 0.0050 | | vpr-1 | |
| cofactor binding | 0.048 | | cel-1 | |
| | | | lsm-3 | |
| | | | gpd-4 | |
| | | | fib-1 | |

**Table 25: EPIG Pattern 8 GO biological processes.**

| GO-Biological Processes | |
|---|---|
| Term | p-value |
| embryonic development ending in birth or egg hatching | 1.37E-28 |
| nematode larval development | 7.10E-14 |
| genitalia development | 1.02E-12 |
| receptor-mediated endocytosis | 2.67E-11 |
| protein catabolic process | 1.30E-08 |
| reproduction | 2.17E-08 |
| ubiquitin-dependent protein catabolic process | 2.72E-08 |
| locomotion | 4.38E-07 |
| determination of adult lifespan | 7.60E-07 |
| growth | 7.81E-07 |
| morphogenesis of an epithelium | 1.02E-06 |
| negative regulation of cell proliferation | 4.61E-05 |
| cell division | 6.63E-05 |
| protein folding | 9.43E-05 |
| tRNA aminoacylation for protein translation | 0.00014 |
| microtubule polymerization or depolymerization | 0.00020 |
| positive regulation of growth rate | 0.00020 |
| proteolysis involved in cellular protein catabolic process | 0.00031 |
| chromosome segregation | 0.00042 |
| rRNA processing | 0.0020 |
| RNA processing | 0.0022 |
| pronuclear migration | 0.0022 |
| RNA interference | 0.0023 |
| protein depolymerization | 0.0024 |
| establishment of mitotic spindle orientation | 0.0025 |
| development of primary male sexual characteristics | 0.0037 |
| negative regulation of multicellular organism growth | 0.0055 |
| gastrulation with mouth forming first | 0.0082 |
| spermatogenesis | 0.0095 |
| regulation of embryonic development | 0.011 |
| response to DNA damage stimulus | 0.012 |
| meiosis | 0.014 |
| embryonic morphogenesis | 0.016 |
| body morphogenesis | 0.026 |
| GPI anchor metabolic process | 0.029 |
| microtubule-based movement | 0.029 |
| molting cycle, collagen and cuticulin-based cuticle | 0.033 |
| protein transport | 0.037 |
| germ cell development | 0.041 |

**Table 26: EPIG pattern 8 bioinformatics analysis.**

| GO-Molecular Function | |
|---|---|
| Term | p-value |
| threonine-type endopeptidase activity | 1.58E-13 |
| ATP binding | 1.51E-10 |
| ATP-dependent helicase activity | 1.23E-06 |
| unfolded protein binding | 4.34E-06 |
| pyrophosphatase activity | 1.79E-05 |
| aminoacyl-tRNA ligase activity | 0.00010 |
| ubiquitin thiolesterase activity | 0.00026 |
| RNA binding | 0.0013 |
| transcription factor binding | 0.0087 |
| nuclease activity | 0.026 |
| magnesium ion binding | 0.039 |

| KEGG | |
|---|---|
| Term | p-value |
| Proteasome | 2.1E-18 |
| Endocytosis | 0.0015 |
| Aminoacyl tRNA biosynthesis | 0.0041 |

**Table 27: EPIG pattern 9 bioinformatics analysis.**

| GO-Biological Processes | |
|---|---|
| Term | p-value |
| tRNA aminoacylation for protein translation | 1.14E-06 |
| lipid glycosylation | 0.0020 |
| positive regulation of growth rate | 0.033 |

| KEGG | |
|---|---|
| Term | p-value |
| Aminoacyl tRNA biosynthesis | 6.2E-07 |

| GO-Molecular Function | |
|---|---|
| Term | p-value |
| aminoacyl-tRNA ligase activity | 2.56E-06 |
| ATP binding | 0.0036 |
| carboxylesterase activity | 0.012 |
| ATPase activity, coupled to transmembrane movement of substances | 0.016 |
| carbohydrate binding | 0.038 |

| Cytoscape |
|---|
| Gene |
| wrs-1 |
| byn-1 |
| C25A1.2 |
| T25G3.3 |
| nol-10 |

**Table 28: EPIG pattern 10 bioinformatics analysis.**

| GO-Biological Processes | |
|---|---|
| Term | p-value |
| lipid metabolic process | 0.00073 |
| positive regulation of programmed cell death | 0.0019 |
| lipid transport | 0.0028 |
| body morphogenesis | 0.0047 |
| cell adhesion | 0.0078 |
| proteolysis | 0.0083 |
| potassium ion transport | 0.038 |

| KEGG | |
|---|---|
| Term | p-value |
| Lysosome | 0.000041 |
| Other glycan degradation | 0.037 |

| GO-Molecular Function | |
|---|---|
| Term | p-value |
| structural constituent of cuticle | 1.18E-06 |
| triglyceride lipase activity | 0.0017 |
| lipid transporter activity | 0.0021 |
| carbon-oxygen lyase activity | 0.0046 |
| receptor binding | 0.015 |
| hydrolase activity, hydrolyzing O-glycosyl compounds | 0.015 |
| voltage-gated potassium channel activity | 0.017 |
| cysteine-type peptidase activity | 0.033 |

| Cytoscape | |
|---|---|
| Gene | |
| C05G5.4 | |
| apx-1 | |
| nhr-111 | |
| pqn-54 | |
| F18E2.1 | |

**Table 29: EPIG pattern 11 bioinformatics analysis.**

| GO-Biological Processes | |
|---|---|
| Term | p-value |
| oviposition | 0.014 |
| transmembrane transport | 0.020 |

| KEGG | |
|---|---|
| Term | p-value |
| Glycosphingolipid biosynthesis | 0.017 |
| Glycosaminoglycan degradation | 0.034 |

| Cytoscape |
|---|
| Gene |
| alp-1 |
| nhr-111 |
| pept-1 |

**Table 30: EPIG pattern 12 bioinformatics analysis.**

| GO-Biological Processes | |
|---|---|
| Term | p-value |
| embryonic development ending in birth or egg hatching | 4.20E-16 |
| cell division | 9.20E-10 |
| morphogenesis of an epithelium | 1.30E-06 |
| mitotic spindle organization | 4.10E-06 |
| embryonic pattern specification | 2.30E-05 |
| hermaphrodite genitalia development | 0.00012 |
| cell fate commitment | 0.00015 |
| gastrulation | 0.00042 |
| gonad development | 0.00055 |
| reproduction | 0.0072 |
| DNA replication | 0.014 |
| meiotic chromosome segregation | 0.024 |
| positive regulation of growth | 0.026 |
| nematode larval development | 0.027 |
| establishment or maintenance of cell polarity | 0.028 |
| growth | 0.049 |

| KEGG | |
|---|---|
| Term | p-value |
| DNA replication | 0.0078 |
| Progesterone-mediated oocyte maturation | 0.013 |
| Pyrimadine metabolism | 0.02 |

| Cytoscape |
|---|
| Gene |
| puf-3 |
| rfc-3 |
| plk-1 |
| skr-2 |
| gei-4 |

## *3.3 Functional analysis of mercury-responsive genes using RNAi*

RNAi was used to investigate the biological function of genes up-regulated by mercury in *C. elegans*. Nematodes were exposed to mercurial alone, gene-specific dsRNA alone (via consumption of bacteria), or both mercurial and gene-specific dsRNA. The effect on growth was assessed using a modified growth assay. A significant gene-mercurial interaction occurs when co-exposure to both dsRNA and mercurial results in an effect on growth that is different than would be predicted based on the additive effects of the mercurial and dsRNA exposures.

The RNAi-sensitive strain rrf-3 was used to increase the sensitivity of the assay. The dose-responses of rrf-3 nematodes to $HgCl_2$ and MeHg were determined using open-vector bacteria (Figure 21).

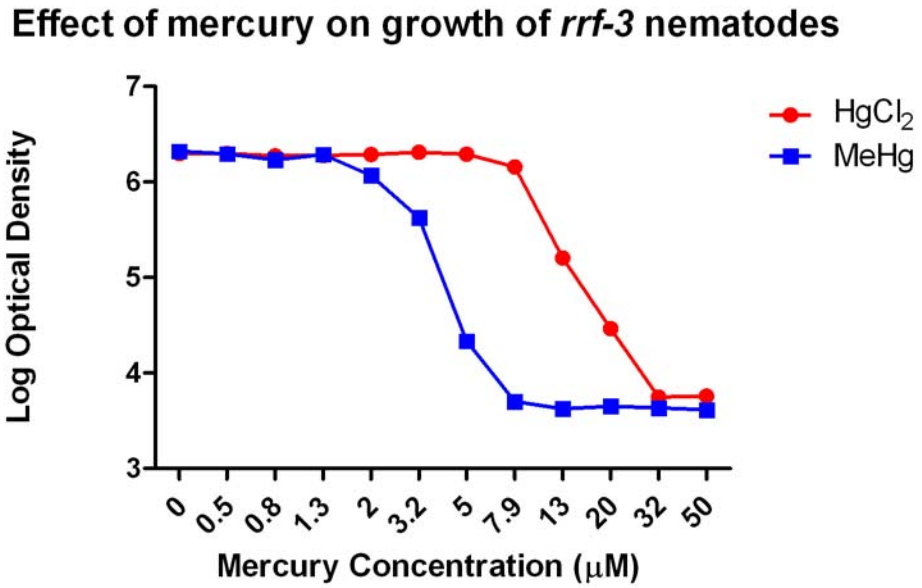



**Figure 21: rrf-3 mercury dose-response.** Results are displayed as mean Log optical density ± SEM.

The EC20 for inorganic mercury was 10.1 μM (9.9-10.3 95% CI), while the EC20 for MeHg was 3.0 μM (2.9-3.1 95% CI). These concentrations were used in the RNAi-mercury growth assay.

All genes up-regulated by $HgCl_2$ and genes up-regulated by sub- and low-toxicity treatments of MeHg were tested in the RNAi screen. As there were 1604 genes that were up-regulated by the high-toxicity MeHg treatment, only genes whose mRNA levels increased 5-fold were included in the experiment. A total of 599 genes were tested. Of these, 258 were only up-regulated by $HgCl_2$, 276 were only up-regulated by MeHg,

and 65 were up-regulated by both $HgCl_2$ and MeHg. Although the majority of genes were differentially expressed in response to only one mercurial, both mercurials were tested for each gene.

An initial screen was performed in which the gene-mercurial interaction was assessed by visual observation. A liberal threshold was used to pass genes onto the next screen. 162 genes were deemed to have a potentially significant gene-mercurial interaction to at least one of the mercurials. Of these 162 genes, 64 were up-regulated only by $HgCl_2$, 45 were only up-regulated by MeHg and 53 were up-regulated by both mercurials.

In the initial screen, bacterial cultures were picked directly from the RNAi library. In order to verify that these bacteria were expressing dsRNA to the appropriate gene, individual clones were sequenced. Of the 162 genes that passed the first screen, 146 were correctly annotated, 9 were incorrectly annotated, and 7 did not encode dsRNA to any gene. The 155 genes for which bacterial clones containing dsRNA to a specific gene were passed on the quantitative screen. A schematic of the RNAi screen is shown in figure 22.

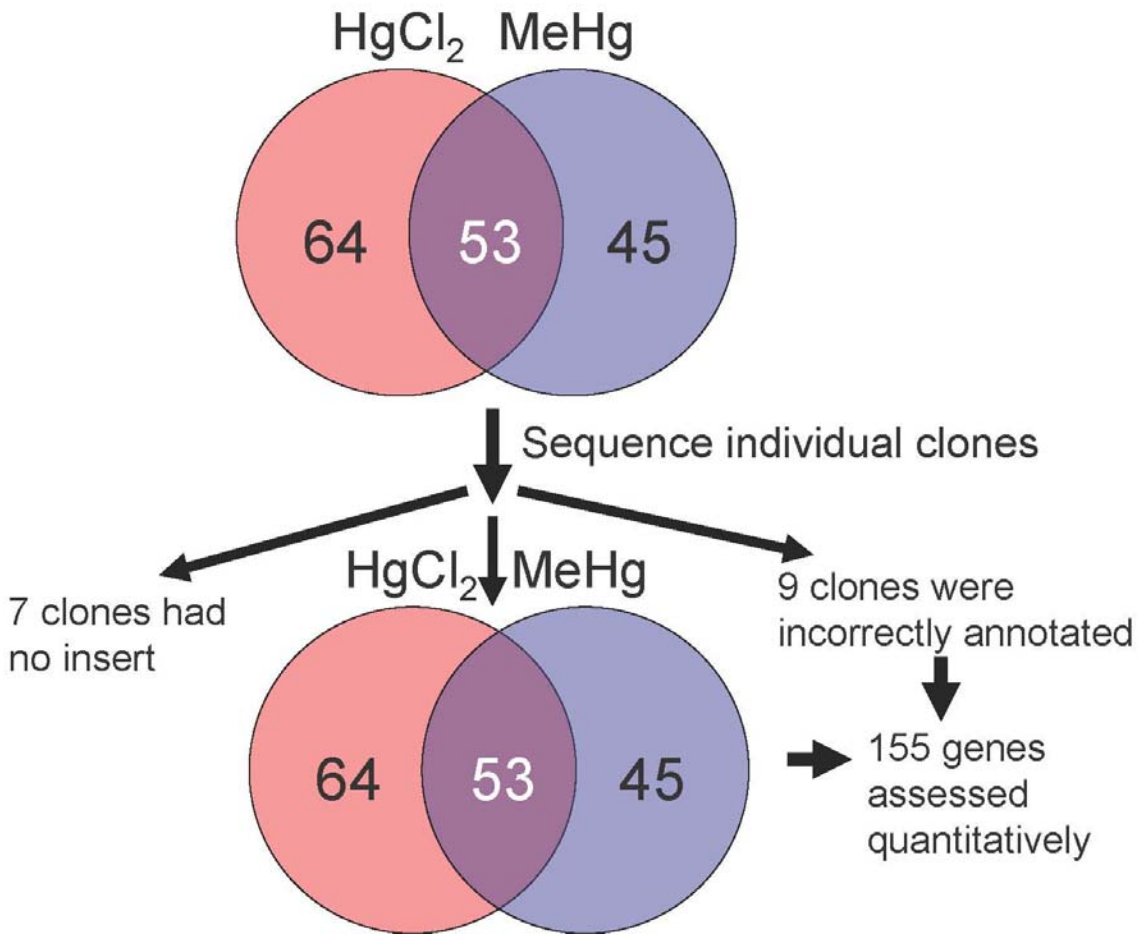
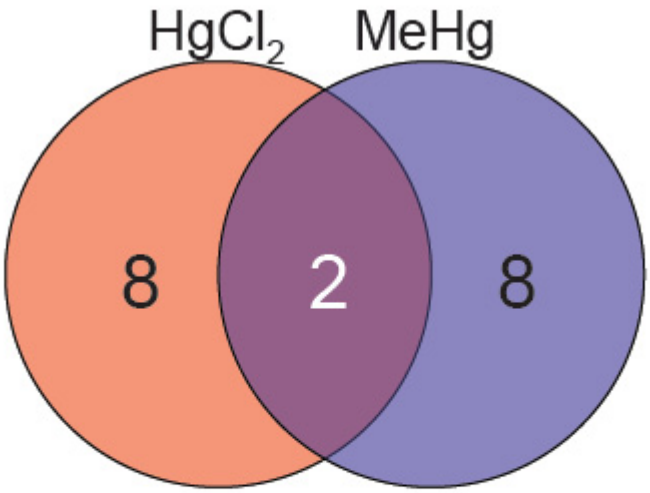



**Figure 22: Schematic of *C. elegans* RNAi screen**

In the quantitative screen, the gene-mercurial interaction was tested for both mercurials and growth was quantitatively measured using the COPAS. For each gene-mercurial combination, an interaction parameter and p-value was calculated. The interaction parameter indicates the difference in size between the predicted combined effect of mercurial exposure and knock-down of the gene on growth, with the actual combined effect of mercurial exposure and knock-down of the gene. Therefore, a

positive interaction parameter indicates that nematodes exposed to both mercurial and dsRNA had greater growth than predicted. In this case, knocking down the gene increased the nematodes' resistance to that mercurial. A negative interaction indicates that nematodes exposed to both mercurial and dsRNA had less growth than predicted. Knocking down the gene decreased the nematodes' resistance to that mercurial. There were 20 significant gene-mercurial interactions (Figure 23).




| Sequence Name | Common Name | Mercurial | Interaction Parameter | p-value |
|---|---|---|---|---|
| F37B12.2 | *gcs-1* | $HgCl_2$ | -228 | 2.4 E-05 |
| | | MeHg | -354 | 2.9 E-11 |
| F14F9.4 | | $HgCl_2$ | 161 | 0.00072 |
| | | MeHg | 121 | 0.0068 |

**Figure 23: Results of quantitative *C. elegans* RNAi screen.**

Of the 20 significant interactions, there were only 2 genes: *gcs-1* and F14F9.4, for which there was a significant gene-mercurial interaction for both mercurials. In all other cases, the gene-mercurial interaction was mercurial-specific. Tables 31 and 32 list the

significant positive and negative interactions, respectively. What was particularly conspicuous is that nearly all of the positive interactions involve $HgCl_2$, while nearly all of the negative interactions involve MeHg.

**Table 31: Positive gene-mercurial interactions in RNAi screen**

| Sequence Name | Gene Name | Mercurial | Interaction p-value | Interaction parameter |
|---|---|---|---|---|
| F14F9.4 | | $HgCl_2$ | 0.00072 | 161 |
| F59D6.2 | | $HgCl_2$ | 0.00036 | 129 |
| B0285.9 | *ckb-2* | $HgCl_2$ | 1.20E-06 | 122 |
| C54D10.8 | | $HgCl_2$ | 0.0022 | 122 |
| F14F9.4 | | MeHg | 0.0068 | 121 |
| C18D4.2 | *fbxa-136* | $HgCl_2$ | 0.0036 | 107 |
| F19C7.5 | | $HgCl_2$ | 3.00E-06 | 95 |
| C16C10.12 | *wht-3* | $HgCl_2$ | 0.00022 | 94 |
| Y39A1B.1 | *clec-163* | $HgCl_2$ | 0.0017 | 78 |
| T09F5.10 | | $HgCl_2$ | 0.0061 | 65 |
| K01D12.1 | | MeHg | 0.0024 | 54 |

**Table 32: Negative gene-mercurial interactions in RNAi screen**

| Sequence Name | Gene Name | Mercurial | Interaction p-value | Interaction parameter |
|---|---|---|---|---|
| F37B12.2 | *gcs-1* | MeHg | 2.9E-11 | -354 |
| F37B12.2 | *gcs-1* | HgCl2 | 2.40E-05 | -228 |
| Y45F10B.1 | *tsp-5* | MeHg | 0.0016 | -153 |
| H23L24.5 | *pme-4* | MeHg | 0.0069 | -148 |
| Y113G7B.1 | *fbxa-116* | MeHg | 0.00057 | -137 |
| *Y69E1A.8* | | MeHg | 0.0021 | -104 |
| T05E7.4 | | MeHg | 0.00013 | -103 |
| T22D1.2 | | MeHg | 0.00079 | -101 |
| F41H10.8 | *elo-6* | MeHg | 0.010 | -99 |

## 3.4 Effect of mercurials on human homologs

To ascertain the evolutionary conservation of genes involved in resistance or susceptibility to mercurials, the significance of gene-mercurial interactions in human cells was tested using the homologs of genes found in the *C. elegans* RNAi experiment. The human homologs of genes for which there was a significant gene-mercurial interaction were tested in cell culture (Table 33).

**Table 33: List of human homologs of *C. elegans* genes that were found to have a significant gene-mercurial interaction.** The interactions listed are the results of the *C. elegans* RNAi screen.

| *C. elegans* gene | Interaction | | Human homologs |
|---|---|---|---|
| *gcs-1* | $HgCl_2$ | -354 | GCLC |
| | MeHg | -228 | |
| *pme-4* | MeHg | -148 | PARG |
| *elo-6* | MeHg | -99 | ELOVL3, ELOVL6 |
| *ckb-2* | $HgCl_2$ | 122 | CHKA, CHKB |
| *wht-3* | $HgCl_2$ | 94 | ABCG2 |
| F59D6.2 | $HgCl_2$ | 129 | BACE1, BACE2 |

ABCG2, also known as the breast cancer resistance protein, is a transporter that is up-regulated in many cancers that exports chemotherapeutics from the cell [119]. High levels of ABCG2 expression are found in the placenta and brain, where it may function to export xenobiotics [119]. BACE1 is the rate limiting enzyme for the production of the β-amyloid peptide {Willem, 2009 #13}. β-amyloid is the major

component of plaques in Alzheimer's disease patients{Willem, 2009 #13}. BACE2 is the homolog of BACE1. Less is known about the function of BACE2, though *bace2-/-* mice have no obvious phenotype, while *bace1-/-* mice die in the first weeks after birth [120]. CHKA and CHKB phosphorylate choline to generate phosphocholine, a precursor of phosphatidylcholine [121]. ELOVL3 and ELOVL6 synthesize saturated and mono-unsaturated long-chain fatty acids [122]. GCLC is the catalytic subunit of the rate limiting enzyme in the synthesis of glutathione [123]. Finally, PARG catalyzes the hydrolysis of poly(ADP-ribose) [124].

### 3.4.1 Effect of mercury on cell lines

Three human-derived cell lines were used in these experiments: SK-N-SH (neuroblastoma), HepG2 (hepatocellular carcinoma) and HEK293 (embryonic kidney). These cell lines were chosen because they represent the primary target organs of each mercurial (brain-MeHg, kidney-$HgCl_2$) and an organ, that while, not particularly susceptible, is exposed to both inorganic and organic mercury (liver). The 24 h dose-response to both mercurials was measured in each cell line using the neutral red assay (Figures 24-26). A NOAEL, EC20 and EC50 were estimated for each mercurial in each cell line and are listed in Table 34.

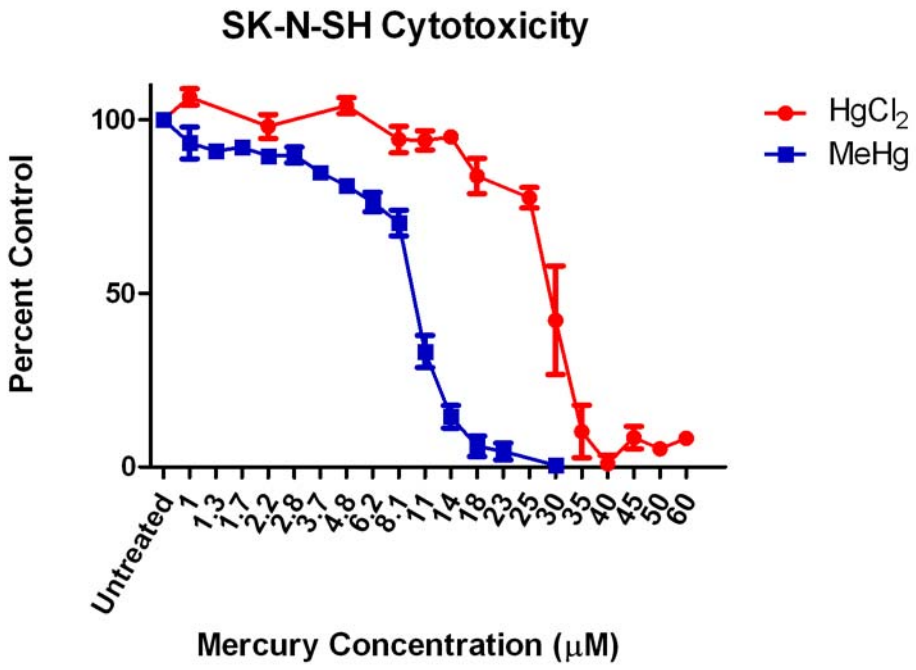



**Figure 24: SK-N-SH cells dose response.** Cells were exposed to either mercurial for 24 h. Cell viability was assessed using the neutral red assay, and mercurial-treated cells were compared to untreated cells to determine the percent control. Results display the mean percent of control ± SEM.

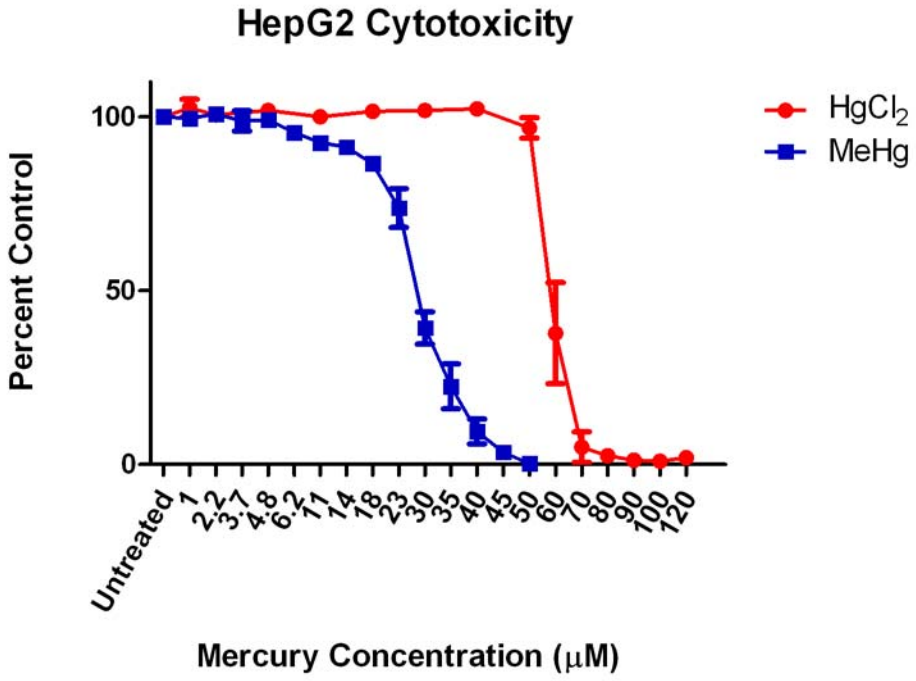



**Figure 25: HepG2 cells dose response.** Cells were exposed to either mercurial for 24 h. Cell viability was assessed using the neutral red assay, and mercurial-treated cells were compared to untreated cells to determine the percent control. Results display the mean percent of control ± SEM.

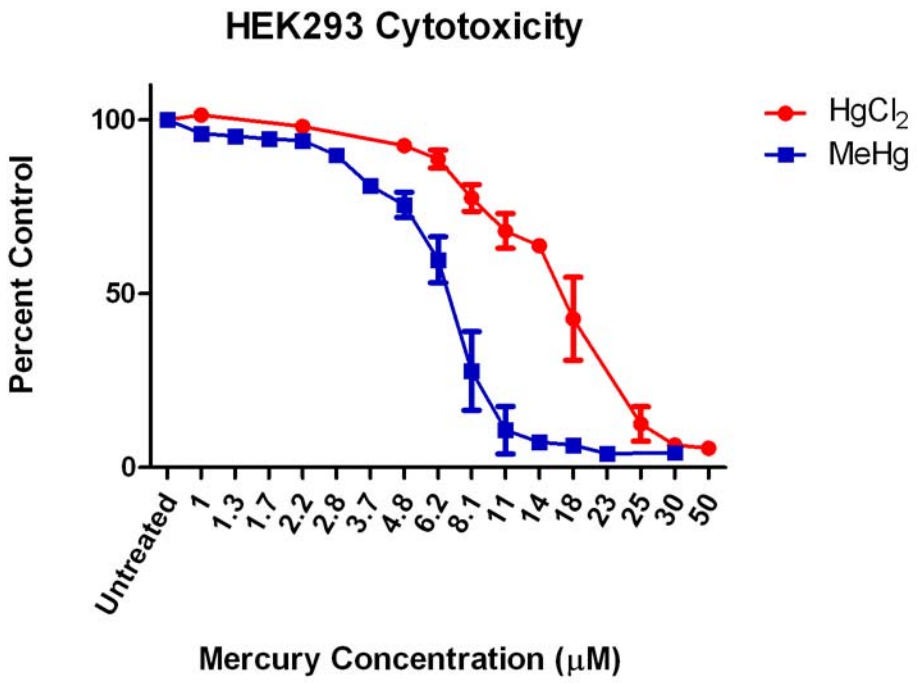



**Figure 26: HEK293 cells dose response.** Cells were exposed to either mercurial for 24 h. Cell viability was assessed using the neutral red assay, and mercurial-treated cells were compared to untreated cells to determine the percent control. Results display the mean percent of control ± SEM.

**Table 34: Estimated NOAEL, EC20, and EC50 in the three cell lines.**

| | SK-N-SH | | HepG2 | | HEK293 | |
|---|---|---|---|---|---|---|
| | $HgCl_2$ | MeHg | $HgCl_2$ | MeHg | $HgCl_2$ | MeHg |
| NOAEL | 14 μM | 1.7 μM | 25 μM | 18 μM | 4.8 μM | 2.2 μM |
| EC20 | 22 μM | 5.1 μM | 50 μM | 27 μM | 7.7 μM | 3.9 μM |
| EC50 | 29 μM | 9.7 μM | 58 μM | 33 μM | 17 μM | 6.8 μM |

### 3.4.2 Effect of mercurials on gene expression

The effect of each mercurial on gene expression was tested for each homolog in each cell line. Cells were exposed to either $HgCl_2$ or MeHg for 24 h at the NOAEL, EC20, and EC50 levels established in the neutral red assay (Figures 25-33). Relative mRNA levels that were significantly different than those found in untreated cells are designated with an asterisk.

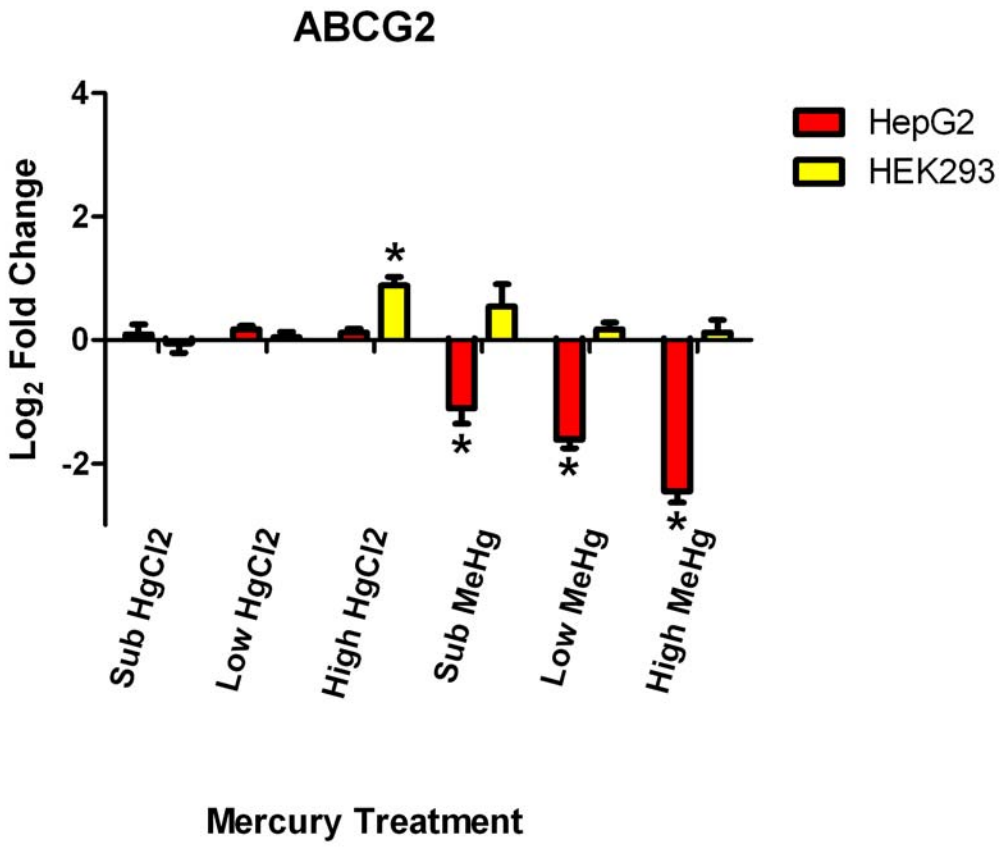



**Figure 27: qRT-PCR of ABCG2.** Sub = NOAEL, Low = EC20 and High = EC50 concentrations in a 24 h exposure.

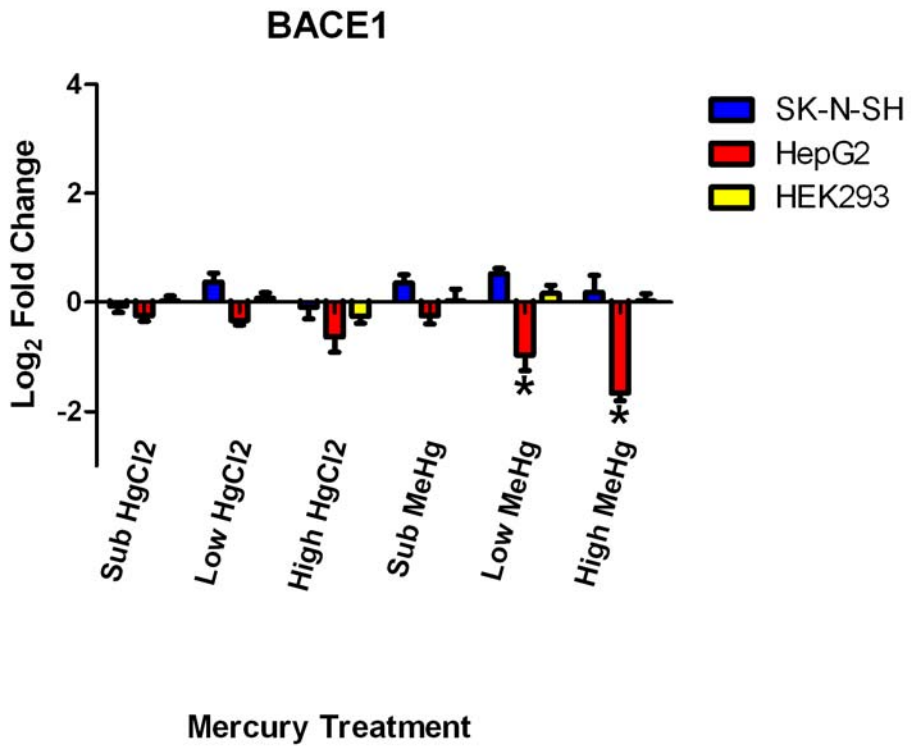



**Figure 28: qRT-PCR of BACE1.** Sub = NOAEL, Low = EC20 and High = EC50 concentrations in a 24 h exposure.

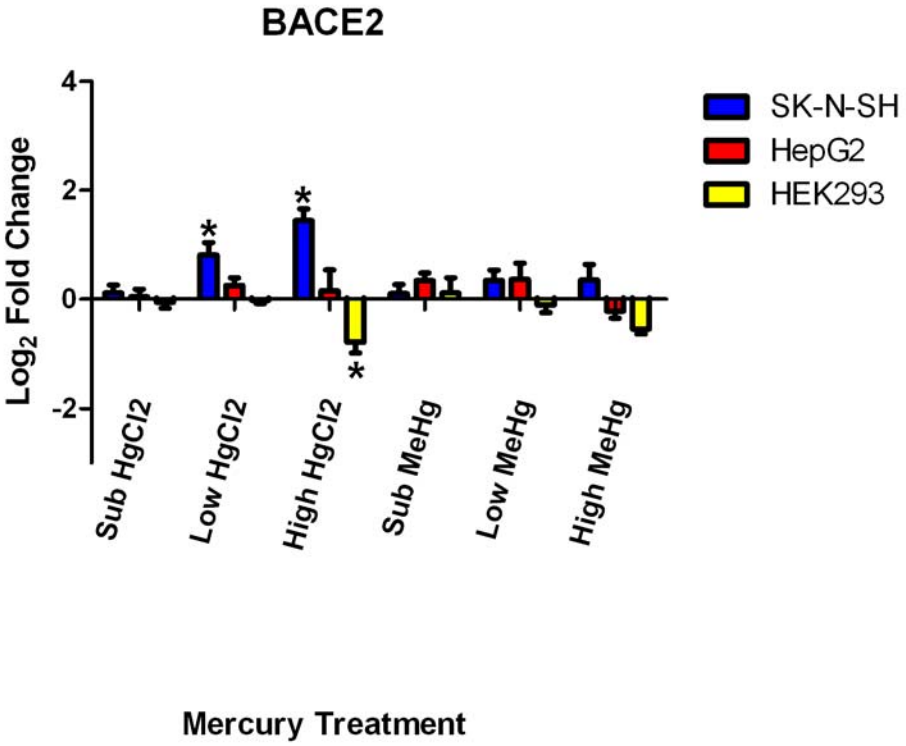



**Figure 29: qRT-PCR of BACE2.** Sub = NOAEL, Low = EC20 and High = EC50 concentrations in a 24 h exposure.

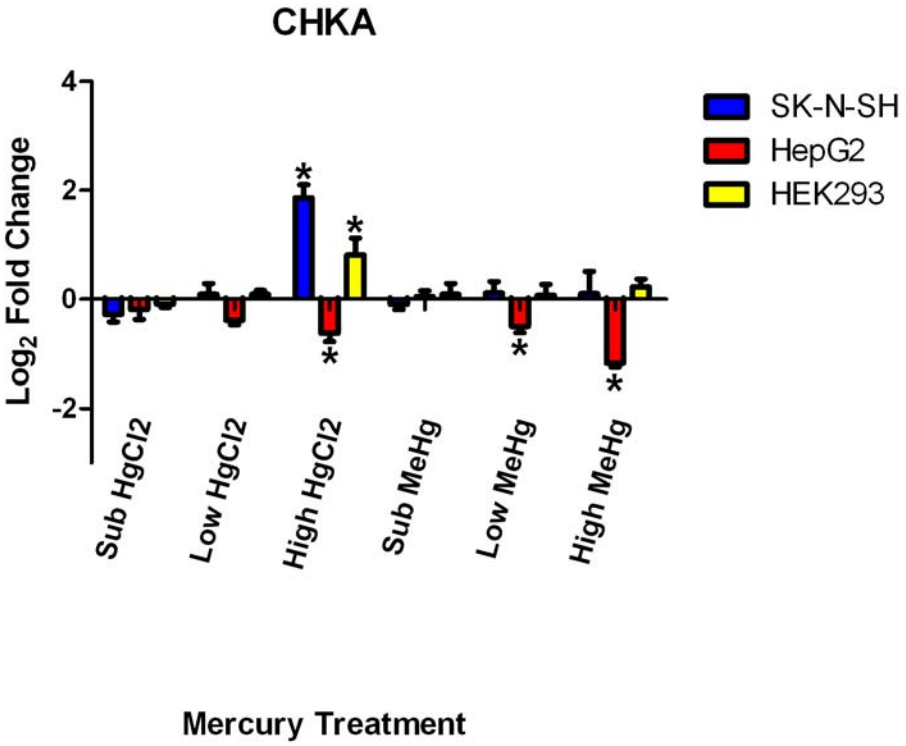



**Figure 30: qRT-PCR of CHKA.** Sub = NOAEL, Low = EC20 and High = EC50 concentrations in a 24 h exposure.

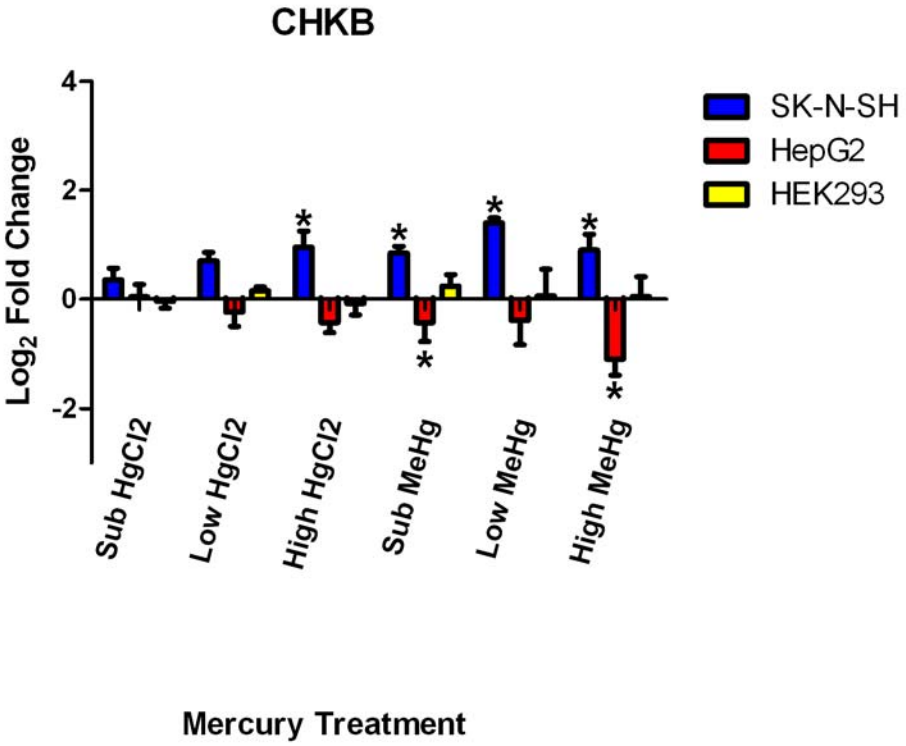



**Figure 31: qRT-PCR of CHKB.** Sub = NOAEL, Low = EC20 and High = EC50 concentrations in a 24 h exposure.

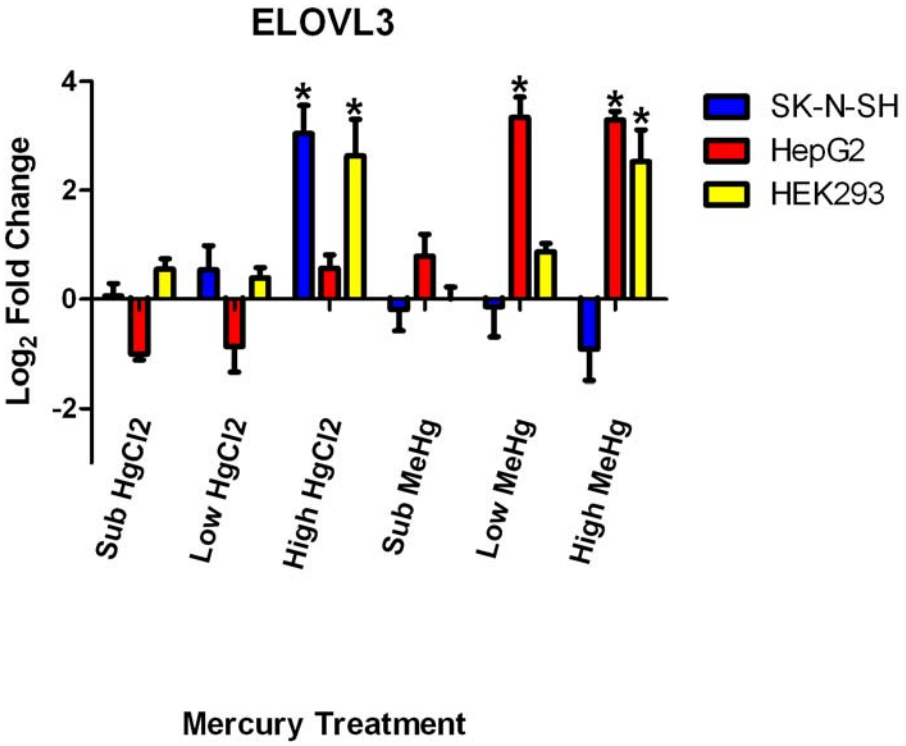



**Figure 32: qRT-PCR of ELOVL3.** Sub = NOAEL, Low = EC20 and High = EC50 concentrations in a 24 h exposure.

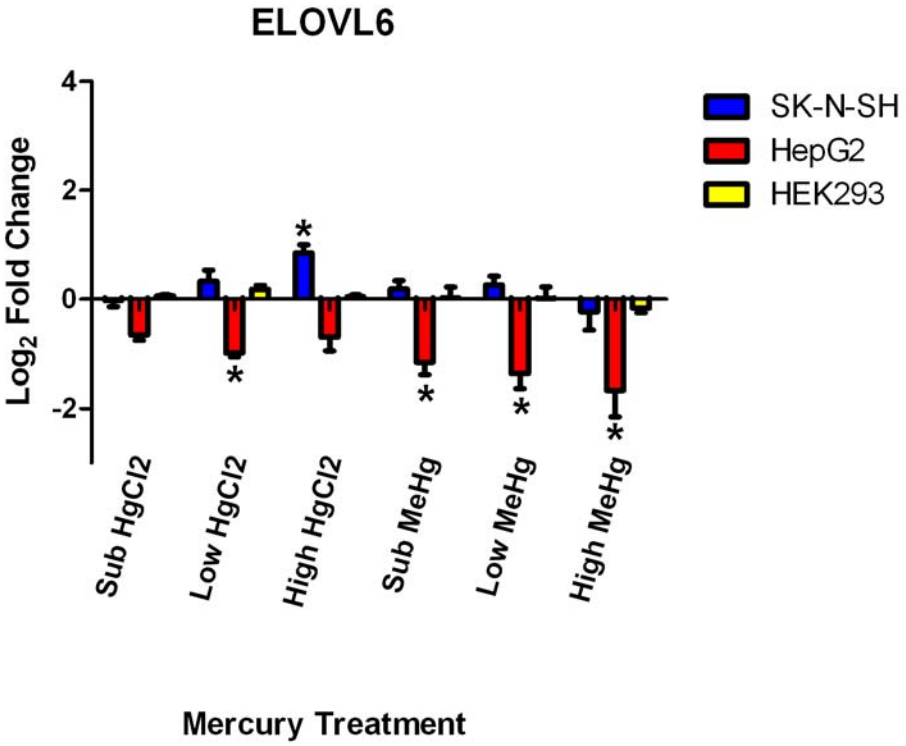



**Figure 33: qRT-PCR of ELOVL6.** Sub = NOAEL, Low = EC20 and High = EC50 concentrations in a 24 h exposure.

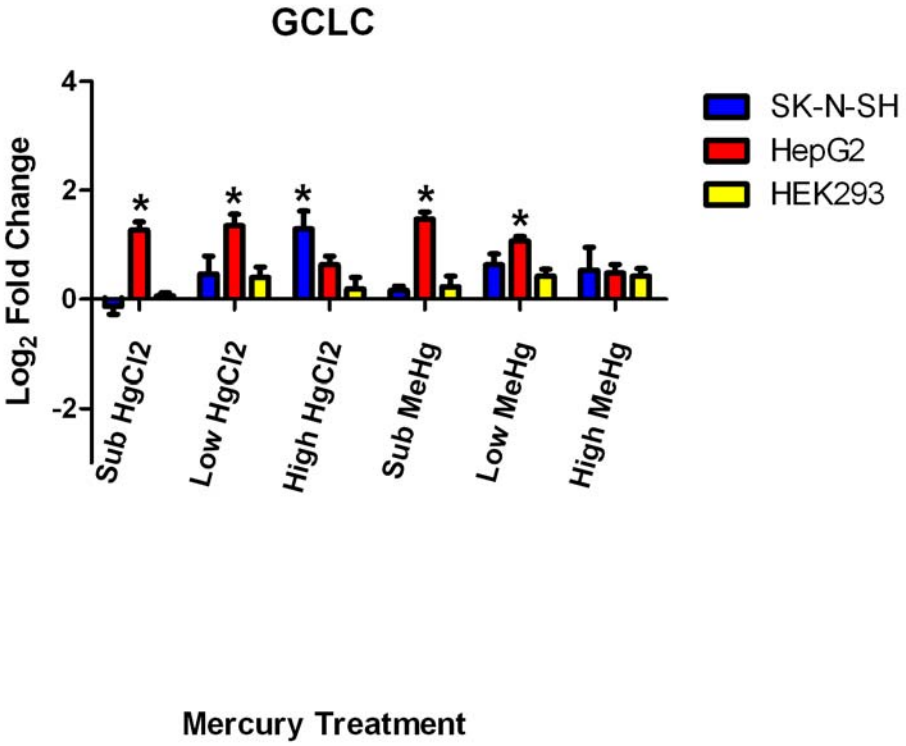



**Figure 34: qRT-PCR of GCLC.** Sub = NOAEL, Low = EC20 and High = EC50 concentrations in a 24 h exposure.

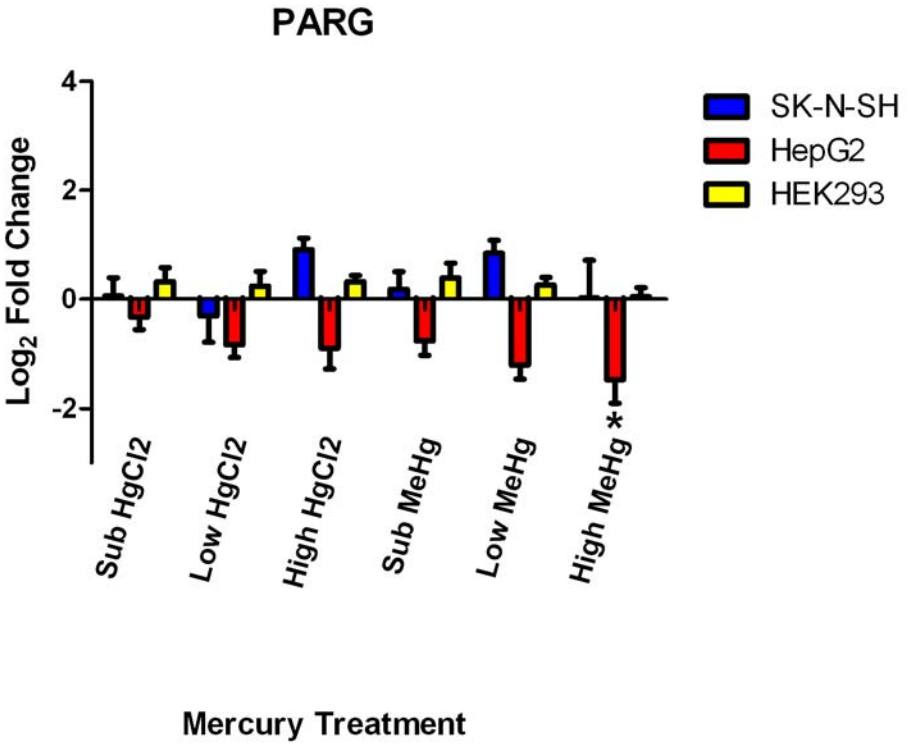



**Figure 35: qRT-PCR of PARG.** Sub = NOAEL, Low = EC20 and High = EC50 concentrations in a 24 h exposure.

Of the 162 cell line-mercurial-toxicity-gene combinations tested, 36 resulted in significant differential expression. There was no detectable ABCG2 expression in the SK-N-SH cells. For every gene, there was at least one cell line-mercurial-toxicity combination that resulted in a significant difference in expression of that gene relative to untreated cells. While the *C. elegans* homologs of these genes were all up-regulated in response to one of the mercurials, many of the human homologs were down-regulated in response to mercurial exposure. This was particularly evident in the HepG2 cells, where PARG, ELOVL6, BACE1, CHKA, CHKB, and ABCG2 are all down-regulated in

response to MeHg exposure. There was a high degree of variability in expression between cell lines. For example, BACE2 was up-regulated by a high concentration of $HgCl_2$ in SK-N-SH cells, but it was down-regulated in HEK293 cells. ELOVL3 was the only gene in which the response to mercurials was similar between cell lines. As was observed in *C. elegans*, the two mercurials had different effects on transcription. For example, in HepG2 cells, both EC20 and EC50 MeHg treatments resulted in a ~10-fold increase in ELOVL3 levels, while $HgCl_2$ exposure had no effect on ELOVL3 mRNA levels. Of the 36 cell line-mercurial-toxicity combinations that resulted in a significant change in mRNA levels, 24 were unique to that cell line-mercurial combination. There were only 6 instances in which both mercurials, at equitoxic levels, induced similar changes in expression of a gene in a cell line. In SK-N-SH cells, CHKB was up-regulated by EC50 exposures to both mercurials, and in HEK293 cells, ELOVL3 was up-regulated by EC50 exposures to both mercurials. In HepG2 cells, GCLC was up-regulated by NOAEL and EC20 $HgCl_2$ and MeHg treatments, while ELOVL6 and CHKA were down-regulated by EC20 and NOAEL treatments, respectively. However, there were no instances in which a gene was significantly up-regulated by one mercurial and significantly down-regulated by the other in any of the cell lines.

### 3.4.3 Gene-Mercurial interaction

RNAi was used to determine if conservation of function exists between the *C. elegans* genes involved in mediating the response to mercurials and their human

homologs. The effect of knocking down each gene on mercurial resistance was tested using both mercurials in the three cell lines. However, it was first necessary to determine the effectiveness of the siRNA in knocking down each of the genes. Each cell line was transfected with siRNA to each gene or a non-homologous siRNA control. The effectiveness of the siRNA was assessed by comparing mRNA levels of cells transfected with siRNA relative to cells transfected with non-homologous siRNA (Figure 36).

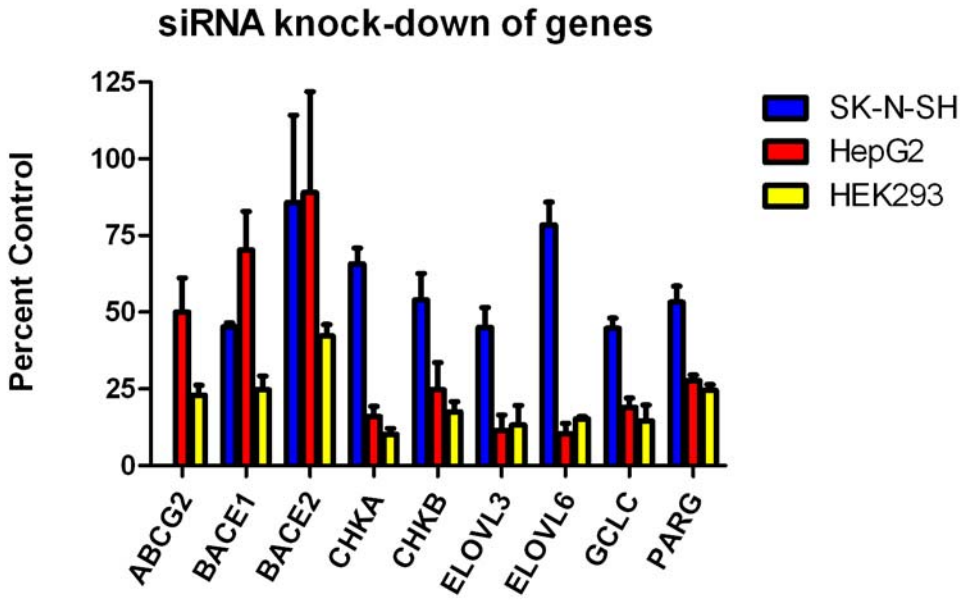



**Figure 36: Effectiveness of siRNA in cells.** Cells were transfected with gene-specific siRNA or non-homologous siRNA (control) and incubated for 24 h. Relative mRNA levels were measured using qRT-PCR. mRNA levels in cells treated with gene-specific siRNA were compared to mRNA levels in control cells to determine percent of control. Results display the mean percent of control ± SEM.

The effectiveness of ABCG2 siRNA was not tested in SK-N-SH cells, as it was demonstrated that ABCG2 expression was not detectable in that cell line. HEK293 cells

exhibited the greatest efficiency of gene knock-down, while SK-N-SH cells exhibited the poorest. Transfection with BACE2 siRNA did not result in a significant knock-down in BACE2 mRNA levels in SK-N-SH or HepG2 cells, so the effect of knocking down BACE2 in these cell lines in response to mercurials was not tested. In all other cases, siRNA treatment resulted in a significant decrease in target mRNA.

Before testing for gene-mercurial interactions, it was necessary to determine if the transfection conditions increased the susceptibility of the cells to mercurials. To determine this, cells were transfected with non-homologous siRNA for 24 hr, and then treated separately with both mercurials for 24 hours. Cytotoxicity was determined using the neutral red assay (Figures 37-39).

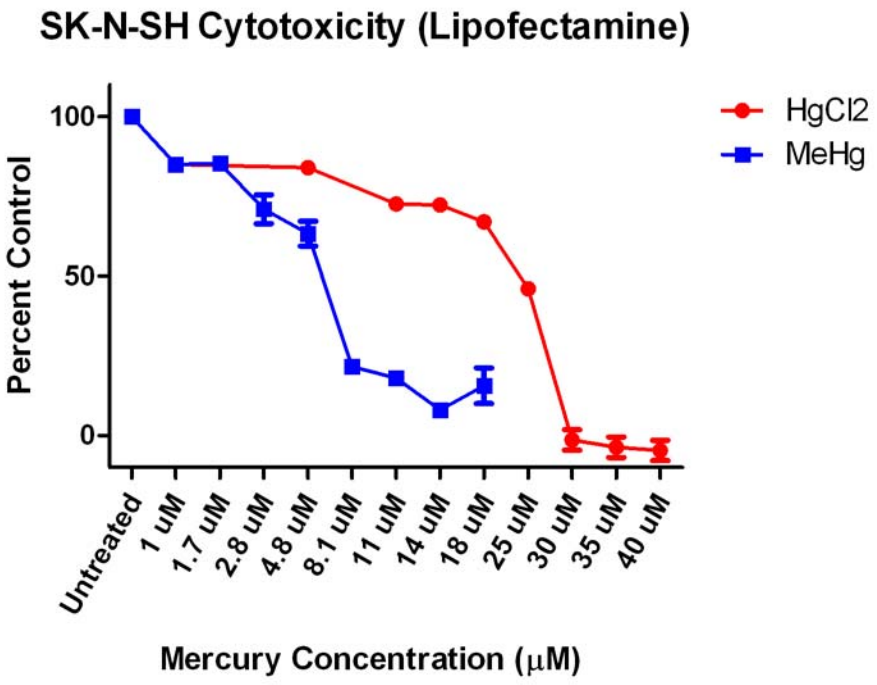



**Figure 37: SK-N-SH dose response under transfection conditions.** Cells were transfected with non-homologous siRNA. 24 h later, cells were exposed to either mercurial for 24 h. Cell viability was assessed using the neutral red assay, and mercurial-treated cells were compared to untreated cells to determine the percent control. Results display the mean percent of control ± SEM.

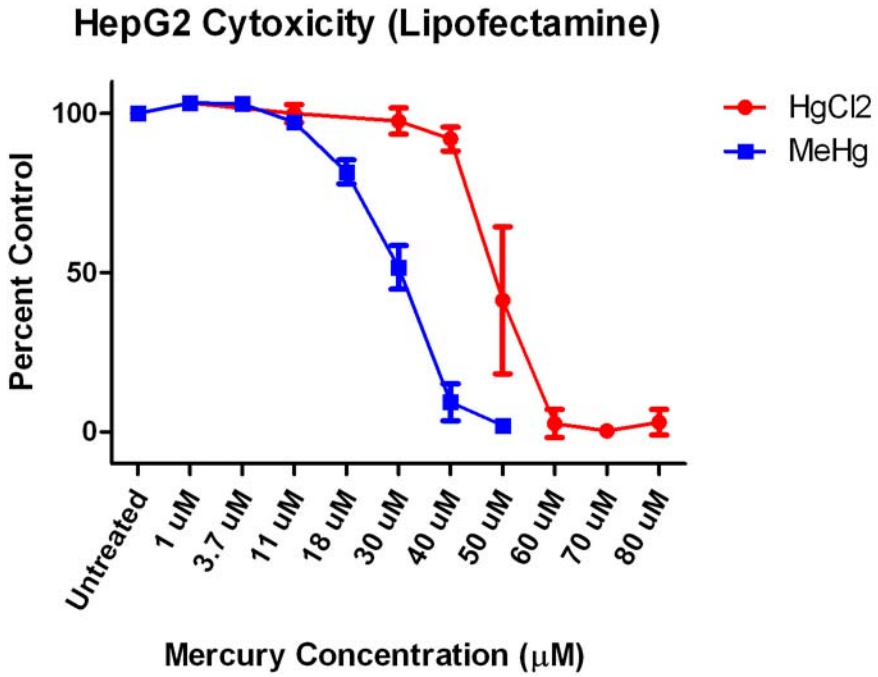



**Figure 38: HepG2 dose response under transfection conditions.** Cells were transfected with non-homologous siRNA. 24 h later, cells were exposed to either mercurial for 24 h. Cell viability was assessed using the neutral red assay, and mercurial-treated cells were compared to untreated cells to determine the percent control. Results display the mean percent of control ± SEM.

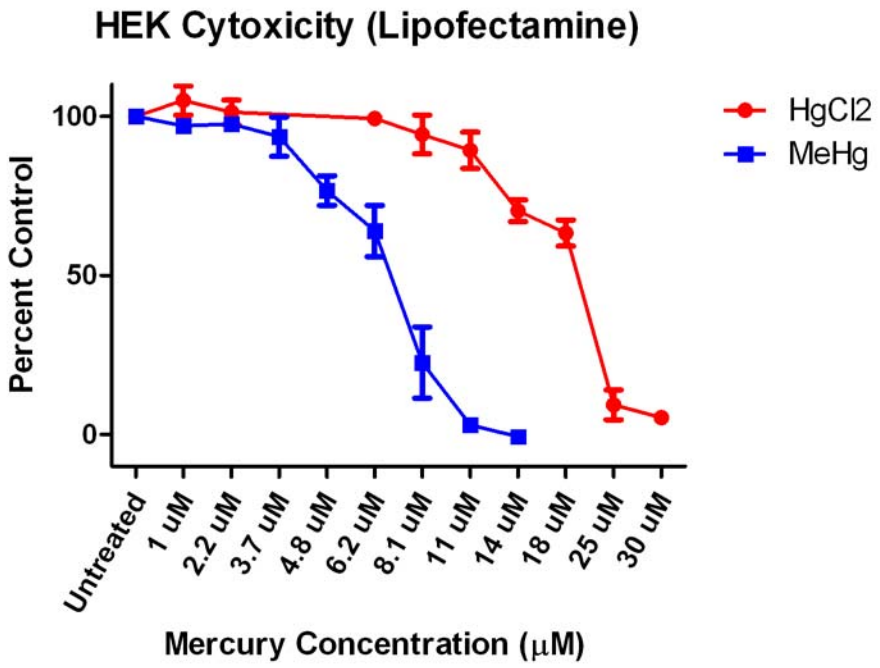



**Figure 39: HEK293 dose response under transfection conditions.** Cells were transfected with non-homologous siRNA. 24 h later, cells were exposed to either mercurial for 24 h. Cell viability was assessed using the neutral red assay, and mercurial-treated cells were compared to untreated cells to determine the percent control. Results display the mean percent of control ± SEM.

While the HEK293 cells showed no enhanced mercury susceptibility under the transfection conditions, the SK-N-SH and HepG2 cells did. The estimated EC50s for each mercurial in each cell line were determined and are listed in Table 35.

**Table 35: Estimated EC50 for cells under transfection conditions.**

| SK-N-SH | | HepG2 | | HEK293 | |
|---|---|---|---|---|---|
| $HgCl_2$ | MeHg | $HgCl_2$ | MeHg | $HgCl_2$ | MeHg |
| 21 μM | 5 μM | 48 μM | 30 μM | 17 μM | 6.8 μM |

These EC50s were then used to assess the effect of knocking down each gene in response to each mercurial in the three cell lines. Cells were transfected with either siRNA to a specific gene or, as a control, non-homologous siRNA. 24 h after the siRNA transfection, HgCl2 or MeHg was added to the cells. 24 h after mercury was added to the cells, cell viability was measured using the neutral red assay. Tables 36-38 list the results of these experiments. The interaction p-value is the result of a 2-way ANOVA which examined if there was a significant gene-mercurial interaction. The RNAi parameter is the percent difference in viability between cells exposed to non-homologous siRNA and cells exposed gene-specific siRNA. The mercury parameter is the percent difference in viability between cells treated with non-homologous siRNA and mercurial and cells treated with gene-specific siRNA and mercurial. The interaction parameter is the result of the mercury parameter subtracted from the RNAi parameter. If the mercury parameter is less than the RNAi parameter, that indicates a negative

interaction. If the mercury parameter is greater than the RNAi parameter, that indicates a positive interaction.

**Table 36: SK-N-SH gene-mercurial interactions.** Significant interactions are in bold

| Gene | Mercurial | Interaction p value | RNAi parameter | Mercury parameter | Interaction parameter |
|---|---|---|---|---|---|
| BACE1 | HgCl2 | 0.057 | 9.7 | 0.68 | -9.0 |
| | MeHg | 0.22 | 8.4 | 4.2 | -4.2 |
| **CHKA** | HgCl2 | 0.088 | -4.8 | -17 | -12 |
| | **MeHg** | **0.037** | **-0.47** | **-12** | **-12** |
| CHKB | HgCl2 | 0.39 | -7.2 | -12 | -4.6 |
| | MeHg | 0.24 | 4.7 | -1.2 | -5.9 |
| **ELOVL3** | **HgCl2** | **0.0066** | **-1.3** | **-11** | **-9.8** |
| | MeHg | 0.53 | -4.9 | -5.4 | -0.58 |
| ELOVL6 | HgCl2 | 0.35 | -19 | -25 | -5.9 |
| | MeHg | 0.071 | -14 | -21 | -7.4 |
| **GCLC** | **HgCl2** | **0.031** | **1.9** | **-6.0** | **-8.0** |
| | MeHg | 0.45 | 3.1 | 2.6 | -0.57 |
| PARG | HgCl2 | 0.15 | -6.5 | -14 | -7.9 |
| | MeHg | 0.61 | -7.3 | -3.6 | 3.7 |

**Table 37: HepG2 gene-mercurial interactions.** Significant interactions are in bold.

| Gene | Mercurial | Interaction p-value | RNAi parameter | Mercury parameter | Interaction parameter |
|---|---|---|---|---|---|
| **ABCG2** | HgCl2 | 0.83 | 2.6 | 1.9 | -0.70 |
| | **MeHg** | **0.038** | **4.5** | **0.40** | **-4.1** |
| BACE1 | HgCl2 | 0.069 | -3.1 | 6.7 | 9.7 |
| | MeHg | 0.68 | 0.70 | -0.50 | -1.2 |
| **CHKA** | HgCl2 | 0.72 | -4.1 | -5.8 | -1.7 |
| | **MeHg** | **0.0028** | **0.62** | **-8.8** | **-9.4** |
| CHKB | HgCl2 | 0.24 | -10 | -2.5 | 7.8 |
| | MeHg | 0.92 | -4.2 | -3.8 | 0.38 |
| ELOVL3 | HgCl2 | 0.76 | -4.2 | -7.0 | -2.7 |
| | MeHg | 0.35 | 0.60 | 2.9 | 2.3 |
| **ELOVL6** | **HgCl2** | **0.0002** | **-8.6** | **17** | **26** |
| | MeHg | 0.57 | -5.0 | -3.8 | 1.2 |
| **GCLC** | HgCl2 | 0.86 | 7.2 | 7.7 | 0.50 |
| | **MeHg** | **0.0004** | **8.3** | **-3.2** | **-12** |
| PARG | HgCl2 | 0.59 | 0.13 | -2.2 | -2.4 |
| | MeHg | 0.96 | 2.1 | 2.3 | 0.20 |

**Table 38: HEK293 gene-mercurial interactions.** Significant interactions are in bold.

| Gene | Mercurial | Interaction p value | RNAi parameter | Mercury parameter | Interaction parameter |
|---|---|---|---|---|---|
| **ABCG2** | HgCl2 | 0.56 | 10 | 4.1 | -6.4 |
| | **MeHg** | **0.0068** | **13** | **-7.8** | **-21** |
| BACE1 | HgCl2 | 0.92 | -2.9 | -6.5 | -3.6 |
| | MeHg | 0.063 | 2.8 | -13 | -16 |
| BACE2 | HgCl2 | 0.87 | -5.6 | -9.1 | -3.5 |
| | MeHg | 0.30 | -7.4 | -17 | -9.8 |
| **CHKA** | HgCl2 | 0.11 | -4.9 | -20 | -15 |
| | **MeHg** | **0.021** | **-5.4** | **-22** | **-16** |
| CHKB | HgCl2 | 0.71 | -20 | -19 | 0.42 |
| | MeHg | 0.78 | -19 | -15 | 4.4 |
| **ELOVL3** | HgCl2 | 0.62 | -0.10 | -8.1 | -8.0 |
| | **MeHg** | **0.0023** | **5.1** | **-18** | **-23** |
| ELOVL6 | HgCl2 | 0.91 | -20 | -25 | -4.7 |
| | MeHg | 0.43 | -18 | -8.0 | 9.7 |
| GCLC | HgCl2 | 0.060 | 7.0 | -15 | -22 |
| | MeHg | 0.82 | 3.2 | 3.4 | 0.23 |
| **PARG** | HgCl2 | 0.20 | 5.8 | -16 | -22 |
| | **MeHg** | **0.0076** | **14** | **-18** | **-32** |

There were 11 significant gene-mercurial interactions. There was not a significant interaction with either mercurial and BACE1, BACE2, or CHKB in any of the cell lines. Of the 11 significant interactions, only knock-down of ELOVL6 in $HgCl_2$-treated HepG2 cells resulted in a positive interaction. This was the only instance in which there was a significant gene-mercurial interaction involving ELOVL6. There was also only one significant PARG-mercurial interaction, though it appears very important in resistance to MeHg in HEK293 cells, as this resulted in the greatest interaction parameter in the experiment. ABCG2 was important in resistance to MeHg in both HepG2 and HEK293

cells, but it was not expressed in SK-N-SH cells. Surprisingly, there was a significant GCLC-mercurial effect only in SK-N-SH cells treated with $HgCl_2$ and HepG2 cells treated with MeHg. As the GCLC homolog *gcs-1* was the most critical resistance gene to both mercurials in *C. elegans*, it was expected that similar results would also be observed in cell culture. There was a significant negative interaction between CHKA and MeHg in all three cell lines, while there was not a significant interaction between CHKA and $HgCl_2$ in any of the cell lines.

# 4. Discussion

This research was conducted to compare the molecular mechanisms by which $HgCl_2$ and MeHg act. The nematode *C. elegans* was used in these studies due to the facility with which both whole organism and molecular events can be studied. The toxicity of both mercurials to *C. elegans* was assessed using multiple assays, and approximately equitoxic $HgCl_2$ and MeHg treatments were used to compare the global effects of the two mercurials on transcription. Bioinformatics analysis revealed large differences in the effects the two mercurials had on transcription. RNAi was used to identify genes involved in the response to mercury. The evolutionary conservation of function of these genes was tested using RNAi of their human homologs in cells exposed to mercury.

## *4.1 Toxicity assays*

In the three toxicity assays (growth, reproduction, stress-response gene expression) MeHg was consistently more toxic than $HgCl_2$. It is possible that the differences in toxicity between the mercurials are due to differences in the absorption and distribution of the mercurials in *C. elegans*. Helmcke et al. found a significant increase in mercury levels in nematodes exposed to 100 μM MeHg for 30 min [102]. Using a mercuric ion-sensitive, hypodermis-expressed GFP, Chapleau et al. demonstrated that mercuric mercury can reach the hypodermis within minutes of

mercury exposure [125]. Therefore, it is clear that both mercurials are capable of being absorbed by *C. elegans*.

MeHg was able to induce changes in stress-response gene expression at lower concentrations than $HgCl_2$. A 24 h exposure to 1.6 μM MeHg resulted in significant increases in both *gcs-1* and *gst-38* mRNA levels. A 16 μM $HgCl_2$ exposure was required for *gcs-1* induction, while an 8 μM $HgCl_2$ exposure was needed to up-regulate *gst-38*. A 10 μM MeHg exposure was required to up-regulate both heat shock proteins, as opposed to a 25 μM $HgCl_2$ exposure. MeHg exposure induced the glutathione-response genes at 5-10-fold lower concentrations than $HgCl_2$, while it induced the heat shock proteins at 2-3-fold lower concentrations than $HgCl_2$. This suggests that MeHg may be more efficient at inducing the glutathione response than $HgCl_2$. The microarray data also suggest this is the case. In the low-toxicity HgCl2 treatment, no glutathione S-transferases were up-regulated, while 13 glutathione S-transferases were up-regulated in the low-toxicity MeHg treatment. Similarly, in the high toxic exposures, there were 19 glutathione S-transferases up-regulated by MeHg, but only 7 up-regulated by $HgCl_2$. These data suggest that MeHg induces oxidative stress at lower levels of overt toxicity in *C. elegans* than $HgCl_2$.

The relative toxicity of MeHg and $HgCl_2$ was different in the growth and reproduction assays. In the growth assay, MeHg was only slightly more toxic than $HgCl_2$, while in the reproduction assay MeHg was approximately 15 times more toxic

than $HgCl_2$. This suggests that, as in humans, there are developmental stages in which MeHg is especially toxic. In the growth assay, nematodes are first exposed to mercury at the L1 larval stage and exposure stops at no later than the L4 stage. In the reproduction assay, nematodes are exposed to mercury beginning in the L4 stage and exposure continues through the reproductive process. There is no overlap in the developmental stages affected by these two assays (Figure 40).

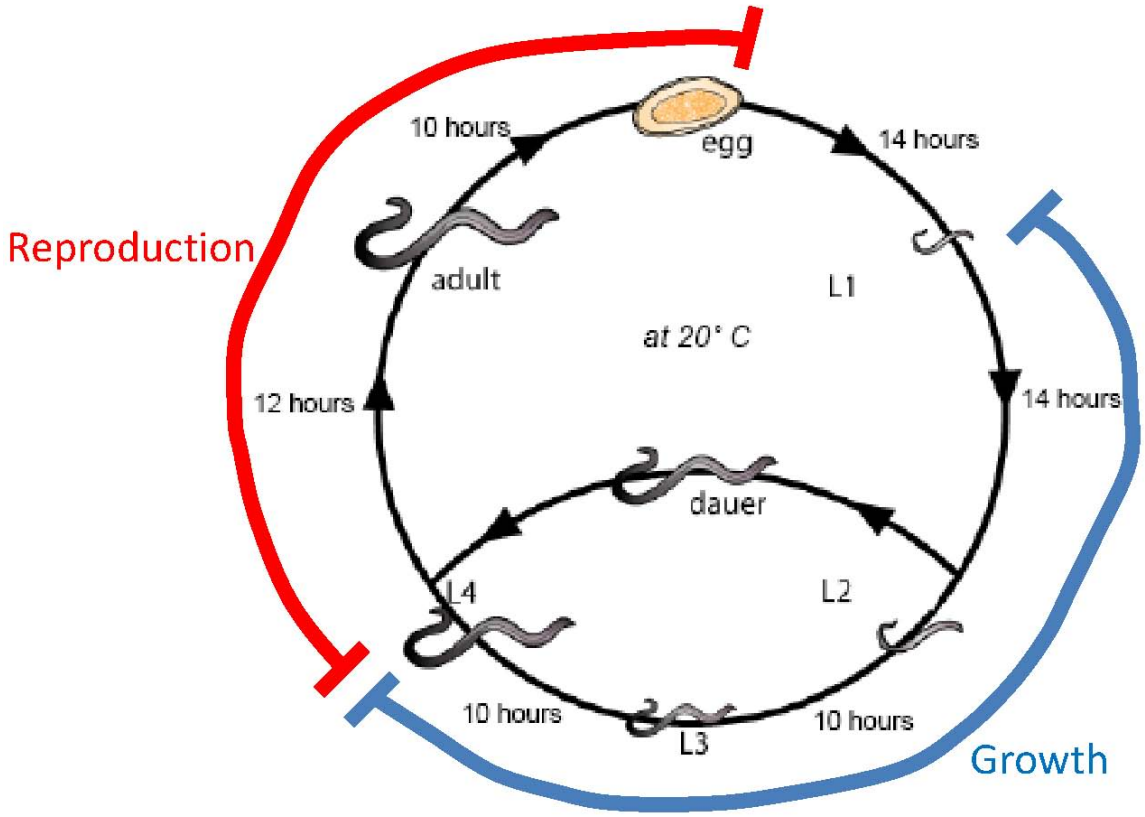



**Figure 40: Comparison of life stages exposed to mercury during the reproduction and growth toxicity assays.**

In mammals, MeHg is more neurotoxic than $HgCl_2$. This may also be the case in *C. elegans*, which would explain why MeHg is more toxic than $HgCl_2$ to *C. elegans* in the reproduction assay. Egg-laying is dependant on neuromuscular activity, and MeHg may have affected this activity. The microarray data suggest a possible contributing mechanism for the difference in $HgCl_2$ and MeHg toxicity in the reproduction assay. EPIG pattern 4 is characterized by genes that are highly up-regulated by toxic $HgCl_2$ levels and are slightly down-regulated in all MeHg exposures. Gene Ontology analysis found an enrichment of several neurotransmission and neuromuscular processes. An interaction analysis using Cytoscape yielded a sub-network consisting of *eat-16* (EATing, abnormal pharyngeal pumping), *gar-3* (G-protein linked Acetylcholine Receptor), and *unc-104* (UNCoordinated), which were in pattern 4, as well as *gpb-2* (G-Protein Beta subunit) and *cat-1* (abnormal CATecholamine distribution) (Figure 41).

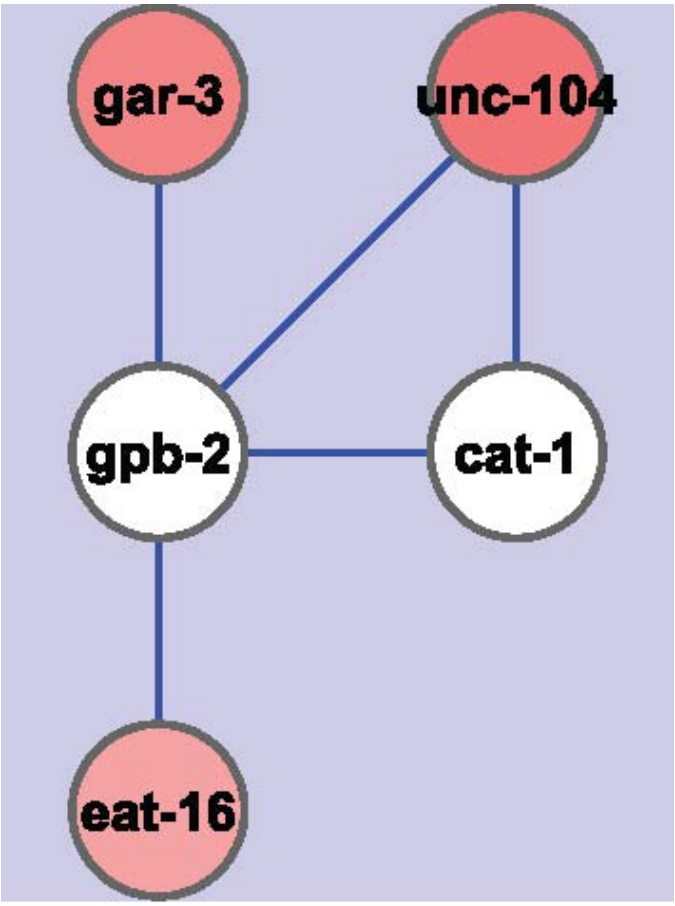



**Figure 41: An enriched sub-network in EPIG pattern 4 that contains genes important in egg-laying.** The darkness of the color of the node is correlated to the magnitude fold-change.

*eat-16*, *gpb-2* and *cat-1* are all important in egg-laying, and *gar-3* likely acts upstream of *gpb-2* [126]. *unc-104* is required for anterograde axonal transport of synaptic vesicles in neuromuscular junctions [126]. Therefore, it is possible that the large discrepancy in reproductive toxicity of the two mercurials is because $HgCl_2$ exposure results in an up-regulation of genes required for egg-laying, while MeHg exposure depresses expression of those genes.

## *4.2 Analysis of microarray results*

Several studies have looked at the effects of either $HgCl_2$ or MeHg on the transcriptome, but this was the first study to directly compare the effects of these two mercurials on the transcriptome. Hierarchical clustering and PCA showed that, particularly under sub- and low-toxic conditions, $HgCl_2$ and MeHg have very different effects on mRNA levels in *C. elegans.* The extent of this difference was unexpected, as the established mechanisms of toxicity of inorganic and methylmercury are largely the same. In higher organisms, the distribution of $HgCl_2$ and MeHg vary, and as the cell culture experiments performed in this study demonstrated, mercury-induced changes in transcription vary by cell type. It is possible that the same situation occurs in *C. elegans*. However, neither mercurial appears to be impeded in its movement through tissues, as an analysis of tissue-specific expression of DEGs did not indicate that major differences existed in the target tissues of the mercurials.

There were several KEGG pathway and GO terms that were enriched in up-regulated genes in both $HgCl_2$ and MeHg high-toxic exposures. When investigating the genes that populated these categories, it was found that the genes were primarily: UDP-glucuronosyl transferases, cytochrome P450s, heat shock proteins and genes involved in redox homeostasis. As these are all stress-response genes, one would expect these genes to be up-regulated by a toxic exposure to most toxicants.

As expected, exposure to $HgCl_2$ increased the abundance of metallothionein transcripts. What was interesting was that exposure to MeHg down-regulated *mtl-2* transcript. Previous studies have found an inconsistent effect of MeHg exposure on metallothionein levels. Kramer et al. found no increase in metallothionein protein levels in cultured astrocytes after a 48 h MeHg exposure [127]. A microarray experiment examining the effect of MeHg exposures in fathead minnows did not result in any differentially expressed metallothioneins [92]. In rats orally administered MeHg, metallothionein protein levels in the brain were not increased, but they increased in the kidney and liver [128]. A microarray study using zebrafish also found the effect of MeHg on metallothioneins was tissue-specific [24]. Mt2 was up-regulated in the liver after 21 and 63 day dietary exposure to 13.5 μg MeHg/g food, but was not differentially expressed in a 7 day exposure or when the MeHg concentration in food was 5 μg/g. However Mt2 was not up-regulated in the brain or skeletal muscle and Mt1 was not up-regulated under any condition. To the best of our knowledge, this is the first report of down-regulation of metallothioneins in *C. elegans* exposed to MeHg.

Metallothioneins mitigate MeHg toxicity in a variety of systems. An increase in metallothionein levels by pre-exposure to inorganic metal or transfection with an MT-1 plasmid was protective against MeHg exposure in rat primary astrocyte cultures [129, 130]. Yoshida *et al*. found that MTI/II knockout mice exposed *in utero* through weaning to MeHg performed worse than wild type mice in behavioral tests as adults [131]. It is

assumed that metallothioneins, due to their large number of sulfhydryl groups, bind MeHg, and mammalian MT1 can bind MeHg [132]. In addition to binding metals, metallothioneins also are important in regulating the redox status of cells [133].

It is possible that *C. elegans* are more reliant on glutathione-mediated detoxification in MeHg exposures than in $HgCl_2$ exposures. This may explain why there were a greater number of glutathione S-transferases up-regulated by MeHg than $HgCl_2$ in the microarrays. Additionally, while knock-down of *gcs-1* made *C. elegans* susceptible to both mercurials, the effect was greater in nematodes exposed to MeHg. The *gcs-1*-HgCl2 interaction parameter was -228, but the *gcs-1*-MeHg interaction parameter was -354. The inability of *C. elegans* to up-regulate metallothioneins in response to MeHg exposure may be one reason why MeHg is more toxic to *C. elegans* than $HgCl_2$.

The gene expression patterns identified by EPIG indicate that the different mercurials have different effects on transcription. Gene Ontology analysis of genes in EPIG patterns provides insight into how the different mercurials affect biological processes. EPIG pattern 8 is a group of 669 genes that were down-regulated in response to $HgCl_2$ and up-regulated in response to MeHg. GO analysis found a significant enrichment of genes included in the ubiquitin-dependent protein catabolic process (Table 25). Similarly, KEGG pathway analysis of this group of genes found a significant enrichment of genes in the proteasome pathway (Table 26). This was the most significantly enriched KEGG pathway identified. Metals bind to proteins, which

disrupts the form and function of these proteins. It is important that these proteins are degraded because the accumulation of damaged proteins can be pathogenic. The proteasome system is protective in yeast exposed to MeHg [134, 135].

GO analysis of genes down-regulated by high-toxic MeHg exposure also resulted in an enrichment of genes involved in the ubiquitin-dependent protein catabolic process (Table 15). An examination of these genes found that they were all components of the ubiquitin ligase complex. In contrast, the genes from EPIG pattern 8 (down-regulated by $HgCl_2$, up-regulated by MeHg) that were assigned to this GO term were all components of the proteasome. Thus, while *C. elegans* exposed to MeHg up-regulate transcription of components of the proteasome, the cell may still experience the stress caused by damaged proteins due to a decrease in the cell's ability to ubiquitinate damaged proteins. Because both ubiquitin ligase complex and proteasome genes were down-regulated by $HgCl_2$, a poorly functioning ubiquitin-proteasome pathway likely also exists in nematodes exposed to $HgCl_2$.

Microarray studies of $HgCl_2$-exposed zebrafish and MeHg-exposed mouse embryos found an enrichment of genes in the Wnt signaling pathway [91, 95]. In this study, there was an enrichment of Wnt signaling pathway genes down-regulated in *C. elegans* exposed to the high-toxic MeHg treatment. By contrast, the DEGs associated with Wnt signaling in MeHg-exposed, gestational day 8 mouse embryos were primarily up-regulated [95]. A study examining the effects of $HgCl_2$ on transcription in the liver of

zebrafish also found an enrichment of DEGs involved in Wnt signaling, though these genes were primarily down-regulated [91]. An examination of the genes in the present study associated with Wnt signaling suggests that Wnt signaling may not have been perturbed by MeHg exposure in *C.elegans*. While the dishevelled-related gene *dsh-1* was down-regulated 2.6-fold in response to high-toxic MeHg exposures, all the other genes associated with Wnt signaling are components of the ubiquitin ligase complex. Likewise, the TGF-β signaling pathway was also an enriched KEGG pathway in genes down-regulated by high-toxic MeHg treatment, but it was populated by the same ubiquitin ligase complex genes.

## 4.3 C. elegans RNAi screen

In the *C. elegans* RNAi screen, the knock-down of 8 genes increased the resistance of nematodes to mercury exposure, while the knock-down of 10 genes increased the tolerance of nematodes to mercury exposure. Of these 18 genes, in only 2 cases were significant gene-mercury interactions observed for both mercurials. One of these genes, *gcs-1*, was the most important gene for resistance to both $HgCl_2$ and MeHg, as knock-down of this gene resulted in the greatest negative interaction for both mercurials. In fact, it was the only resistance gene found for $HgCl_2$. While there have been no published results investigating the importance of *gcs-1* to mercury resistance in *C. elegans*, it is well known that glutathione is important for resistance to mercurials in other systems. In humans, polymorphisms in GCLC and glutathione S-transferases

result in increased mercury body burdens [136, 137]. In the *C. elegans* RNAi screen there was a positive gene-mercury interaction for F14F9.4 in response to both mercurials. The function of F14F9.4 is unknown. It is not clear why knock-down of this gene would improve *C. elegans* resistance to mercury, though this is an area for future research.

The knock-down of 7 genes other than *gcs-1* also resulted in increased susceptibility to MeHg exposure. Interestingly, although 22 glutathione S-transferases were up-regulated in at least one condition in the microarray, none were found to be critical in resistance to either mercurial. This is likely due to redundancy of function among GSTs. *pme-4* (Poly(ADP-ribose) Metabolism Enzyme) catalyzes the catabolism of poly-ADP-ribose to ADP-ribose, and is required for resistance to ionizing radiation [138]. *pme-4* is primarily expressed in the cytoplasm of neurons, and is predicted to be critical in preventing neurodegeneration [126]. MeHg is a well-established neurotoxicant, so *pme-4* may be critical in maintaining neuron viability in MeHg-exposed nematodes. *elo-6* was found to be important in resistance to MeHg even though it was down-regulated 2.5-fold in the high-toxic MeHg exposure. *elo-6* (fatty acid ELOngation) is a long-chain fatty acid elongation enzyme that plays an essential role in growth of *C. elegans* [139]. *elo-6* is a paralog of *elo-1* and *elo-2*, each of which encode a poly-unsaturated fatty acid elongase. There is a large body of evidence that suggests co-exposure to poly-unsaturated fatty acids mitigates MeHg toxicity in humans [140] [141]. However, while the specific enzymatic activity of elo-6 has not been established, the

Ensembl project found *elo-6* to have greater homology to the human genes ELOVL3 and ELOVL6, which are involved in the elongation of saturated and monounsaturated fatty acids.

Several of the remaining genes are poorly characterized, so it is unclear how these genes would be involved in resistance to MeHg. *fbxa-116* (F-box A protein) contains an F-box motif, which is predicted to mediate protein-protein interactions. *C. elegans* have 224 F-box A genes, compared to 38 in humans. It is not clear why there are more F-box genes in *C. elegans*, or why this particular one is important in MeHg resistance. *tsp-5* is tetraspanin family integral membrane protein. There are no known or predicted functions for the remaining 3 genes: T05E7.4, T22D1.2, Y69E1A.8.

The knock-down of 9 genes other than F14F9.4 was found to improve nematode resistance to mercury. Of these 9 genes, there is no known or predicted function for the following 3 genes: F19C7.5, C54D10.8 and T09F5.10. Of the remaining 6 genes, one was an F-box gene (*fbxa-136*) and one was a C-type lectin (*clec-163*), though no additional information is available about these genes. K01D12.1 is not well characterized, but was assigned to the secreted small molecules methylase KOG (eukaryotic Orthologous Groups) by NCBI. F59D6.2 is a poorly characterized gene, but it is homologous to the human genes BACE1 and BACE2.

*ckb-2* and *wht-3* have been characterized. *ckb-2* is a choline kinase. *ckb-2* was up-regulated in the unfolded-protein response, particularly when exposed to tunicamycin,

an antibiotic that inhibits N-glycosylation. [142] This activation is dependent on *xbp-1* [143]. Unfortunately, the function of *ckb-2* in the unfolded protein response in unknown, so it is not clear why knock down of this gene would improve the resistance of nematodes to mercury. *ckb-2* was not highly regulated by mercury exposure, as it was up-regulated 2.4-fold in the high-toxicity $HgCl_2$ exposure, and was unaffected under all other conditions. *wht-3* is an ATP-binding cassette (ABC) protein. ABC transporters use energy from the hydrolysis of ATP to move small molecules across membranes. It was shown that *wht-3* is required for RNAi in the germ line of *C. elegans*, so *wht-3* may be a dsRNA and miRNA transporter [144]. Only 9 of the 49 ABC transporters tested were essential for RNAi in the germ line, so this does not appear to be a characteristic common among *C. elegans* ABC transporters [145].

$HgCl_2$ and MeHg differed in the number of positive and negative interactions. Of the 10 significant gene-MeHg interactions, 8 resulted in increased susceptibility to MeHg. Conversely, of the 10 significant gene-$HgCl_2$ interactions, 9 resulted in increased tolerance to $HgCl_2$. This difference in effect of the gene-mercury interactions between the two mercurials and the similar gene-mercury interaction for only 2 of 18 genes is further evidence that the two mercurials act differently at the molecular level.

## *4.4 Effects of mercury in cell culture*

### 4.4.1 Toxicity of mercurials to cells

The three cell lines used in these experiments were chosen because they represent tissues exposed to mercurials in mammals. SK-N-SH cells are neuroblastoma-derived; the brain is the primary target organ of MeHg. HEK cells are human embryonic kidney cells; the kidney is the primary target organ of inorganic mercury. HepG2 cells are derived from a hepatocellular carcinoma. After the kidney, the liver accumulates the most inorganic mercury. As ingestion is the primary route of exposure to MeHg, the liver is exposed to virtually all MeHg that enters the body. Despite exposure to both mercurials, the liver is not generally considered to be a major target organ for toxicity to either mercurial. The cytotoxicity assays demonstrated that liver cells were more resistant to mercury exposure. The $HgCl_2$ EC50 for HepG2 cells was twice as high as that for SK-N-SH, and 3 times as high as the $HgCl_2$ EC50 for HEK293 cells. There was an even greater difference between cell lines when comparing the MeHg EC50s. The MeHg EC50 for HepG2 cells was three times as that for SK-N-SH cells and 5 times as that for HEK293 cells.

### 4.4.2 Mammalian cell qRT-PCR and gene-mercury interactions

Changes in gene expression in cells exposed to mercury were not predictive of which gene-mercury combinations would result in significant interactions. Neither BACE1 nor BACE2 were involved in any significant gene-mercury interactions,

although BACE1 was down-regulated by MeHg in HepG2 cells, and $HgCl_2$ exposure up-regulated BACE2 in SK-N-SH cells and down-regulated BACE2 in HEK293 cells. CHKB was up-regulated at NOAEL, EC20, and EC50 treatments of MeHg in SK-N-SH cells, but there was not a significant BACE1-MeHg interaction in these cells. ELOVL3 was highly up-regulated (11-fold EC20, 10-fold EC50) in MeHg-exposed HepG2 cells, but ELOVL3 RNAi did not affect the viability of HepG2 cells exposed to MeHg. These data indicate that genes that are up-regulated in response to mercury exposure are not necessarily essential for the cellular response to mercury. The small number (18 out of 599) of genes that were both up-regulated in *C. elegans* exposed to mercury and had significant gene-mercury interactions also indicate that most differentially expressed genes are not essential to the organismal response to mercury.

These experiments yielded some interesting results. While GCLC was up-regulated in response to sub- and low-toxic treatments to both mercurials in HepG2 cells and high-toxicity $HgCl_2$ treatment in SK-N-SH, it was not differentially expressed under any of the tested conditions in HEK293 cells. HEK293 cells were found to be the most sensitive to both $HgCl_2$ and MeHg. The inability to up-regulate GCLC in response to mercurial exposure may play a role in that sensitivity. While GCLC siRNA resulted in a significant negative interaction with $HgCl_2$ in SK-N-SH and MeHg in HepG2 cells, the results were nowhere near as dramatic as what was observed *in vivo* in *C. elegans*. In *C. elegans*, the magnitude of the *gcs-1*-$HgCl_2$ interaction parameter was 2-fold greater and

the *gcs-1*-MeHg interaction was 3-fold greater than the gene-mercury interaction parameter for any other resistance gene. By contrast, the GCLC-$HgCl_2$ interaction parameter in SK-N-SH cells was the second smallest and the GCLC-MeHg interaction parameter was the fourth smallest of the 11 significant gene-mercury interactions. This difference is likely due to species differences in the glutamate cysteine ligase enzymes. While the *C. elegans* enzyme *gcs-1* acts as a monomer, the mammalian enzyme has two subunits: GCLC and GCLM. GCLC is the catalytic subunit and GCLM is a modifier subunit. While GCLC is able to function without GCLM, its enzymatic activity is greatly increased when coupled to GCLM [146]. In mice, GCLM is limiting in most tissues [147]. It is therefore possible that using siRNA against GCLC did not significantly reduce glutathione levels in the cells tested. The discrepancy in the results between *C. elegans* and mammalian cells probably don't indicate a difference in the importance of glutathione to mercury resistance, but rather a difference in glutathione levels in the different systems.

Co-exposure of PARG siRNA and MeHg resulted in the largest observed gene-mercury interaction, which indicates that PARG is critical in the resistance of HEK293 cells to MeHg exposure. However, MeHg exposure did not affect mRNA levels of PARG in HEK293 cells. PARP (poly-ADP-ribose polymerase) is an enzyme that adds ADP-ribose to proteins (as many as 200 units), while PARG cleaves poly-ADP-ribose to ADP-ribose monomers [148]. The maintenance of poly-ADP-ribose on proteins is a very

dynamic process, as the half-life of ADP-ribose polymers is estimated to be less than 1 minute [149]. In cases of severe stress, PARP can become highly activated, which leads to over-production of poly-ADP-ribose and cell death [149]. Treatment with the PARP inhibitor 3,4-dihydro-5-[4-(1-piperidinyl)-butoxy]-1(2*H*)-isoquinolinone (DPQ) decreased MeHg-induced cell death in a dose-dependent manner [150]. This suggests that exposure to MeHg increases PARP activity, and that PARG is necessary to maintain poly-ADP-ribose homeostasis. *pme-4*, the *C. elegans* PARG homolog, is important in maintaining neuronal viability, so it is somewhat surprising that PARG would be important in resistance to mercury in HEK293 cells, but not SK-N-SH cells. SK-N-SH cells are a transformed cell line, so PARG may protective against mercury induced neurotoxicity *in vivo* in mammals. *pme-4* may also be expressed in other tissues in *C. elegans* response to mercury exposure.

ELOVL3 was highly up-regulated by $HgCl_2$ in SK-N-SH cells and by MeHg in HEK293 cells. RNAi of ELOVL3 increased the susceptibility of those cells to those mercurials, respectively. The only condition in which RNAi improved mercury resistance was knock-down of ELOVL6 in $HgCl_2$ exposed HepG2 cells. $HgCl_2$ induced a 2.0-fold down-regulation of ELOVL6 in the EC20 treatment, so HepG2 cells down-regulated a gene that was harmful to the mercury response. MeHg exposure induced a much greater decrease (2.4-fold in EC20, 2.8-fold in EC50 treatment) in ELOVL6 in

HepG2 cells than $HgCl_2$ did. However, there was not a significant ELOVL6-MeHg interaction in these cells.

As in *C. elegans*, $HgCl_2$ and MeHg have different effects on transcription in cells. ELOVL3 is the most highly up-regulated gene in HepG2 cells exposed to MeHg, but it was not differentially expressed in HepG2 cells exposed to $HgCl_2$. Conversely, ELOVL3 was the most highly up-regulated gene in SK-N-SH cells in response to $HgCl_2$, but it was not differentially expressed in SK-N-SH cells exposed to MeHg. BACE1 was down-regulated by EC20 and EC50 MeHg exposures in HepG2 cells, but $HgCl_2$ had no effect on BACE1 mRNA levels. BACE2 was up-regulated in SK-N-SH cells at EC20 and EC50 $HgCl_2$ treatments, whereas MeHg had no effect. Finally, in HepG2 cells, ABCG2 was down-regulated by MeHg in a dose-dependent manner, while $HgCl_2$ had no effect on steady state ABCG2 mRNA levels. While there are many instances in which a gene was differentially expressed in response to only one mercurial, there were no instances in which a gene was up-regulated by one mercurial and down-regulated by the other. However, the different effects that $HgCl_2$ and MeHg have on transcription in cultured cells is further evidence that the differences in toxicity between the two mercurials is not simply due to differences in tissue distribution.

## 4.5 Summary

This project compares the toxicological and molecular responses to inorganic and organic mercury in *C. elegans*. While MeHg was more toxic than $HgCl_2$ in the toxicity assays, the difference in relative toxicity varied by assay. In particular, MeHg was 15 times more toxic than $HgCl_2$ in the reproduction assay, but ~10% more toxic than MeHg in the growth assay. Since the different assays measured the effects of $HgCl_2$ and MeHg on different biological processes, these data suggest that the two mercurials have both unique and similar mechanisms of toxicity.

The extent to which inorganic and organic mercury act differently at the molecular level was investigated by assessing the effect of equi-toxic treatments of the two mercurials on the transcriptome. At equi-toxic concentrations, MeHg exposure resulted in a greater number of differentially expressed genes and a higher percentage of down-regulated genes than $HgCl_2$ exposure. PCA and hierarchical clustering and EPIG indicate that the two mercurials have different effects on the transcriptome. GO and KEGG pathway analysis demonstrated that, while stress-response genes are up-regulated in high-toxic exposures to both mercurials, the two mercurials likely affect different biological processes. These data provide strong evidence that inorganic and organic mercury act differently at the molecular level.

RNAi was used to examine the biological function of 599 genes up-regulated by one or both mercurials in response to mercurial exposure. Of these 599 genes, there was

a significant gene-mercurial interaction for both mercurials in only 2 genes. A significant gene-mercurial interaction was observed for one of the mercurials in 16 genes. These data indicate that the genes necessary for the nematodes' response to mercurials differ by mercury species. $HgCl_2$ and MeHg also had different effects on transcription in human cells. While different genes were essential for the mercurial response in different cell lines, there was not a significant gene-mercury interaction for both mercurials with the same gene in any cell line. This study demonstrates that inorganic and organic mercury act differently at the molecular level.

# 5. Future Directions

## *5.1 Mercury and choline kinase α*

In the mammalian cell culture experiments, choline kinase-$\alpha$ (CHKA) RNAi coupled with MeHg exposure resulted in a significant negative interaction in all three cell lines, which indicates that CHKA is important in resistance to MeHg exposure. While there were no significant negative CHKA-$HgCl_2$ interactions, the magnitude of the CHKA-$HgCl_2$ interaction parameter was close to that of the CHKA-MeHg interaction parameter in both SK-N-SH and HEK293 cells. CHKA catalyzes the phosphorylation of choline. Therefore, knock-down of CHKA likely results in reduced levels of phosphocholine.

In mice exposed to $CCl_4$, choline kinase enzymatic activity increased, which suggests that cells may increase phosphocholine levels in times of stress [151]. The stress-responsive increase in Chka (mouse homolog of human CHKA) is regulated at the transcriptional level. Chka contains a xenobiotic response element and an AP-1 binding element in its promoter [152] [153]. Yalcin et al. found that siRNA targeting CHKA in immortalized bronchial epithelial cells decreased intracellular phosphocholine and phosphatidic acid, which abrogated MAPK and PI3K/Akt signaling [154]. They hypothesized that it was necessary to maintain the intracellular pool of phosphocholine in order to generate phosphatidic acid, which is critical in activation the MAPK and

PI3K/Akt signaling pathways (Figure 44). Both of these are critical survival signaling pathways.

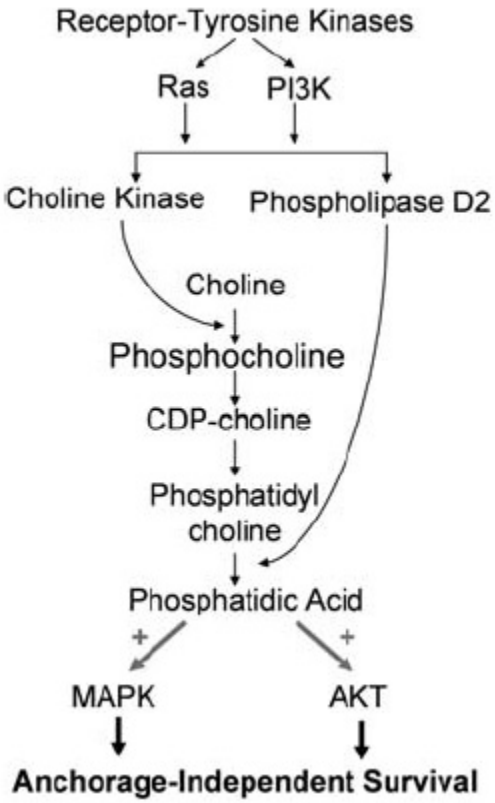
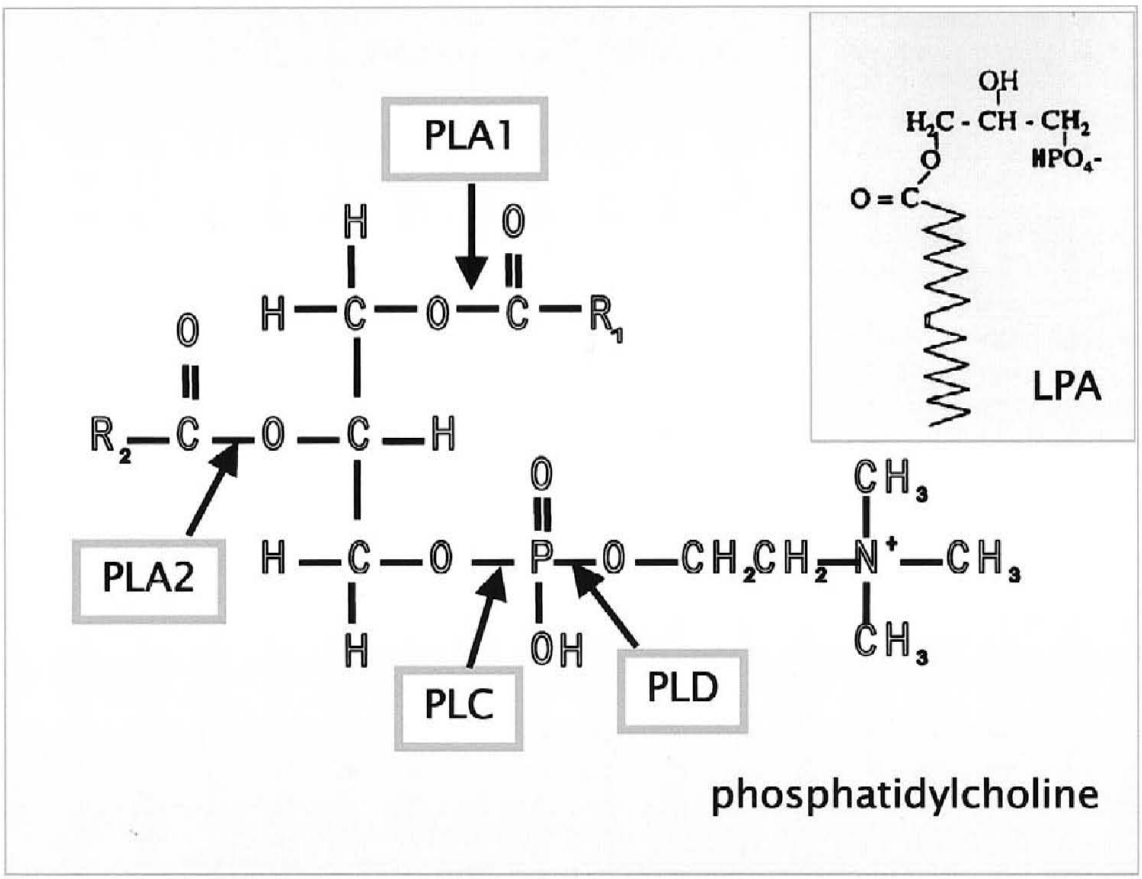



**Figure 42: Proposed mechanism indicating how phosphocholine and phosphatidic acid are critical to survival of neoplastic cells.**

It is possible that the MAPK and PI3K/Akt pathways are activated as a protective response in cells exposed to mercury. The MAPK signaling pathway is activated in mercury exposure [78] [155] [156] [157]. Surprisingly little work has been done to investigate the effects of mercury on PI3K/Akt signaling, but Ya et al. showed that both $HgCl_2$ and MeHg activated PI3K/Akt signaling in pancreatic β-cells [158].

Phosphatidic acid is generated through the cleavage of phosphatidylcholine by phospholipase D (PLD). Both $HgCl_2$ and MeHg increased PLD activity in bovine pulmonary artery endothelial cells [159]. However, both HgCl2 and MeHg also increased phospholipase $A_2$ (PLA2) activity, and MeHg increased phospholipase C (PLC) activity [160] [161] [162]. These phospholipases cleave phosphatidylcholine at different sites, and only PLD generates phosphatidic acid (Figure 45).




Budnik L T , Mukhopadhyay A K Biol Reprod 2002;66:859-865

**Figure 43: Site of cleavage of phospholipases on phosphatidylcholine**

A chemical inhibitor of PLC reversed toxicity in MeHg-exposed canine kidney cells, and a chemical inhibitor of PLA2 attenuated $HgCl_2$ toxicity in bovine pulmonary artery endothelial cells [160, 162]. MeHg exposure also decreased cellular phosphatidylcholine levels, which was mitigated by inhibiting PLC [162]. The effect of PLD inhibition on mercury toxicity has not been investigated.

These data suggest a model in which mercury exposure activates phospholipase D, which cleaves phosphatidylcholine to generate phosphatidic acid, which activates the MAPK and PI3K/Akt survival signaling pathways. The concomitant activation of PLA2 and PLC by mercury competes with PLD for phosphatidylcholine, which decreases activation of the MAPK and PI3K/Akt survival signaling pathways. In this model, inhibition of PLD should increase mercury toxicity. To test this model, we will test the effect of inhibiting PLA2, PLC and PLD on cell viability, phosphatidic acid levels and activation of the MAPK and PI3K/Akt signaling pathways. We hypothesize that, in MeHg-exposed cells, inhibition of PLA2 and PLC will increase cell viability, phosphatidic acid levels and activation of the MAPK and PI3K/Akt signaling pathways. We further predict that inhibition of PLD will decrease cell viability, phosphatidic acid levels and activation of the MAPK and PI3K/Akt signaling pathways. Finally, we will add phosphatidic acid to cells in which PLD is inhibited to rescue the increased MeHg toxicity.

## *5.2 The effects of inorganic and organic mercury in yeast*

In the present study, the effect of $HgCl_2$ and MeHg on transcription was investigated. The importance of genes up-regulated by mercury was then investigated using RNAi to knock-down those genes. This method limited the search for genes important in the mercury response to those that were differentially regulated by mercury. It is likely that there were genes (e.g. transcription factors), critical to the mercury response, that were not differentially expressed by mercury. These genes would have been missed in this screen.

In order to expand on the findings in this study, a similar study using *Saccharomyces cerevisiae* (yeast) will be carried out. A yeast library that contains deletion mutants of ~4700 non-essential genes will be screened for genes essential for growth in the presence of mercury. The effects of exposure to low- and high-toxic concentrations of HgCl2 and MeHg on growth will be assessed by measuring growth over a 45 h period. A dose-response time course measuring the effect of HgCl2 and MeHg on wild-type yeast growth is shown in figure (46 and 47).

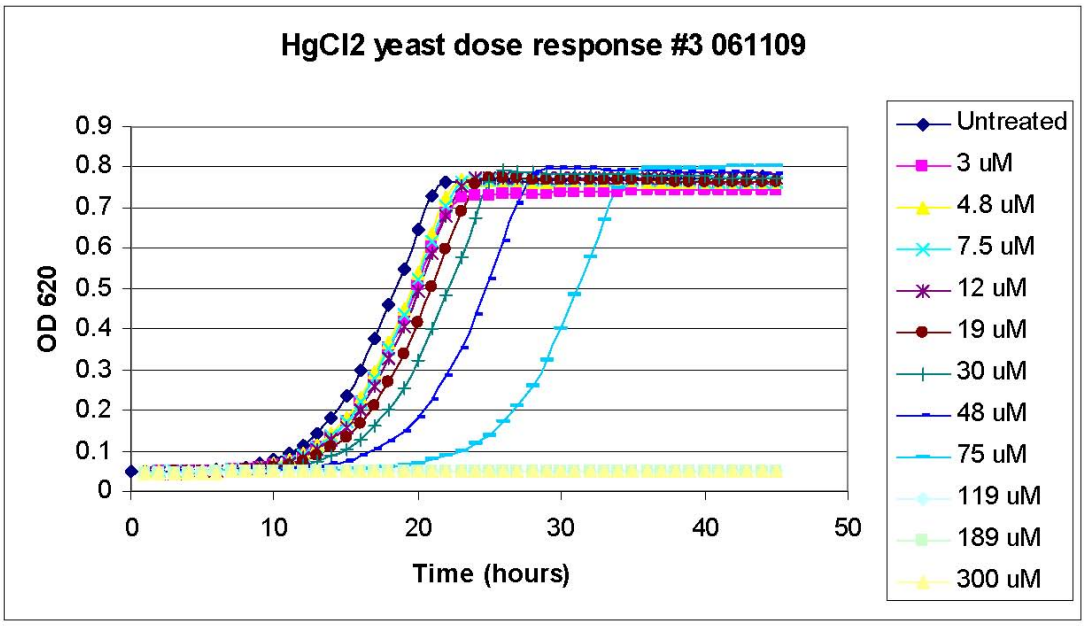



**Figure 44: Dose-response time course of wild type yeast exposed to $HgCl_2$**

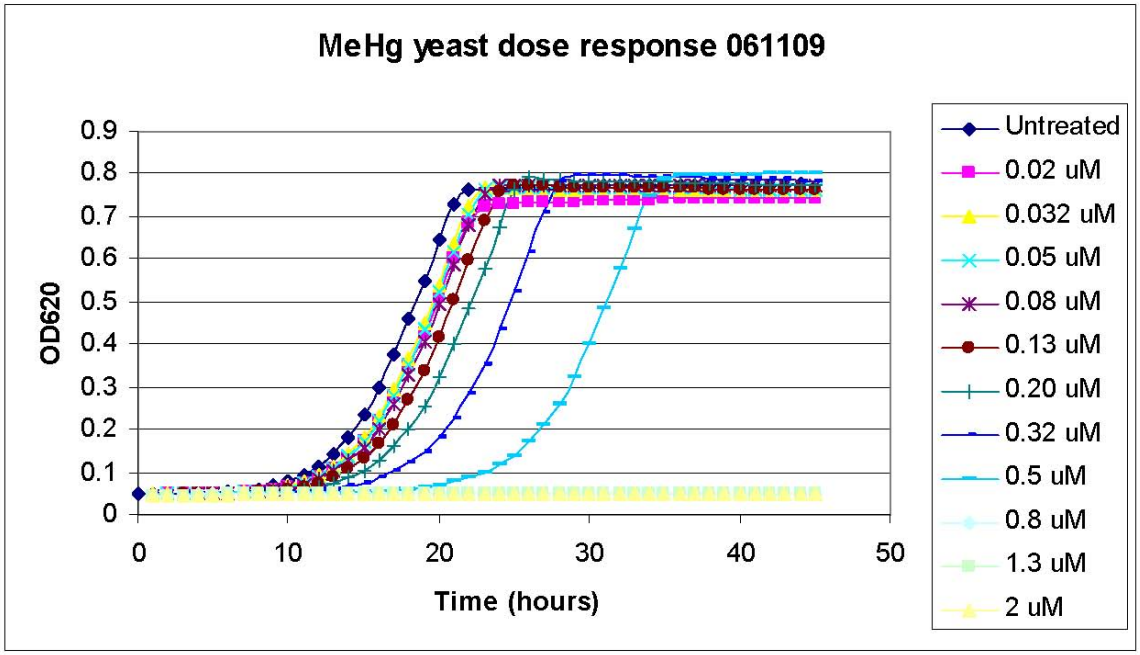



**Figure 45: Dose-response time course of wild type yeast expolsed to MeHg**

To compare the toxicities of the two mercurials, a Hill model was fit to the data sets for both HgCl2 and MeHg for time points in the log phase of growth (12-26 hours). An EC50 was then calculated for each time point. The EC50 for both mercurials were compared at each time point to determine a toxic equivalency factor (TEF) (i.e. what is the fold-change in the EC50 between the two mercurials). The TEF was 149 (145-153 95%CI) and did not change significantly with time ($p > 0.32$). MeHg is 149 times more toxic to yeast than $HgCl_2$, which is a much greater difference than was observed in *C. elegans.* The mercury concentrations that will be used in the yeast knockout screen are listed in table 39.

**Table 39: Concentrations used in yeast studies**

| Mercurial | Concentration (μM) | |
|---|---|---|
| | Low dose | High dose |
| $HgCl_2$ | 21 | 63 |
| MeHg | 0.14 | 0.42 |

A microarray study will be performed to determine the global transcriptional effects of exposure to inorganic and organic mercury in yeast. To facilitate a comparison between the yeast deletome and transcriptome, yeast will be exposed to the same mercury concentrations and growth conditions as in the deletion study. In the present study, the examination of genes involved in the mercury response was limited to those genes that were differentially expressed in *C. elegans* exposed to mercury. The yeast microarray study will identify the genes that are differentially expressed in response to mercurial exposure and the yeast deletome study will identify the genes that are important to the organismal response to mercurial exposure. A more complete picture of the molecular effects of inorganic and organic mercury will be determined by examining these two datasets. Unlike *C. elegans*, the entire yeast protein interactome is known [163]. This will allow for a more complete bioinformatics analysis, particular in regards to signaling pathways. Evolutionarily-conserved responses to inorganic and organic mercury will be elucidated by comparing the results of the yeast microarray and deletome with the results of the *C. elegans* microarray and RNAi experiments.

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